Dyskeratosis congenita mutations in dyskerin SUMOylation consensus sites lead to impaired telomerase RNA accumulation and telomere defects

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Mutations in the dyskerin gene (DKC1) cause X-linked dyskeratosis congenita (DC), a rare and fatal premature aging syndrome characterized by defective telomere maintenance. Dyskerin is a highly conserved nucleolar protein, and a component of the human telomerase complex that is essential for human telomerase RNA (hTR) stability. However, its regulation remains poorly understood. Here, we report that dyskerin can be modified by small ubiquitin-like modifiers (SUMOs). We find that human DC-causing mutations in highly conserved dyskerin SUMOylation consensus sites lead to impaired hTR accumulation, telomerase activity and telomere maintenance. Finally, we show that modification of dyskerin by SUMOylation is required for its stability. Our findings provide the first evidence that dyskerin stability is regulated by SUMOylation and that mutations altering dyskerin SUMOylation can lead to defects in telomere maintenance that are characteristics of DC.

INTRODUCTION

Telomerase is a specialized H/ACA box ribonucleoprotein (RNP) required for the synthesis of telomeres, the nucleoprotein structures that cap the ends of linear eukaryotic chromosomes and protect them from degradation, end-to-end fusions and inappropriate DNA repair responses (1). Telomere synthesis by telomerase in germ cells, stem cells and highly proliferative cells also compensate for telomere loss caused by the end-replication problem (2). The active core of telomerase is composed of a catalytic subunit called telomerase reverse transcriptase (TERT) and a telomerase RNA (TR) which provides the template for telomere synthesis (3). TR accumulation and telomerase RNP assembly are regulated by the H/ACA box RNPs dyskerin, NHP2 and NOP10 (4–7). Two telomerase associated ATPases, pontin and reptin, are also necessary for the stability of dyskerin and TR in vivo (8). The most recently identified telomerase subunit TCAB1 is involved in the subcellular localization and trafficking of the telomerase complex (9).

Dyskeratosis congenita (DC) is clinically characterized by a triad of nail dystrophy, reticular skin pigmentation and oral leukoplakia (10,11). Patients with dyskeratosis congenita have a predisposition to develop pulmonary fibrosis and a variety of malignancies but in 70% of cases, bone marrow failure is the primary cause of early mortality (12). The X-linked form of the disease is the most common and tends to be associated with a more severe phenotype and earlier onset of the disease. X-linked dyskeratosis congenita is caused by mutations in the DKC1 gene which encodes the protein dyskerin (also known as NAP57 and Cbf5p) (13), a highly conserved essential nucleolar protein required for telomerase RNA (TR) stability, telomerase activity and telomere maintenance (5,14). Other modes of DC inheritance include autosomal dominant, autosomal recessive and sporadic autosomal and are due to mutations in genes that encode components regulating telomerase or telomere function (TERT, TR, NOP10, NHP2, TCAB1 and TIN2) (10).

Several dyskerin mutations have been identified and most of them are grouped into two major clusters comprising amino acids 31–72 and 314–420 (15). Interestingly, the two clusters of mutations are located outside the catalytic pseudouridine synthase TruB domain. Modeling of the clusters of dyskerin mutations on the archaeal H/ACA RNP complex (consisting of dyskerin, NHP2, NOP10, GAR1 and a H/ACA RNA) structure

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revealed that the mutations localize spatially to a single domain that does not overlap with the binding sites for GAR1, NOP10 and NHP2 (16). Accordingly, a number of dyskerin mutations do not affect the assembly of the dyskerin/NOP10/NHP2 pre-RNP complex in vitro (17). Additionally, dyskerin mutations do not map to the H/ACA box RNA binding sites in a model structure, and dyskerin mutations do not alter the association of the pre-RNP complex with the hTR H/ACA motif (16,17). It has been shown that various DKC1 mutations impair telomerase and telomere function by reducing steady-state hTR accumulation in vivo (14,18). However, to date, the mechanisms regulating dyskerin have not been characterized.

