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Three novel truncating *TINF2* mutations causing severe dyskeratosis congenita in early childhood

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Abstract

Dyskeratosis congenita (DC) is a telomere-biology disorder characterized by a mucocutaneous triad, aplastic anemia, and predisposition to cancer. Mutations in a narrow segment of *TINF2* exon 6 have been recognized to cause often-severe DC that is either sporadic or autosomal dominant. We describe three children with very early presentations of DC, including one with the severe variant known as Revesz syndrome. Whereas most *TINF2* mutations reported to date are missense changes, each of our patients carried a novel heterozygous nonsense or frameshift mutation, revealing a new 5' boundary to the affected gene segment in patients with DC. Examination of patient-derived lymphoblastoid cell lines revealed stable expression of the predicted truncated TIN2 proteins. In co-immunoprecipitation assays, the ability of a truncation mutant to interact with TRF1 was severely impaired, whereas the ability of the most common DC-associated mutant was much less affected. This suggests that disruption of TIN2-TRF1 interaction may contribute to the severe clinical phenotype observed in the context of the TIN2 truncation mutation but is unlikely to be the primary cause of telomere shortening associated with the more prevalent TIN2 missense mutations. Telomere flow-FISH analysis of one pedigree demonstrated the dramatic effect a *de novo* nonsense *TINF2* mutation had on telomere length in early development. These cases underscore the severe manifestations of truncating *TINF2* mutations.

Keywords

Aplastic anemia; bone marrow failure; dyskeratosis congenita; Revesz syndrome; TIN2; *TINF2*; telomere

INTRODUCTION

Dyskeratosis congenita (DC; MIM #s 127550, 305000, 224230) is an inherited bone marrow failure and cancer predisposition syndrome that classically is defined by a triad of oral

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Conflict of interest statements

Ghadir S. Sasa, M.D. denies presence of any potential conflict.

Albert Ribes-Zamora denies presence of any potential conflict.

Nya D. Nelson denies presence of any potential conflict.

Alison A. Bertuch, M.D., Ph.D. denies presence of any potential conflict.

During the submission of our manuscript, the mutation harbored by Patient 1 was reported by Vulliamy, *et al.* (28), who received our patient's sample for mutation testing while we were independently sequencing *TINF2*.

leukoplakia, nail dystrophy, and abnormal skin pigmentation (1, 2). DC is heterogeneous in presentation and can have variable additional manifestations, such as pulmonary fibrosis, liver disease, enteropathy, and urogenital abnormalities (3). Two rare, severe variants of DC have been described, Revesz syndrome (RS; MIM #268130) and Hoyeraal-Hreidarsson syndrome (HHS; MIM #300240) (1, 2). Individuals with RS manifest the additional defining feature of bilateral exudative retinopathy. Other features of RS include intrauterine growth retardation, intracranial calcification and cerebellar hypoplasia (4). HHS is characterized by cerebellar hypoplasia along with microcephaly, developmental delay, immunodeficiency, intrauterine growth retardation, and bone marrow failure (5, 6).

At the molecular level, most patients with DC have very short telomeres, best characterized in leukocytes, and defined as a length less than the first percentile for age (7-9). To date, approximately 50% of affected individuals have genetic alterations in one of six genes affecting telomere biology (1). Five of those genes affect components of telomerase, the enzyme responsible for the maintenance of telomere length. The sixth gene implicated in DC, *TINF2*, encodes TIN2, a member of the telomere-associated shelterin complex, which plays integral roles in the structure and function of telomeres (10). Interestingly, patients harboring *TINF2* mutations are reported to have extremely short telomeres, correlating clinically with the frequently early age of presentation (less than 10 years) and severe manifestations of the disease (11, 12). The described mutations are all heterozygous, and map to a short, highly conserved segment of exon 6 of unknown function (Fig. 1A). Of the 40 reported probands with *TINF2*-associated DC or one of its variants, 35 harbored missense mutations, whereas only five probands had a truncating mutation (Fig. 1A and Table 1) (10-13). Two different *TINF2* mutations have been reported in five individuals with RS (11, 12). Here, we describe three children with novel nonsense/frameshift *TINF2* mutations who developed severe aplastic anemia (SAA) and other manifestations of DC at an early age. We demonstrate expression of the expected truncated TIN2 proteins, which, with the severe telomere shortening observed in the patients, indicates that loss of the TIN2 C-terminus results in significant deleterious effects. We further show that one of the truncation mutants has markedly reduced interaction with the shelterin complex member TRF1, whereas the most commonly found missense mutation does not, revealing a specific loss of function of the TIN2 truncation.

