SUMOylation- and GAR1-Dependent Regulation of Dyskerin Nuclear and Subnuclear Localization

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ABSTRACT The nuclear and subnuclear compartmentalization of the telomerase-associated protein and H/ACA ribonucleoprotein component dyskerin is an important although incompletely understood aspect of H/ACA ribonucleoprotein function. Four SUMOylation sites were previously identified in the C-terminal nuclear/nucleolar localization signal (N/NoLS) of dyskerin. We found that a cytoplasmic localized C-terminal truncation variant of dyskerin lacking most of the C-terminal N/NoLS represents an under-SUMOylated variant of dyskerin compared to wild-type dyskerin. We demonstrate that mimicking constitutive SUMOylation of dyskerin using a SUMO3 fusion construct can drive nuclear accumulation of this variant and that the SUMO site K467 in this N/NoLS is particularly important for the subnuclear localization of dyskerin to the nucleolus in a mature H/ACA complex assembly- and SUMO-dependent manner. We also characterize a novel SUMO-interacting motif in the mature H/ACA complex component GAR1 that mediates the interaction between dyskerin and GAR1. Mislocalization of dyskerin, either in the cytoplasm or excluded from the nucleolus, disrupts dyskerin function and leads to reduced interaction of dyskerin with the telomerase RNA. These data indicate a role for dyskerin C-terminal N/NoLS SUMOylation in regulating the nuclear and subnuclear localization of dyskerin, which is essential for dyskerin function as both a telomerase-associated protein and as an H/ACA ribonucleoprotein.

KEYWORDS GAR1, SUMOylation, dyskerin, nuclear localization, subnuclear localization, telomerase

The H/ACA ribonucleoprotein (RNP) complex is responsible for pseudouridine synthesis at specific bases in ribosomal RNA (rRNA) and small nuclear RNA (snRNA) in subnuclear compartments, specifically the nucleolus and Cajal bodies (CBs), respectively (1–5). The protein components of this complex at maturity are dyskerin (6–8), NOP10, NHP2 (9), and GAR1 (1, 10). The mature H/ACA complex assembles with non-coding RNA (ncRNA) members of the H/ACA family, such as small nucleolar RNAs (snoRNAs) and small Cajal body-specific RNAs (scaRNAs) that provide target pseudouridine synthesis specificity to dyskerin, the pseudouridine synthase of the H/ACA complex. The H/ACA motif is also a conserved biogenesis domain in telomerase RNAs of metazoans (11), including the human telomerase RNA (hTR), which relies on the H/ACA complex proteins for stability, processing, and function (12–16).

While hTR has no known target for guiding pseudouridine synthesis by dyskerin, the importance of the H/ACA complex in hTR biogenesis is demonstrated by mutations causing the premature aging disease and telomere syndrome dyskeratosis congenita (DC), with reported patient mutations in the genes encoding each protein component

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of the mature complex, excluding GAR1, as well as in the H/ACA biogenesis domain of hTR itself (17). Patients with DC have characteristic accelerated telomere shortening, which leads to pathology in proliferative tissues and results in bone marrow failure as the leading cause of mortality in this disease (18–21). In particular, DC patients with mutations disrupting the H/ACA complex components or H/ACA domain of hTR have reduced hTR accumulation, which drives telomerase activity defects and accelerated telomere shortening (12). There have also been several reports of DC mutations in the H/ACA complex components affecting H/ACA RNA biogenesis beyond hTR (22, 23), and the essentiality of dyskerin and the H/ACA complex is also likely due to their importance in rRNA and snRNA posttranscriptional modification. The *dkc1* gene encoding dyskerin is a core essential gene that is highly conserved, with phylogenetic roots in bacteria and archaea. Knockout of this gene is lethal in fungi (6), flies (24, 25), mice (26), and human cells (27, 28). Although X-linked DC (X-DC) is a commonly inherited form of the disease caused by mutations in *dkc1*, a complete deletion or loss of the gene has never been reported in X-DC, further demonstrating the essentiality of dyskerin.