SUMOylation is a post-translational modification which results in the covalent attachment of a small ubiquitin-like modifier (SUMO) protein to target proteins (19). SUMO transfer is catalyzed by a cascade of enzymes involving ATP, an E1 SUMO-activating enzyme (SAE1/SAE2), an E2 conjugating enzyme (Ubc9) and an E3 SUMO ligase. SUMOylation occurs on target lysine residues usually found in the consensus SUMOylation motif YKXE. In humans, there are three different functional SUMO isoforms: SUMO1, SUMO2 and SUMO3. SUMO2 and SUMO3 share only ~50% homology with SUMO1, but they share ~97% homology with each other and hence are often referred to as SUMO2/3. Similarly to ubiquitin, SUMO2/3 has the ability to form polymeric chains through a conserved lysine (K11) (20). SUMO1 lacks this residue and thus the ability to form chains, although it can terminate and cap poly-SUMO2/3 chains (21). SUMO modification regulates many cellular processes including protein–protein interactions, transcription, cellular localization and the response to DNA damage (19). Recently, a role for SUMOylation in the biogenesis and function of RNPs was reported (22). Interestingly, SUMOylation of the core box C/D RNP protein NOP58 was found to be important for its binding to small nucleolar RNAs (snoRNAs) and for their localization to the nucleolus. As dyskerin was also identified as a nucleolar SUMOylation candidate in this and another study (22,23), we sought to determine the role of SUMOylation in the biogenesis and function of the H/ACA box RNP protein dyskerin in telomerase regulation, telomere maintenance and its implication in DC.

RESULTS

Dyskerin is SUMOylated in vitro and in vivo

Several putative dyskerin SUMOylation sites were identified using the SUMOsp 2.0 program, a site-specific predictor of SUMOylation sites (24) (Fig. 1A). Among them, K39 is located at the N-terminus of dyskerin and has been found to be mutated to glutamic acid (K39E) in DC. K43 is located four residues downstream of K39 but no disease-associated mutation has been found for this residue. K16 and K448 are comprised within two nuclear localization signals (NLSs) in dyskerin, located at the N-terminus and C-terminus, respectively (25). K39, located in the N-terminal FLIKPE motif, matches with the consensus hydrophobic cluster SUMOylation motif (HCSM) ΨΨΨKXE where three hydrophobic residues replace the single hydrophobic residue present in the conventional consensus SUMOylation motif ΨΨΨKXE (26). This extended SUMOylation motif has been shown to enhance the SUMOylation efficiency of zinc finger and BTB domain protein 1 and Ran GTPase-activating protein 1 (RanGAP1) (26). Strikingly, every amino acid of the dyskerin HCSM sequence is mutated in DC. The single amino acid substitutions F36V, I38T/M, K39E, P40R, E41K and L37 deletion (L37del) are all associated with X-linked recessive DC or Hoyeraal–Hreidarsson syndrome (13,27,28). Moreover, the FLIKPE motif of dyskerin is highly conserved among organisms suggesting an important function for this motif (Fig. 1A). We hypothesized that dyskerin is SUMOylated and that mutations in the HCSM of dyskerin contribute to DC by impairing dyskerin SUMOylation.

To assess whether dyskerin could be a substrate for SUMO modification, in vitro translated and [35S]-methionine (Met)-labeled FLAG tagged wild-type (WT)-dyskerin (FLAG-dyskerinWT) was subjected to an in vitro SUMOylation assay using purified recombinant E1 (SAE1/2), E2 (UBC9) and SUMO1 or SUMO2 (29,30) (Fig. 1B). The migration of in vitro expressed 35S-Met-labeled FLAG-dyskerinWT in the absence of SUMO, UBC9 and SAE1/2 (Lanes 1 and 6) is indicated. Higher-molecular weight species corresponding to SUMO1- and SUMO2-modified dyskerin were detected in reactions containing E1, E2 and SUMO (Fig. 1B). The formation of SUMO–dyskerin conjugates was dependent on the addition of SAE1/2, UBC9 and SUMO since no slowly migrating species were observed when any one of the components was omitted from the reactions. Modification of dyskerin was extensive in the presence of either SUMO1 or SUMO2, suggesting that dyskerin can be modified in several residues by both SUMO1 and SUMO2 (Fig. 1B).

We next performed lysine to arginine mutational analyses to identify the SUMOylated lysines. We analyzed a series of mutant forms of FLAG-dyskerin corresponding to each putative SUMOylation site by generating single K39R, K43R, K46R, K16R and K448R lysine to arginine mutations. We also generated double K16R/K39R, K16R/K43R, K39R/K448R and K43R/K448R mutants (Fig. 1A). The conserved glutamic acid residue in the consensus ΨΨΨKXE motif is known to be important for the efficiency of SUMO addition to the nearby lysine (31–33). Similarly, the hydrophobic cluster preceding the lysine in the extended ΨΨΨΨΨKXE motif also enhances SUMOylation (26). We therefore included an additional variant of FLAG-dyskerin containing the DC-associated mutation L37del to test whether this deletion might act by impairing SUMOylation of K39 (Fig. 1A).