METHODS

Ethics and patient ascertainment

All described probands and family members were enrolled on a research protocol that was approved by the Baylor College of Medicine Institutional Review Board. The subjects were ascertained through clinical encounters over a two year period at Texas Children's Cancer Center and Hematology Service, a tertiary referral center. The probands represent three of the four patients found to have *TINF2* mutations, the fourth patient having the most common mutation, TIN2p.R282H.

Molecular genetic studies

Peripheral blood was obtained from Patients 1 and 2 and the sister of Patient 1, and DNA extracted using Puregene Blood Core Kit B (Qiagen). Saliva was collected from the parents of Patient 1 and DNA extracted using the Oragene-DNA sample collection kit (DNA Genotek). We performed polymerase chain reaction amplification of *TINF2* exon 6 using published primers (11), followed by bidirectional sequencing and comparison to the coding sequence of *TINF2* isoform 1 (NM_001099274.1) (14). The patients' mutations were independently confirmed through clinically certified labs (GeneDx, Gaithersburg, MD, or Ambry Genetics, Aliso Viejo, CA). Biologic samples of Patient 3 (skin fibroblast culture)

and Patient 2's mother (peripheral blood) were obtained and subjected to *TINF2* exon 6 sequencing solely by Ambry Genetics.

Telomere length analysis

Telomere flow-FISH (15) analyses of Patient 1 and his family and Patient 2 were performed by Repeat Diagnostics (Vancouver, BC).

Western blotting for TIN2

EBV-transformed lymphoblastoid cell lines (LCLs) were generated from Patient 1, Patient 1's sister, and Patient 2 by the tissue culture core laboratory within the Department of Molecular and Human Genetics, Baylor College of Medicine. We prepared small scale whole cell lysates by combining cytoplasmic and nuclear fractions as previously described (16). Protein was measured using Pierce BCA protein assay kit (Thermo Scientific), and specific protein expression analyzed by western blotting under the following conditions: 50 µg of total protein was loaded onto NuPage 4-12% bis-tris gel (Invitrogen), and TIN2 probed using a 1:2000 dilution of polyclonal rabbit anti-TIN2 antibody #865, which targets full-length TIN2 (kindly provided by Dr. Titia De Lange, Rockefeller University) (17). Beta actin served as the loading control, and was assayed using monoclonal antibody AC-15 (Sigma-Aldrich) at 1:5000 dilution. Analysis of TIN2 protein in Patient 3's skin fibroblasts was not possible because of poor growth of the culture

Plasmids

TINF2 short isoform cDNA sequence derived from pLPC-N-FH2-TIN2 plasmid (17) (kindly provided by Titia De Lange, Rockefeller University) was amplified and cloned into pcDNA3.1, creating pAB625, which was subsequently used for mutagenesis and co-immunoprecipitation experiments. Myc-TRF1 plasmid was generously provided by Ming Lei (University of Michigan) (18).

Mutagenesis

TINF2 mutations, c.805C>T/p.Q269X and c.845G>A /p.R282H, were introduced into pAB625 using oligonucleotide single-stranded mutagenesis (19) to give rise to pAB631 and pAB629, respectively. Mutations were confirmed by sequencing.

Co-immunoprecipitation assays

HEK293T cells (2×10^6) were transfected with 4 µg of either Myc-TRF1 plasmid, pAB625 (FLAG-TINF2), pAB629 (FLAG-TINF2-R282H) or pAB631 (FLAG-TINF2-Q269X) using Lipofectamine LTX with PLUS Reagent (Invitrogen) according to the manufacturer's protocols. Extracts were made by adding 500 µl RIPA buffer (50 mM Tris-HCL pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS) and 100 µl of Protease Inhibitor cocktail III (Calbiochem) to a frozen pellet for 30 min on ice. Immunoprecipitations were performed by mixing 750 µg of Myc-TRF1 plasmid transfected HEK293T cell extracts with 750 µg of either pAB625, pAB629 or pAB631 transfected cell extracts. Unmixed controls of 750 µg of Myc-TRF1 plasmid or pAB625 transfected cell extracts were also prepared. Mixtures were rotated 2 hrs at 4°C and subsequently 10 µg of -myc antibody 9E-10 (Sigma-Aldrich) was added to each reaction for 1 hr. One hundred fifty microliters of RIPA washed Protein G Plus-Agarose beads were added to each reaction and were rotated overnight at 4°C. Beads were collected by mild centrifugation, washed 4 times with 500 µl of RIPA buffer and finally resuspended in 40 µl of RIPA buffer and 20 µl of SDS-PAGE buffer. Forty microliters of each reaction were separated on 10% SDS-polyacrylamide gels and proteins analyzed by sequential western blotting using -FLAG M2 monoclonal antibody (Sigma-Aldrich; 1:1000) and -Myc antibody 9E-10 (Sigma-Aldrich;