The compartmentalization of dyskerin and the H/ACA complex is an important though incompletely understood aspect of H/ACA RNP function. Dyskerin has been reported to rely on two nuclear/nucleolar localization sequences (N/NoLSs) for complete nuclear import and retention, as well as for nucleolar accumulation (29). With the exception of GAR1, the H/ACA RNP components are present at sites of transcription of H/ACA RNAs in the nucleoplasm, along with the assembly factor NAF1, which is replaced by GAR1 upon complex maturation (30–32). Mature H/ACA complexes localize in the dense fibrillar component (DFC) of the nucleolus and in the Cajal bodies (7), where they guide posttranscriptional modification of rRNA and snRNA, respectively, dependent upon the H/ACA RNA with which the complex is assembled. The stepwise assembly of H/ACA RNPs has been proposed to play a role in localization of the complex to its sites of function (32). Although the mechanism governing subnuclear compartmentalization of the mature H/ACA complex remains incompletely characterized, it is likely to rely on regulation of miscibility with these discrete membrane-free regions of the nucleus. This has been recently demonstrated for other nucleolar proteins resident in the DFC such as fibrillarin, which relies on an intrinsically disordered glycine- and arginine-rich (GAR) domain and RNA interactions for miscibility with the DFC (33, 34).

The posttranslational modification SUMOylation has been demonstrated to affect nuclear and subnuclear localization of a number of protein targets, including resident proteins of the nucleolus (35–37). This modification involves conjugation of small ubiquitin-like modifier (SUMO) protein to lysine residues of target proteins in an E1-activating (SAE1/SAE2) and E2-conjugating (Ubc9) enzyme-dependent manner, often with the help of one of many E3 SUMO ligases, and promoted by a SUMOylation consensus motif in target proteins (ψ KXE/D, where ψ is a hydrophobic residue and X is any residue) (38). Although SUMOylation has been reported to regulate various functions of target proteins, a key aspect of SUMOylation is mediating protein-protein interactions between SUMO targets and proteins containing SUMO-interacting motifs (SIMs) which noncovalently bind SUMO (39–41). SUMOylation is a reversible modification, with several identified SUMO-specific proteases cleaving immediately after the C-terminal diglycine repeat in SUMO moieties and therefore being responsible both for maturation of free SUMO and for removal of SUMO from target lysines (42–45). Typically, at steady state, only a small proportion of a SUMO target is conjugated to SUMO moieties. We previously demonstrated that dyskerin is a SUMOylation target of SUMO1 and SUMO2/3 isoforms and that substituting either of two N-terminal X-DC-implicated lysine residues to arginine reduces the proportion of SUMOylated dyskerin in cells, leading to reductions in hTR, reduced telomerase activity, and accelerated telomere shortening (46). We have since shown that these two X-DC residues impact the

dyskerin-hTR interaction, although the SUMO dependence of this interaction was not investigated (16).

Here, we further investigate a regulatory role for SUMOylation of dyskerin. Using mutational analyses and SUMO fusion constructs, we demonstrate that the C-terminal N/NoLS of dyskerin is a SUMO3 target and that mimicking constitutive SUMOylation of a cytoplasmic truncation variant of dyskerin is sufficient to drive nuclear accumulation but not proper subnuclear localization of dyskerin. We also demonstrate that the nucleolar localization of dyskerin is mediated by the SUMO3 site K467 in this C-terminal N/NoLS and that K467 is required for the interaction between dyskerin and GAR1 in a SUMO3-dependent manner, and we identify here a SIM in GAR1 that is important for this interaction.