We next tested whether dyskerin could be a substrate for SUMOylation in vivo. HEK293 cells were transfected with plasmids encoding FLAG-dyskerinWT or the FLAG-dyskerin variants: L37del, K39R, K43R and a 6His-SUMO1-encoding plasmid, followed by denaturing Ni2+-NTA agarose bead pull-downs. Input (middle panel) and eluted (top panel) proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted using anti-FLAG (Fig. 2A) or anti-His (Supplementary Material, Fig. S1A) antibodies. Dyskerin is predicted to be 58 kDa; however, we occasionally observe dyskerin migrating at a higher molecular weight, suggestive of post-translational modifications. FLAG-dyskerin present in the input material was
detectable at a molecular weight of $\approx 70$ kDa. Notably, a higher molecular weight His6-SUMO1 modified FLAG-dyskerin WT (Lane 2, $\approx 90$ kDa) was enriched by the pull-down, indicating that dyskerin can be SUMOylated in vivo by SUMO1. The absence of a 90 kDa species in the input is consistent with steady-state levels of SUMOylated proteins being typically $5\%$ (34,35). Control pull-downs were performed in the absence of FLAG-dyskerin (Lane 1), His6-SUMO1 (Lane 7) or both (Lane 6). SUMOylation of the FLAG-dyskerin variants was reduced compared with FLAG-dyskerin WT, most notably FLAG-dyskerin K39R (Lane 4) but also FLAG-dyskerin K43R (Lane 5). We conclude that dyskerin is SUMOylated in vivo on key lysine residues and that K39R is a major site for SUMOylation by SUMO1.

Altering dyskerin SUMOylation at K39 and mutations in highly conserved dyskerin SUMOylation consensus sites can lead to defects in telomere maintenance

To determine the role of dyskerin SUMOylation in telomere length maintenance, we used shRNA lentiviruses specific to the DCK1 3′UTR to stably knockdown endogenous dyskerin in HEK293 cells (Supplementary Material, Fig. S1B). We then tested whether stable expression of FLAG-dyskerin WT, FLAG-dyskerin K39R, FLAG-dyskerin K43R or empty vector could rescue dyskerin knockdown. We also stably expressed mutant FLAG-dyskerin L37del as a control for short telomeres (5). The telomere length was analyzed using quantitative fluorescence in situ hybridization (Q-FISH) after 18–40 population doublings (PDs) (late PD) (Fig. 3; Supplementary Material, Figs. S2 and S3). ShRNA knockdown of dyskerin (labeled vector) reduced the telomere length of HEK293 cells when compared with the
telomere length of cells infected with a control shRNA (labeled control), as shown by a shift in the telomere length distribution toward shorter telomeres (Fig. 3A). We then asked whether stable expression of FLAG-dyskerinWT or mutant FLAG-dyskerin rescued the short telomere phenotype associated with dyskerin loss of function. Stable expression of FLAG-dyskerinWT rescued the dyskerin knockdown phenotype and even slightly increased the telomere length when compared with control cells, as shown by a shift in the telomere length distribution toward longer telomeres and increase in the frequency of very long [>300 arbitrary units of fluorescence (a.u.f.)] telomeres. Stable expression of the FLAG-dyskerin del37L, K39R and K43R variants was not able to rescue the telomere shortening observed in the empty vector-containing knockdown cells. The telomere shortening in the HEK293 dyskerin knockdown cells (labeled vector) and the knockdown cells stably expressing the FLAG-dyskerin variants was also obvious from the higher occurrence of telomere signal-free ends (SFEs, chromosome ends lacking detectable telomere sequences) in these cells (Fig. 3B). We conclude that mutations altering dyskerin