1:5000). Input extract western blots were performed by loading 50 µg of each reaction collected after the 2 hr mixing period. In addition to -FLAG and -Myc antibodies, the membrane was probed with -actin AC-15 antibody (Sigma-Aldrich; 1:5000).

PATIENTS AND RESULTS

Patient 1

A 4-year-old Hispanic male was referred to us for evaluation of pancytopenia with macrocytic anemia [white blood count (WBC) 2,970/µl; absolute neutrophil count (ANC) 1,160/µl; hemoglobin 8.3 gm/dl; platelet count 36,000/µl; mean corpuscular volume (MCV) 96.8 fl]. Bone marrow aspiration and biopsy revealed 15 to 30% cellularity, with maturing trilineage hematopoiesis and normal cytogenetics. Past medical history was significant for an undescended testis and phimosis. His family history was noncontributory. Physical findings of dystrophic nails, oral leukoplakia, and subtle lacy hyperpigmentation involving the neck, inguinal, and scrotal areas established the diagnosis of classical DC. Within weeks of presentation, the patient became dependent on platelet transfusions, and his ANC decreased to 240/µl, at which point he received matched related donor hematopoietic stem cell transplantation (HSCT).

Consistent with the clinical diagnosis of DC, telomere flow-FISH analysis demonstrated telomere lengths well below the first percentile for all WBC subsets tested (Fig. 2) (7). *TINF2* exon 6 sequence analysis revealed a novel heterozygous mutation, *TINF2*c.805C>T, *TINF2* p.Q269X (Fig. 1). *TINF2* sequences and telomere lengths of the parents and sibling were normal (Figs. 1B and 2), indicating that the patient carried a *de novo* *TINF2* mutation.

Patient 2

A 21-month-old Hispanic male, with an unremarkable family history, was referred for further evaluation of SAA. He was a product of a normal pregnancy but at age 9 months was diagnosed with bilateral exudative retinopathy requiring multiple laser surgeries and resulting in loss of vision in one eye. He was found to be thrombocytopenic (platelets of 41,000 /µl) at 13 months of age and, during the following five months, developed macrocytic anemia (hemoglobin nadir 5.8 gm/dl, MCV 102 fl) and neutropenia (ANC 390/µl). His platelets concurrently decreased to 12,000/µl. His bone marrow showed 20 to 40% cellularity with marked myeloid and megakaryocytic hypoplasia, and normal cytogenetics. At that time, he was noted to have nail dystrophy and oral leukoplakia. On presentation to our clinic at age 21 months, his weight and head circumference were below the third percentile. In addition, he was delayed in gross motor and language areas, and he had a wide-based gait with poor coordination suggestive of cerebellar dysfunction. Brain magnetic resonance imaging confirmed cerebellar hypoplasia. Given the above findings, he was diagnosed with RS, a severe variant of DC. Due to the severity of his bone marrow failure, the patient underwent matched umbilical cord blood transplantation following reduced intensity conditioning (RIC). He experienced multiple complications resulting in his death on day +92 after transplant.

Telomere length analysis prior to umbilical cord transplant demonstrated very short telomeres in all WBC subsets, with median lengths of 2.3 to 2.8 kilobases. *TINF2* exon 6 sequencing revealed a novel heterozygous deletion, *TINF2*c.839delA (Fig. 1B), resulting in a frameshift and a premature stop codon, which could result in the expression of a truncated variant of TIN2, p.K280RfsX36 (Fig. 1A). His mother's *TINF2* sequence analysis was normal (Fig. 1B); his father was unavailable for testing but reportedly had no medical problems.