RESULTS

The C-terminal nuclear/nucleolar localization sequence of dyskerin is a SUMOylation target. Many proteome-wide studies performed in human cell lines have identified dyskerin as a target of SUMOylation, both by SUMO1 and by SUMO2/3 (47–55). In compiling the results of these studies, it is evident that dyskerin is a highly decorated target for SUMOylation, with 24 sites identified by mass spectrometry (MS) analyses (Fig. 1A). For the purpose of this study, we focused on four SUMO2/3 sites in particular due to the placement of these lysines in the C-terminal N/NoLS (K467, K468, K498, and K507), which was previously reported to mediate efficient localization of dyskerin to the nucleus alone and in combination with an N-terminal N/NoLS (29) and consists of a lysine/glutamine rich (K/E-rich) tail conserved in eukaryotes (Fig. 1A). Importantly, truncation of the C-terminal N/NoLS by replacing K446 with a stop codon (X), and thus removal of all four SUMO3 sites and the lysine-rich (K-rich) clusters in which they are situated, substantially reduces the amount of SUMOylated dyskerin detectable by immunoblotting following Ni-nitrilotriacetic acid (NTA) purification from HEK293 cells expressing FLAG-tagged dyskerin and 6×His-SUMO3 (Fig. 1B, wild type versus K446X). Indeed, while FLAG-tagged wild-type dyskerin colocalizes with the nucleolar marker fibrillarin in HEK293 cells assessed by immunofluorescence (IF), the FLAG-tagged K446X accumulates predominantly in the cytoplasm (Fig. 1C, top and middle panels). Interestingly, mimicking constitutive SUMOylation of K446X by fusing a SUMO3 moiety to the N terminus of this dyskerin variant allows for detection of high-molecular-weight products by Ni-NTA from HEK293 cells coexpressing FLAG-tagged SUMO3-K446X and 6×His-SUMO3, indicating that this fusion protein is highly SUMOylated (Fig. 1B). This SUMO3 fusion is also sufficient to drive the K446X truncation variant into the nucleus (Fig. 1C, bottom two panels). However, the SUMO3 fusion variant remains excluded from the nucleolar compartment, suggesting that mimicking permanent SUMOylation of dyskerin disrupts proper subnuclear localization. This hypothesis is supported by our observation that fusion of SUMO3 to either the N terminus or the C terminus of FLAG-tagged wild-type (WT) dyskerin also leads to disrupted subnuclear localization (Fig. 2A). SUMO3-K446X and SUMO3-wild-type dyskerin form distinct puncta in the nucleoplasm (Fig. 1C and Fig. 2A). We examined whether some of these puncta may represent CBs given the expected localization of WT dyskerin to CBs and the observed occasional overlap of these puncta with fibrillarin puncta outside of nucleolar clusters (Fig. 1C, arrowheads). When assessed for colocalization with the CB marker coilin or the CB and nuclear gem marker SMN by IF, the localization of nucleoplasmic puncta of SUMO3 fusions (WT and K446X) to CBs or nuclear gems was impaired (Fig. 2B and Fig. 2C and D, arrows) compared to unfused WT dyskerin (Fig. 2B and Fig. 2C and D, arrowheads). These data suggest that the C-terminal N/NoLS of dyskerin regulates nuclear localization in a SUMO3-dependent manner, though the reversibility of SUMOylation after nuclear import is likely important for mediating proper subnuclear trafficking of dyskerin. We speculate that the dyskerin puncta formed by SUMO3 fusion are most likely nucleoplasmic aggregates driven by the permanent nature of the SUMO3 fusion. This would be consistent with a previous proposal that “balanced SUMOylation levels” may be required for nucleolar regulation (56).