Figure 2. Dyskerin is SUMOylated in vivo and K39 is a major sumoylation site. (A) FLAG-dyskerinWT (Lane 2) or the FLAG-dyskerin variants: L37del (Lane 3), K39R (Lane 4), K43R (Lane 5), and 6His-SUMO1 were expressed in HEK293 cells. HEK293 cells were also transfected with a pcDNA vector only (Lane 6), pcDNA vector and plasmid encoding FLAG-dyskerinWT (Lane 7) or pcDNA vector and plasmid encoding 6His-SUMO1 (Lane 1) as controls. His-SUMO-modified proteins were enriched after lysis under denaturing conditions using Ni-NTA agarose beads. Input (middle panel) and eluted (top panel) proteins were separated by SDS–PAGE and immunoblotted using an anti-FLAG antibody. Lower panel represents an immunoblot using an anti-tubulin antibody. Levels of 6His-SUMO1-modified FLAG-dyskerin variants were quantified from two experiments in comparison with levels of 6His-SUMO1-modified FLAG-dyskerinWT (set at 1) and quantifications are indicated underneath the upper panel. (B) In vitro expressed 35S-Met-labeled FLAG-dyskerinWT, L37del, K39R, K43R, K46R, K16R, K448R mutants and K16R/K39R, K16R/K43R, K39R/K448R, K43R/K448R double mutants were incubated with SUMO1 or SUMO2, UBC9 and SAE1/2 in the presence of ATP and the products were analyzed as in Fig. 1B. The migration of in vitro expressed 35S-methione-labeled dyskerin is indicated. Double mutations greatly impair modification by SUMO1 or SUMO2 (Lanes 23, 24, 26, 27, 29, 30, 33 compared with Lanes 2 and 3). All reactions were performed at the same time and separated on gels simultaneously.
Figure 3. Mutations in dyskerin SUMOylation consensus sites lead to telomere shortening and increased SFEs (A) Histograms of telomere length distribution in cells expressing a control shRNA (control) or in cells knocked down for endogenous dyskerin and transfected with vector, FLAG-dyskerin WT, L37del, K39R or K46R dyskerin variants (late PD). Telomere lengths are expressed as arbitrary units of fluorescence (a.u.f.). A shift to the left in the length distribution of dyskerin mutant cells indicates telomere shortening. The total number of telomeres analyzed (ranging from 2880 to 3312), mean telomere length ± standard error of the mean are indicated. A nonparametric ANOVA test, the Kruskall–Wallis test (P < 0.0001), indicated telomere shortening in the vector, L37del, K39R and K46R mutants compared with the control and WT. This was confirmed in post-hoc between-group analysis with Bonferroni-corrected Mann-Whitney’s U test which indicated that the differences were significant (P < 0.0001) for each pair of comparisons. The P-values in the figure refer to comparisons of the different variants with the control or dyskerin WT. Note the greater abundance of short (<100 a.u.f.) telomeres in the vector, L37del, K39R and K46R mutants compared with the control and WT. This was confirmed in post-hoc between-group analysis with Bonferroni-corrected Mann-Whitney’s U test which indicated that the differences were significant (P < 0.0001) for each pair of comparisons. The P-values in the figure refer to comparisons of the different variants with the control or dyskerin WT. Note the greater abundance of short (<100 a.u.f.) telomeres in the vector, L37del, K39R and K46R mutants compared with the control and WT. Telomeres were hybridized with a Cy3-conjugated PNA probe (Cy3-[CCCTAA]3). A minimum of nine metaphases per clone were analyzed. (B) Telomere SFEs are increased in the lysine-to-arginine dyskerin mutants. Expression of WT dyskerin rescues the SFEs in dyskerin knockdown cells. Error bars indicate standard deviation. (***P < 0.001, **P < 0.01, *P < 0.05 and n.s. P > 0.05).
SUMOylation at K39 and mutations in highly conserved dyskerin SUMOylation consensus sites can lead to defects in telomere maintenance that are characteristic of DC.

Impaired dyskerin SUMOylation can lead to reduced telomerase activity and telomere RNA levels