Patient 3

A Caucasian female, with an unremarkable family history, was first noted to have leukopenia, macrocytic anemia, and thrombocytopenia at 21 months of age (WBC 3,600/ μ l, ANC 1,800/ μ l, hemoglobin 10.1 gm/dl, MCV 100 fl, and platelets 31,000/ μ l). Over the next two years, her anemia and thrombocytopenia progressed (hemoglobin 4.1 gm/dl and platelets 6,000/ μ l) and she developed neutropenia (ANC 400/ μ l). She was referred to our clinic at age 3.5 years for further management. Her examination was normal with no mucocutaneous findings. Bone marrow evaluation revealed 5% cellularity with no dysplastic or cytogenetic abnormalities. Testing for Fanconi anemia and paroxysmal nocturnal hemoglobinuria were negative. Telomere length testing was not clinically available at that time. She was diagnosed with SAA and underwent a 6/6 HLA-matched sibling donor HSCT using RIC. Shortly after undergoing HSCT, she developed skin hyperpigmentation, nail dystrophy progressing to vanishing nails, and oral leukoplakia. These changes initially were thought to be graft-versus-host disease, but they did not improve with multiple immunosuppressive therapies. A diagnosis of DC was entertained. *DKC1* and *TERC* sequencing of a buccal sample were normal. Additional features of DC, such as epiphora, esophageal stricture, and osteopenia-related fractures, developed. Other complications of DC became apparent when the patient turned 10 years old (seven years post HSCT). She had progressive interstitial lung disease with fibrosis, as well as gastrointestinal bleeding secondary to enteropathy and non-cirrhotic portal hypertension. She eventually developed hepatopulmonary syndrome and died at the age of 12 years from multi-organ failure.

After identification of *TINF2*'s involvement in DC, a skin biopsy was obtained from the patient and *TINF2* exon 6 sequencing from a skin fibroblast culture was performed, demonstrating a novel heterozygous mutation, *TINF2*c.811C>T (Fig. 1B), which would potentially lead to expression of a truncated protein, p.Q271X (Fig. 1A). Parental testing was not performed.

Mutant TIN2 proteins are expressed in cells

To address the question of whether the expected truncated TIN2 proteins were expressed, western blotting of LCL whole cell lysates from Patients 1 and 2 was performed. This revealed stable expression of truncated TIN2 proteins of the predicted sizes of approximately 30 kD and 35 kD, respectively, in addition to expression of full length TIN2 (~39 kD) (Fig. 3A). It was consistently noted that the amount of the mutant protein was less than the full length TIN2 (see also Supplemental Figure 1), possibly indicating that the truncated proteins were less stable. Alternatively, this could be due to the lack of TIN2's normal C terminus, reducing polyclonal antibody recognition of the truncated protein.

TIN2p.Q269X has much reduced binding to TRF1

TIN2 has four known binding partners, three members of the shelterin complex, TRF1, TRF2, and TPP1, and a component of cohesin, SA1. TIN2 interacts with TRF2, TPP1 and SA1 via its N terminal domain, whereas the region that interacts with TRF1 is more central (20-23). Crystal structural analysis of an internal TIN2 peptide (256-276) bound to TRF1 demonstrated that the C-terminal boundary of interaction on TIN2 is aa 267 (18), the penultimate residue in TIN2p.Q269X. Therefore, we reasoned that the truncation mutants, and possibly previously described TIN2 missense mutants, might have allosteric effects on TRF1 binding. To address this question, we incubated extracts prepared from HEK293T cells transfected with Myc-TRF1 with extracts from cells transfected with FLAG-TIN2S, FLAG-TIN2p.Q269X or FLAG-TIN2p.R282H (the most common missense mutant found in patients) and then immunoprecipitated TRF1 with α -Myc antibody. We found TIN2p.Q269X was markedly impaired for its ability to interact with TRF1, whereas TIN2p.R282H retained substantial binding activity (Fig. 3B). Similar results were obtained

with extracts from co-transfected cells and when TIN2 was immunoprecipitated (data not shown).