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FIG 2 Permanent SUMO3 fusion disrupts dyskerin subnuclear localization and causes dyskerin nucleoplasmic aggregate formation. (A) Localization of FLAG-tagged WT, N-terminal SUMO3 fusion WT dyskerin (SUMO3-WT), and C-terminal SUMO3 fusion WT dyskerin (WT-SUMO3) was assessed by indirect immunofluorescence (Cy3 is shown in red). Fibrillarin was used as a nucleolar marker (FITC shown in green), and the nucleus is indicated by DAPI staining of nuclear DNA (in blue). (B) Colocalization of FLAG-tagged WT dyskerin, N-terminal SUMO3 fusion WT dyskerin (SUMO3-WT), and N-terminal SUMO3 fusion K446X dyskerin variant (SUMO3-K446X) with the Cajal body marker coilin or nuclear gem marker SMN was assessed by indirect immunofluorescence in HEK293 cells transiently expressing FLAG-tagged dyskerin. The percentage of nuclei with ≥ 1 colocalization focus was scored for each FLAG-tagged dyskerin protein. (C) Representative images of the quantification in panel B for dyskerin and coilin colocalization. (D) Representative images of the quantification in panel B for dyskerin and SMN colocalization. Nucleoplasmic FLAG-dyskerin foci that colocalize with nucleoplasmic compartment markers are indicated by arrowheads, while arrows indicate nucleoplasmic FLAG-dyskerin foci that do not colocalize with nucleoplasmic compartment markers. All scale bars indicate 10 μm .

lysine residues, a stop codon was introduced at A481 in the FLAG-dyskerin construct, thus removing the entire second K-rich cluster while leaving the first intact. In contrast to the K446X variant, FLAG-tagged A481X efficiently localizes in the nucleus and the nucleolus, as observed by colocalization with fibrillarin assessed by IF (Fig. 3A). This suggests that the second K-rich cluster, thus the SUMO3 sites within it are not critical regulators of dyskerin nuclear localization *per se*, and these data are consistent with previous localization analysis of a truncation variant at D493 (29). However, full-length FLAG-tagged dyskerin in which the SUMO3 site K467 in the first K-rich cluster is substituted to an arginine (K467R) displays an apparent nucleolar exclusion/nucleoplasmic accumulation phenotype when assessed by IF (Fig. 3A). This is in contrast to the K468R variant that localizes comparably to wild-type dyskerin (Fig. 3A). Substituting both of

FIG 3 Nuclear and subnuclear localization of dyskerin is mediated by SUMO3 sites in the C-terminal nuclear/nucleolar localization sequence. (A) FLAG-dyskerin was transiently expressed in HEK293 cells, and localization was assessed in fixed cells by indirect immunofluorescence. Representative images of the most prevalent localization phenotype of FLAG-dyskerin (wild type [WT], dyskerin truncation variant A481X, and substitution variants K467R, K468R, and double K467/468R [Cy3 is shown in red]) and the nucleolar marker fibrillarin (FITC is shown in green) are shown. Nucleolar localization is represented for WT and K468R, concomitant nucleoplasmic and nucleolar is represented by A481X, and nucleoplasmic localization is represented by K467R and K467/468R. The nucleus is indicated by DAPI staining of nuclear DNA (in blue). (B) Quantification of localization phenotype scoring for FLAG-dyskerin WT and localization variants as a percentage of FLAG-positive HEK293 cells is indicated (≥ 50 cells per condition were counted, in experiment replicates [$n=3$]). (C) Examples of how the localization phenotype was scored. Nucleolar localization was determined by colocalization with clustered fibrillarin signal, nucleoplasmic localization was determined by colocalization with DAPI outside the clustered fibrillarin signal, and cytoplasmic localization was determined by concentrated signal outside and surrounding DAPI. The FLAG-tagged dyskerin construct expressed in each representative image is labeled below each localization phenotype. (D) Localization of eGFP and eGFP-tagged WT dyskerin (in green) was assessed in fixed HEK293 cells following transient transfection, using a FITC filter.