DC patients with point mutations in DKC1 exhibit reduced telomerase activity and decreased hTR levels (5,14,18). To determine how defects in dyskerin SUMOylation and mutations in highly conserved dyskerin SUMOylation consensus sites might lead to telomere length shortening, we assessed telomerase activity and hTR levels in cells stably knocked down for dyskerin and expressing FLAG-dyskerinWT or variants (Fig. 4A–C). Cells at the same late PD as analyzed for the telomere length were used. Telomerase activity was considerably reduced in the FLAG-dyskerin L37del, K39R and K43R expressing cell lines when compared with the control cells (Fig. 4A and B). Stable expression of FLAG-dyskerinWT rescued dyskerin knockdown and increased telomerase activity, in accordance with the increased telomere length observed previously (Fig. 3A). We quantified hTR levels in the dyskerin knockdown and rescue cell lines using a quantitative real-time PCR (qRT–PCR). We found a marked decrease in the expression of hTR in FLAG-dyskerin del37L, K39R and K43R expressing cells, while the FLAG-dyskerinWT expressing cells showed increased levels of hTR compared with hTR levels in cells expressing a control shRNA (Fig. 4C). We thus conclude that SUMOylation of dyskerin is likely required for hTR accumulation and subsequent telomerase activity. Surprisingly, hTR levels were increased in the empty rescue vector clones compared with the control cells, suggestive of an eventual loss of shRNA-mediated knockdown of dyskerin at late passage (occurring approximately between PD12 and PD24). Indeed, immunoblot analysis of dyskerin expression in HEK293 dyskerin shRNA knockdown cells and empty vector rescue cells at late passage (PD24–26) revealed that RNA interference had been lost with increasing PDs (Supplementary Material, Fig. S1C, Lane 2). We ignore to what extent dyskerin knockdown may eventually have also been lost in the knockdown cells expressing FLAG-dyskerin variants, and might have led to an underestimation of the effects on telomerase activity, hTR levels and telomere lengths. However, at the tested PDs between 24 and 26, Western blot analysis indicates that DKC1 shRNA expression has not been lost in the knockdown cells expressing FLAG-dyskerin variants since endogenous dyskerin is not observed (Supplementary Material, Fig. S1C, Lanes 3–6), perhaps due to some residual functional activity of the dyskerin variants. Moreover, the observed telomere length phenotype is a reflection of telomere length changes occurring since early PDs, and thus is representative of the ‘long term’ effects of the dyskerin variants.

SUMOylation at K39 and mutations in highly conserved dyskerin SUMOylation consensus sites can lead to defects in telomere maintenance that are characteristic of DC.

We utilized an alternative approach to address the role of dyskerin SUMOylation in hTR accumulation. We speculated that increased SUMOylation on other lysines might rescue the defect of the dyskerinK39R variant. We created an N-terminal SUMO3 fusion with the dyskerinK39R variant (Fig. 4D), and addressed whether forced SUMOylation of this dyskerin mutant could prevent the decreased levels of hTR observed in the dyskerin knockdown cells stably expressing dyskerinK39R (Fig. 4C and E). Such an approach has previously been used to increase levels of constitutively SUMOylated proteins (36,37). We transiently expressed SUMO3-dyskerinK39R, or dyskerinK39R (as a control) in the dyskerin knockdown cells stably expressing dyskerinK39R. We confirmed that expression of the SUMO3-dyskerinK39R fusion protein leads to its SUMOylation, as evident from the presence of higher-molecular weight dyskerin species (Supplementary Material, Fig. S4). By a qRT–PCR, we observed that hTR levels were 48% in the control dyskerin knockdown cells stably (and also transiently) expressing dyskerinK39R when compared with HEK293 cells transiently transfected with vector (set at 100%), levels comparable with those found in dyskerin knockdown cells transfected with vector (36%) (Fig. 4E), and to levels previously reported in dyskerin knockdown HEK293 cells (40%) (38). However, SUMO3-dyskerinK39R expression in the dyskerin knockdown cells stably expressing dyskerinK39R almost fully rescued the expression levels of hTR (91%) (Fig. 4E), indicating that SUMOylation of dyskerin at K39 regulates hTR accumulation.

SUMOylation regulates dyskerin stability

DC patients with decreased levels of dyskerin (52–72% of control) exhibit decreased hTR levels (39). Since one known role of SUMOylation is protein stabilization (40), we examined the role of SUMOylation in dyskerin stability under conditions in which protein synthesis is inhibited by cycloheximide (CHX) (Fig. 4F). Dyskerin coexpression with either SUMO1 or SUMO3 increased the stability of dyskerin, with a predominant effect in the presence of SUMO3. Dyskerin is stabilized by SUMOylation even upon 0 h CHX treatment, as observed by higher levels of dyskerin in cells expressing SUMO1 or SUMO3 compared with cells transfected with a vector. These results are consistent with our observation that protein levels of FLAG-dyskerinK39R and K43R variants expressed in the cells stably knocked down for dyskerin are lower than protein levels of FLAG-dyskerinWT in the same cells, despite similar levels of FLAG-dyskerin mRNA (Supplementary Material, Fig. S1C and D).