DISCUSSION

TINF2 mutations have been found to account for 11 to 24% of cases with DC (24). Missense mutations are most common, both in terms of distinct mutations and number of affected probands, whereas only one nonsense mutation and three frameshift mutations have previously been identified (Table 1) (10-13). Our patients carried novel nonsense and frameshift mutations, thereby expanding the number of distinct DC-associated *TINF2* mutations to a total of 18. RS is a severe and rare variant of DC associated with the additional morbidities of retinopathy and central nervous system involvement (1, 2, 4). *TINF2* is the only gene discovered to be implicated in this subset of patients with DC, and to date, two mutations (namely, *TINF2*c.838A>T; TIN2p.K280X and *TINF2*c.845G>A; TIN2p.R282H) have been reported (Table 1). Patient 2's mutation (*TINF2*c.839delA; TIN2p.K280RfsX36) constitutes the third mutation causing RS. Therefore, we propose that *TINF2* should be the first DC-related gene sequenced in such patients.

The severity of DC has not been specifically graded, but has been generally linked to the age of onset of aplastic anemia and the number of associated somatic abnormalities. Patients manifesting prior to 10 years of age are considered to have the severest form (25). Alternatively, patients with the two variants of DC, RS and HHS, are referred to as having severe disease (1, 2). DC has been known to show phenotypic variability even within groups carrying the same genetic mutation (26), possibly reflecting added genetic and/or environmental modifiers on the dysfunctional telomere maintenance. Hence, establishing genotype-phenotype correlations has been difficult and complex. However, Walne, *et al.*, observed that most individuals with *TINF2* mutations tended to develop severe DC, with approximately two-thirds developing SAA in the first decade of life (11). Focusing on the small subgroup of probands with nonsense or frameshift *TINF2* mutations (including those in our series), we noted that all patients uniformly presented with very early DC, as early as 1 year of age. In addition to Patient 2 presenting with RS, Patient 1 displayed evidence of organ involvement at birth (undescended testis, phimosis), suggesting a pivotal role of *TINF2* in early development.

Heretofore, all DC-associated *TINF2* mutations map to a short segment of exon 6 that would consequently affect amino acids (aa) 280-298 of the transcribed protein, TIN2 (Fig. 1A) (10, 11). Two of our described mutations (*TINF2*c.805C>T; TIN2p.Q269X and *TINF2*c.811C>T; TIN2p.Q271X) lie closer to the N-terminus and would, therefore, extend the affected segment to aa 269. The function of this segment remains unidentified. Notably, however, the TIN2p.Q269X truncation immediately abuts the residues known to mediate an interaction between TIN2 and TRF1 (18). We found the TIN2p.Q269X mutant is unable to bind TRF1 efficiently, whereas the TIN2p.R282H mutant retains substantial binding. Because patients harboring a TIN2p.R282H mutation have markedly short telomeres and manifest all of the features of DC, it is unlikely that disrupted TRF1 binding is the main factor driving disease pathogenesis. Supporting this notion is the observation that missense mutation of residues known to directly interact with TRF1 has not been reported. Reduction in TIN2-TRF1 binding may, however, contribute to the severe phenotype observed in some patients such as our patient bearing the TIN2p.Q269X truncation mutation.

It is notable that N-terminal nonsense or frameshift mutations have not been reported in patients, suggesting that haploinsufficiency of an N terminal function (e.g., TRF2 binding) does not underlie the telomere dysfunction. Moreover, in our patient LCLs bearing more centrally located nonsense or frameshift mutations, truncated proteins were readily observed

(Fig. 3A). Whether the result in solely haploinsufficiency of C terminal functions or dominant negative effects remains to be determined.