these lysines to arginine (K467/468R) leads to a localization phenotype similar to the single K467R substitution variant (Fig. 3A). FLAG-positive cells were scored based on localization phenotype as a percentage of FLAG-positive cells counted, and localization of each FLAG-tagged dyskerin (wild type or variant) was assessed from three independent experimental replicates (Fig. 3B and C). Importantly, while the major localization phenotype of the K446X truncation variant is cytoplasmic, FLAG signal in cells expressing this variant was also observed in both the cytoplasm and nucleolar fraction concomitantly (Fig. 3B, blue bar). This is consistent with previous time course experiments demonstrating through microinjection of EGFP-tagged K446X into cells that this truncation impairs but does not entirely prevent nuclear and subnuclear localization of dyskerin (29). While still able to localize within the nucleus and to the nucleolus, the truncation variant of dyskerin at A481, A481X, does display an increase in concomitant nucleoplasmic and nucleolar localization of dyskerin compared to the wild type, suggesting that this truncation modestly affects localization of dyskerin, albeit to a lesser extent than K446X or K467R (Fig. 3B, brown bar). Indeed, K467R has a substantial reduction in nucleolar and corresponding increase in nucleoplasmic localization compared to wild-type dyskerin, as assessed by exclusion from colocalization with fibrillarin signal, but colocalization with DAPI (4',6'-diamidino-2-phenylindole) signal (Fig. 3B, red and black bars, respectively). In contrast, no substantial differences in localization were observed between K468R and the wild type (Fig. 3B). The double substitution variant K467/468R does not differ in localization compared to the single K467R variant and thus has a reduction in nucleolar and increase in nucleoplasmic localization compared to wild-type dyskerin (Fig. 3B). As previously shown (Fig. 1C), fusing K446X to SUMO3 is sufficient to drive this truncation into the nucleus, but this fusion variant has reduced nucleolar localization compared to wild-type dyskerin (Fig. 2B). However, SUMO3 fusion of dyskerin differs from K467R in nucleolar exclusion, as SUMO3-K446X and SUMO3-wild-type dyskerin form distinct aggregates in the nucleoplasm while K467R localization in the nucleoplasm is diffuse (Fig. 1C and D and Fig. 2A). Importantly, the FLAG tag does not disrupt localization of wild-type dyskerin, since eGFP-tagged wild-type dyskerin (Fig. 2D) displays localization patterns comparable to those of exogenously expressed FLAG-tagged wild-type dyskerin. Taken together, these data tell us that in addition to SUMO3 mediating nuclear localization of the K446X truncation of dyskerin, the SUMO3 site K467 plays an important regulatory role for the nucleolar localization of dyskerin.

Nuclear and subnuclear localization of dyskerin affects mature H/ACA RNP assembly. As a functional readout for H/ACA complex assembly and localization, coimmunoprecipitation (co-IP) of FLAG-tagged dyskerin and interacting components was performed from HEK293 cell lysate. Following FLAG-IP, interactions of FLAG-tagged dyskerin wild type and N/NoLS variants were assessed by immunoblotting for endogenous H/ACA RNP assembly factors and components. Comparable to wild-type dyskerin, all FLAG-tagged N/NoLS variants were able to interact with the pre-H/ACA RNP component NAF1 and the pre-H/ACA RNP and mature H/ACA RNP components NOP10 and NHP2 (Fig. 4A). Strikingly, the N/NoLS variants with nucleolar exclusion phenotypes (K467R and K467/468R) were unable to interact with the mature H/ACA RNP component GAR1 (Fig. 4B). Importantly, disruption of either nuclear or subnuclear localization of dyskerin leads to impaired hTR-dyskerin interaction as measured by qPCR following RNA extraction and reverse transcription from IP fractions; neither FLAG-tagged K467R nor K446X interacted with hTR relative to wild-type dyskerin (Fig. 4C). This is in contrast to the N/NoLS variants with little to no localization defects, K468R and A481X, which do not display defective interactions with hTR relative to wild-type dyskerin (Fig. 4C). We examined whether mislocalization of dyskerin affects the interaction of dyskerin with H/ACA RNAs other than hTR by the same method and observed that relative to wild-type dyskerin, the K467R and K446X variants also have interaction defects with H/ACA snoRNAs E2, U64, and U85 (Fig. 4D). These data indicate that proper localization of dyskerin is tied to H/ACA RNP complex assembly, connect GAR1-dyskerin interaction