DISCUSSION

Collectively, our findings provide the first evidence that dyskerin stability is regulated by SUMOylation (Fig. 4F, S1C). We propose that impaired dyskerin SUMOylation can lead to decreased dyskerin stability and levels, decreased levels of hTR and functional telomerase, resulting in telomere shortening characteristic of DC. Our data suggest that K39 is one of the major sites for SUMOylation of dyskerin by SUMO1 (Fig. 2A). However, the extensive SUMOylation pattern in in vitro SUMOylation assays with both SUMO1 and SUMO2 (Fig. 1C), and the stabilization of dyskerin by both SUMO1 and SUMO3 suggest the presence of several SUMOylation sites in dyskerin, including K43, and indicate that dyskerin SUMOylation is likely regulated by SUMO1 and SUMO2/3. Typically SUMO2/3, but not SUMO1 form chains efficiently (20,21). In vitro, it is not uncommon to observe multiple forms of substrates incubated with SUMO1 or SUMO2/3, representative of a combination of molecules in a single chain and single molecules at multiple sites for SUMO2/3 and perhaps single...
Figure 4. Downregulation of hTR in dyskerin knockdown cells can be rescued by a SUMO-fused dyskerin mutant and dyskerin stability is increased by SUMOylation. (A) Analysis of telomerase activity by TRAP in HEK293 cells expressing a control shRNA or in HEK293 cells knocked down for endogenous dyskerin and transfected with a vector or expressing FLAG-dyskerin WT, or FLAG-dyskerin variants, L37del, K39R or K43R. Two clones (late PD) for each vector, dyskerin WT and dyskerin variants were analyzed. IC internal control. Neg. negative control. (B) Quantification of telomerase activity calculated as the intensity of the telomerase products relative to the intensity of the PCR amplification of the IC from three different experiments + SEM. (C) qPCR analysis of hTR transcripts in HEK293 cells expressing a control shRNA or in cells knocked down for endogenous dyskerin and transfected with a vector, or expressing FLAG-dyskerin WT or FLAG-dyskerin variants, L37del, K39R or K46R. Two clones (late PD) for each vector, dyskerin WT and dyskerin variants were analyzed. Both vector clones have lost dyskerin shRNA knockdown expression (Supplementary Material, Fig. S1C, data not shown). Data are normalized to ActB and relative to control from three different experiments + SEM. (D) A schematic of the human dyskerin protein illustrating the SUMOylation at the K39 residue (top), the lysine-to-arginine mutation at Position 39 (middle) and the N-terminal SUMO3 fusion of the K39R dyskerin variant (bottom). Circles (S) represent SUMOylation. Predicted sizes of FLAG-dyskerin and SUMO-dyskerin are 58 and 70 kDa, respectively. (E) qPCR analysis of hTR transcripts in HEK293 transfected with pcDNA3.1, HEK293 cells expressing dyskerin shRNA and transfected with a vector (early PD), HEK293 dyskerin knockdown cells stably expressing dyskerinK39R (early PD) and transiently expressing FLAG-dyskerinK39R, HEK293 cells transiently expressing SUMO3-dyskerinK39R and HEK293 dyskerin knockdown cells stably expressing dyskerinK39R (early PD) and transiently expressing SUMO3-dyskerinK39R. Data are normalized to ActB and relative to control from a minimum of three different experiments + SEM. (F) HEK293 cells were transiently transfected with FLAG-dyskerin WT and vector, 6His-SUMO1 or 6His-SUMO3 plasmids. Cells were treated with CHX, harvested at various times (0, 2, 4 and 6 h) and extracted proteins were separated by SDS-PAGE and immunoblotted using anti-dyskerin (upper panel) or anti-tubulin antibodies (lower panel).
molecules added at multiple sites for SUMO1 (41). In cells, SUMOylation of dyskerin is not completely abolished by mutation at one residue (K39R or K43R), thus it is likely that the single SUMOylated form that we observe represents a single SUMO1 molecule added at an alternative site (for example, at K39 in the K43R variant).

We report that mutation of two residues previously found to be mutated in DC, K39 and K43, but which remained uncharacterized to date, can elicit extensive telomere shortening, impaired telomerase activity and decreased levels of hTR, similarly to mutated in DC, K39 and K43, but which remained uncharacterized to date, can elicit extensive telomere shortening, impaired telomerase activity and decreased levels of hTR, similarly to the previously characterized L37del (Figs. 3 and 4, Supplementary Material, Fig. S2 and 3). Interestingly, these phenotypes are observed even in the presence of partial SUMOylation, suggesting that even a mild impairment in SUMOylation can lead to DC phenotypes, and/or that other post-translational modifications on these residues are also required for dyskerin function. Accordingly, K39 was identified as a potential ubiquitination site in global ubiquitin-modified proteome analyses (42).