Finally, telomere length is inherited as a quantitative trait and correlates significantly with paternal, but not maternal, telomere length (27). Patient 1 had a documented *de novo* mutation (Fig. 1B), and his extremely short telomeres (Fig. 2), in contrast to his father's, demonstrate the drastic negative impact that a heterozygous nonsense *TINF2* mutation can have on telomere length in a single generation, similar to what has been reported with missense *TINF2* mutations (10, 11). This raises the possibility that *TINF2* mutations result in telomere shortening through increased telomere attrition rather than or in addition to altered lengthening mechanisms. Further research is needed to elucidate the exact pathophysiology underlying *TINF2*'s deleterious effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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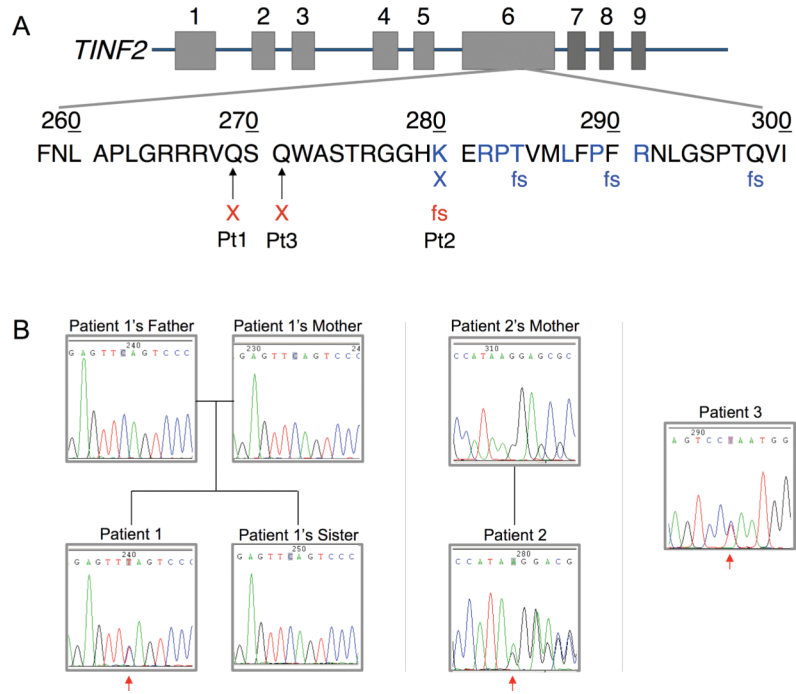


Figure 1. A) Localization of *TINF2* mutations. The genomic *TINF2* structure is depicted with exons as rectangles. The *TINF2* short isoform contains exons 1-6, whereas the long isoform includes exons 1-9 (29). Residues previously reported to be affected by missense mutations are in blue (10, 11, 13, 30). X and fs are placed below sites of known nonsense and frameshift mutations, respectively. Previously reported nonsense (X) and frameshift (fs) mutations are in blue, and new mutations in red. B) *TINF2* sequence chromatograms. Chromatograms are of the described patients and some family members, as labeled. An arrow points to the reported heterozygous change in each patient. (Chromatograms of Patient 2's mother and Patient 3 are courtesy of Ambry Genetics, Aliso Viejo, CA)

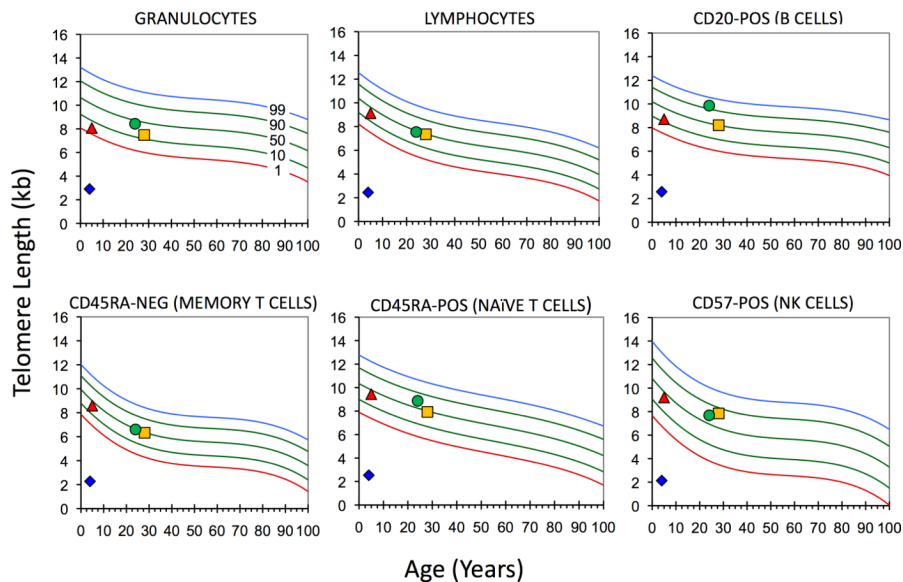


Figure 2. Dramatic telomere shortening in one generation in the presence of a *TINF2c.805C>T*: *TIN2p.Q269X* heterozygous mutation. WBC subset telomere flow-FISH analysis (15) performed on Patient 1, his parents and sibling, as indicated. Proband, diamond; Sister, triangle; Mother, circle; and Father, square. (Courtesy of Repeat Diagnostics, Vancouver, BC)

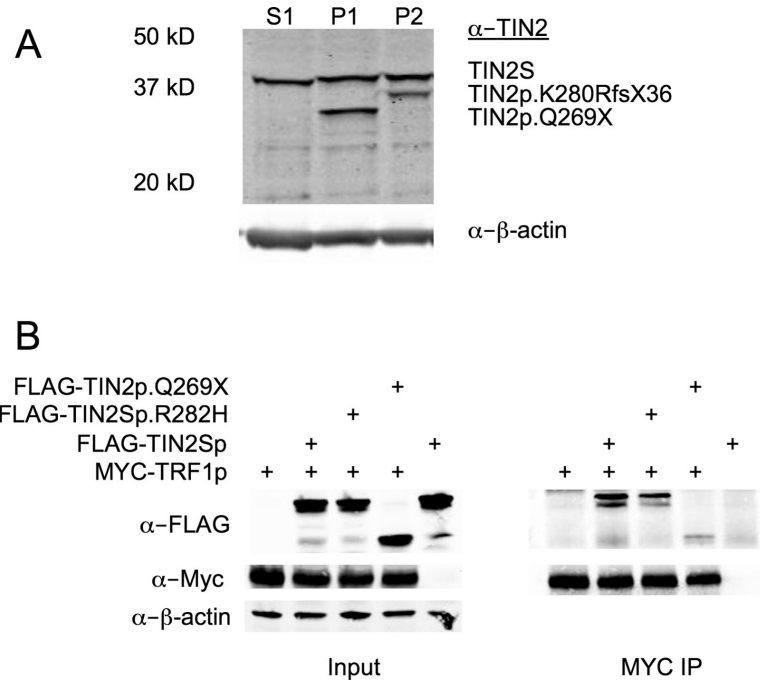


Figure 3.
 A) Stable expression of truncated TIN2 proteins. Western blotting of whole cell extracts from LCLs of Patient 1's sister, Patient 1, and Patient 2 (Lanes S1, P1, and P2, respectively). The positions of the full length short isoform of TIN2 in all, and the truncated TIN2 in Patients 1 and 2 are denoted by arrows. Beta actin served as the loading control. (n.b., The slight difference in migration in S1 as compared to P1 and P2 was not consistently seen; e.g., see Supplemental Figure 1) B) TIN2p.Q269X has markedly reduced binding to TRF1. Co-immunoprecipitations of mixtures of extracts from transfected HEK293T cells analyzed by western blotting (MYC IP). Mixtures prior to immunoprecipitation are shown (Input).

Table 1

DC-associated *TINF2* mutations

<i>TINF2</i> base change	TIN2 amino acid change	Mutation type	Associated phenotype(s)	Number of reported probands (ref)
Previously reported mutations				
c.845G>A	R282H	Missense	DC DC/RS DC/HH/RS HH Ataxia-pancytopenia	12 (10, 11) 2 (10, 11) 1 (11) 2 (11) 1 (13)
c.844C>T	R282C	Missense	DC	7 (11)
c.838A>G [*]	K280E	Missense	DC	1 (10)
c.844C>A [†]	R282S	Missense	DC	1 (10)
c.847C>T	P283S	Missense	DC/HH	1 (11)
c.847C>G	P283A	Missense	DC	1 (11)
c.848C>A	P283H	Missense	DC	1 (11)
c.850A>G	T284A	Missense	DC	1 (11)
c.860T>C	L287P	Missense	DC	1 (11)
c.865_866delinsAG	P289S	Missense	DC	1 (11)
c.871A>G	R291G	Missense	DC	1 (11)
c.838A>T	K280X	Nonsense	DC/HH/RS HH/RS	1 1 (11)
c.867_868insC	F290LfsX2	Frameshift	DC	1 (11)
c.892delC	Q298RfsX19	Frameshift	DC	1 (11)
c.849_850insC	T284HfsX8	Frameshift	DC	1 (11)
Mutations reported in this series				
c.805C>T	Q269X	Nonsense	DC	1
c.811C>T	Q271X	Nonsense	DC	1
c.839delA	K280RfsX36	Frameshift	DC/RS	1

Abbreviations are as follows: DC, dyskeratosis congenita; RS, Revesz syndrome; HH, Hoyeraal- Hreidarsson syndrome.

^{*} Mutation reported as EX6+234A>G in original publication (10).

[†] Mutation reported as EX6+240C>A in original publication (10).