Dyskerin SUMOylation may alter its interactions with hTR or dyskerin-associated proteins which regulate dyskerin stability or biogenesis of H/ACA RNPs, including GAR1, NHP2, NOP10, Nopp140, NAF1, SHQ1, repinit, pontin and TCAB1. Certain dyskerin mutant proteins, including A353V, G402E and T49M, have impaired interactions with hTR (38). Pontin and repinit are two telomerase-associated ATPases necessary for the stability of dyskerin and TR in vivo (8). TCAB1 is involved in the subcellular localization and trafficking of the telomerase complex (9). NAF1 is an RNP assembly factor that is exchanged for the mature RNP component GAR1 (43). Another protein which binds dyskerin independently of the H/ACA RNP proteins NOP10, GAR1 and NHP2 is SHQ1 (43). SHQ1 is an early assembly factor which binds to newly synthesized dyskerin, stabilizing and preventing it from misfolding and degradation, and is localized to the nucleoplasm and excluded from nucleoli and Cajal bodies which are sites of mature H/ACA RNPs (44,45). The C-terminal domain of SHQ1 interacts with the RNA-binding domain of dyskerin and some DC-causing dyskerin mutations in the pseudouridine synthase and archaeosine transglycosylase (PUA) domain were shown to modulate SHQ1 binding (46,47). Thus, these mutations affect the pre-RNP assembly of the H/ACA complex, suggesting that the stability of all H/ACA RNPs, and not just the telomerase RNP, could be altered (47). However, decreased levels of other small RNAs or alterations in their pseudouridylation in X-linked DC patient cells have not typically been found (5,14,18,48). Nonetheless, to date, none of the DC mutations in dyskerin SUMOylation consensus sites have been characterized for their interactions with hTR or dyskerin-interacting proteins, nor have patient cells harboring these mutations been examined for defects in levels or modifications of other small RNAs.

Our study provides one of only a few examples of human disease-causing mutations occurring within a SUMOylation consensus sequence (33,49). Recent genome-wide linkage analysis among DC individuals implicated the DKCI locus in the absence of DKCI mutations. Affected family members showed reduced levels of dyskerin protein to 50–72% of the normal level and reduced levels of hTR, implying that dyskerin protein levels can alone be responsible for DC. Accordingly, the major impact of SUMO3 on dyskerin stability in our study strongly suggests that SUMOylation is required for maintaining proper amounts of dyskerin in the cell, and that impairment in dyskerin stability can lead to the phenotypes of DC. Overall, our study advances our understanding of how dyskerin is regulated by SUMOylation, and importantly might lead to new treatments for DC.

MATERIALS AND METHODS

Cell culture and plasmids

Human embryonic kidney HEK293 cells were maintained in minimum essential medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), penicillin and streptomycin.

The plasmid pCDNA3.1-FLAG-dyskerinWT was obtained from François Dragon (Université du Québec à Montréal). Dyskerin point mutations or deletions were created in pCDNA3.1-FLAG-dyskerin using site-directed mutagenesis and specific primers. The constructs pCDNA3.1-6His-SUMO1 and pCDNA3.1-6His-SUMO3 were obtained from Frédéric Antoine Mallette (Université de Montréal). The SUMO-dyskerinK39R fusion construct was made by inserting SUMO3 (amplified with appropriate primers from pCDNA3.1-6His-SUMO3) in frame between the FLAG-tag and dyskerinK39R in pCDNA3.1-FLAG-dyskerinK39R.

RNA interference and rescue experiment

For dyskerin knockdown, 293T cells were co-transfected with a DKCI 3’UTR-specific shRNA-plKO.1 plasmid construct (TRCN0000039738, Open Biosystems) or a negative control scrambled shRNA-plKO.1 plasmid construct (plasmid 1864, Addgene) and the packaging plKO.1 constructs pMD2.G (plasmids 12259, Addgene) and psPAX2 (plasmid 12260, Addgene) using FuGENE HD (Roche). The supernatant containing lentiviruses was collected, filtered through a 0.45 μm filter and stored in aliquots at −80°C or immediately used to infect HEK293 cells in the presence of 8 μg/ml hexadimethrine bromide (Millipore). Twenty-four hours after infection, stable cells were selected using 1 μg/ml puromycin (Gibco). Seven days later, stable dyskerin knockdown cells were transfected with rescue pCDNA3.1-FLAG-dyskerin variants using FuGene HD (Roche). Stable rescue cells were selected 24 h after transfection with 500 μg/ml G418 for 14 days. Total RNA was isolated and positive clones were screened using PCR and primers specific for exogenous DKCI (F:5’-TGCCACCATGACTACAAGAGACGA-3’ and R:5’-GCAGCGGGACACTCCACAACCCCA-3’).

Nickel affinity purification, immunoblots and protein stability assay

For nickel affinity purification of His-SUMO modified dyskerin, HEK293 cells were transiently transfected with plasmids encoding FLAG-dyskerinWT or the FLAG-dyskerin variants: L37del, K39R, K43R and a 6His-SUMO1-encoding plasmid. Nickel affinity purification, immunoblots and protein stability assays were performed as previously described (50). Input and eluted proteins were resolved by SDS–PAGE, transferred to nitrocellulose and immunoblotted with an antibody against FLAG (Sigma M2) or His (Clonetech, #631212). For protein stability assays,
CHX (Sigma-Aldrich, C7698) at a final concentration of 100 µg/ml was used for the indicated times. Cells were harvested in Laemmlı buffer, immublloted by SDS–PAGE, transferred to nitrocellulose and immublloted with a dyskerin antibody (H-300, Santa Cruz). Cell fractions were immublloted with SUMO1 (Santa Cruz) or SUMO2/3 antibodies (Zymed). Equal loading of proteins analyzed by Western blot analysis was confirmed by immublloting with a tubulin antibody (Sigma).

**In vitro SUMOylation and telomerase assays**

35S-Met-labeled FLAG-dyskerin variants were obtained by *in vitro* transcription/translation using the T7-coupled transcription/translation rabbit reticulocyte lysate (RLL) system (Promega) in the presence of [35S]methionine. *In vitro* SUMOylation reactions were carried out as previously described using an E1 enzyme (120 ng SAE1/SAE2, Alexis Biochemicals), E2 enzyme (720 ng Ubc9, Alexis Biochemicals), SUMO-1 or SUMO-2 protein (500 ng, Enzo Life Sciences) and 1 µl 35S-Met-labeled RRL substrate in SUMOylation buffer [50 mM Tris–HCl pH 7.5, 5 mM MgCl2, 5 mM dithiothreitol (DTT) and protease inhibitors] in an ATP-regenerating system [2 mM ATP (Sigma), 10 mM creatine phosphate (Sigma), 3.5 U/ml creatine kinase (Sigma) and 0.6 U/ml inorganic pyrophosphatase (Sigma)] and incubated for 6 h at 37 °C before separation on a NuPAGE Novex 4–12% Bis-Tris gel (Invitrogen). Gels were dried on a gel dryer (Biorad) for 2 h and exposed to a phosphorimagor (Storm 840, GE Healthcare Life Science). Telomere repeat amplification protocol (TRAP) assays were performed as previously described using cell extracts (51).

**Q-FISH**

Metaphase spreads and Q-FISH analyses were performed as described (52). Metaphase spreads from colcemid-arrested cells were prepared according to the standard cytogenetic methods. Cells were fixed in 4% formaldehyde in phosphate buffer-furred saline (PBS) for 2 min, then washed three times in 1 X PBS for 5 min. Slides were treated in a preheated solution of 1 mg/ml pepsin in 10 mM glycine (pH 2.0) for 10 min at 37°C, washed twice in 1 X PBS for 2 min and then subjected to a second round of formaldehyde fixation and 1 X PBS washes. After dehydration through an ethanol series, cells were hybridized with a Zeiss M1 fluorescence microscope (100 x). Telomere lengths were measured with the TFL-Telo v2.0 software kindly provided by Peter Lansdorp (British Columbia Cancer Center, Vancouver, Canada).

**Real-time PCR**

Total RNA was prepared using the RNeasy kit (QIAGEN) according to the manufacturer’s protocol. Complementary DNA was obtained from reverse transcription of 1 µg total RNA using SuperScript II (Invitrogen). Real-time qPCR analyses were conducted using a Power SYBR Green master mix (ABI) in 7500 fast real-time PCR system (ABI) for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. *hTR* (F3B: 5′-TCTAACCCTAAGTGACTGAGGGGC G TAG-3′ R3C: 5′-GGTTTGCCTGACTACAAAGAGCAGGA G-3′). Data were analyzed using the 7500 analysis software v2.0.4 (ABI) and the comparative ΔΔCT method. Exogenous expression of mRNA encoding FLAG-dyskerin WT and variants was also quantified by a real-time qPCR with the following primers: F:5′-TGCCACCATGAGACTCACAAGAGCAGG-3′ and R-DKC1qPCR: 5′-CCACGTGACGTTCCAATT-3′.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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