FIG 4 Dyskerin nuclear and nucleolar localization is linked to mature H/ACA complex assembly and function. Interactions of FLAG-dyskerin WT and localization variants with endogenous pre-H/ACA and mature H/ACA ribonucleoprotein complex components were assessed by coimmunoprecipitation (IP) from HEK293 cell lysates. (A) Assembly of the H/ACA pre-RNP complex involving NAF1, NHP2, and NOP10 was investigated by immunoblotting for the endogenous H/ACA pre-RNP components and FLAG-dyskerin proteins following IP. (B) Interaction of dyskerin with the mature H/ACA complex component GAR1 was examined following IP by immunoblotting for endogenous GAR1 and FLAG-dyskerin. Localization variants that are excluded from the nucleolus (K467R and K467/468R) do not interact with GAR1. Immunoblotting targets are indicated to the right of each panel as "WB: α target," and a list of antibodies can be found in Materials and Methods. A nonspecific band revealed by the anti-GAR1 antibody at 25 kDa is indicated by an asterisk. Each co-IP and immunoblotting experiment was performed in experimental replicate a minimum of $n = 2$; representative blots are shown. (C) Dyskerin-hTR interactions were assessed by IP of FLAG-tagged dyskerin, followed by RNA extraction and qPCR. Relative to wild-type IP fractions, dyskerin variants with substantial localization defects (K467R and K446X) display significantly reduced enrichment of hTR following IP. HEK293 cells lacking FLAG-tagged dyskerin (indicated as mock) were used as a negative control for RNA binding to the FLAG antibody and/or protein G-Sepharose. Mock-treated cells were subjected to the same IP protocol detailed for fractions containing FLAG-tagged dyskerin. These data (Continued on next page)

defects to the nucleolar exclusion of dyskerin, and demonstrate that improper dyskerin localization disrupts the ability of dyskerin to interact with H/ACA RNAs.

Defects in dyskerin localization affect telomerase activity. We sought to determine whether H/ACA complex assembly defects disrupted dyskerin function in the context of telomerase activity. In order to assess telomerase activity, endogenous dyskerin was depleted via siRNA targeting the 3' untranslated region (UTR) of dyskerin in HEK293 cells with or without stable expression of FLAG-tagged dyskerin wild type or N/NoLS variants. After 72 h of depletion, the telomerase activity was measured in the cell lysate using Q-TRAP. HEK293 cells depleted of endogenous dyskerin have significantly reduced telomerase activity compared to untreated (mock) cells, cells treated with a scramble small interfering RNA (siRNA), and cells with stable exogenous expression of FLAG-tagged wild-type dyskerin that are depleted of endogenous dyskerin (Fig. 4E). In addition, HEK293 cells with stable expression of FLAG-tagged dyskerin localization variants that have defective localization (K467R, K467/8R, and K446X) display significantly reduced telomerase activity following depletion of endogenous dyskerin. In contrast, cells with stable expression of FLAG-tagged dyskerin localization variants that are competent for nuclear and nucleolar localization (K468R and A481X) display telomerase activity similar to cells with stable expression of FLAG-tagged wild-type dyskerin following depletion of endogenous dyskerin.

GAR1 interaction with dyskerin mediates nucleolar localization in a SUMO-dependent manner. While the dyskerin variants that are excluded from the nucleolus do not interact with GAR1, the mainly cytoplasmic K446X truncation which is competent for nucleolar localization is capable of interacting with endogenous GAR1 (Fig. 3B and Fig. 4B). Due to this observation that nucleolar localization of dyskerin is connected to the dyskerin-GAR1 interaction and the SUMO3 site K467, we sought to determine whether the interaction between GAR1 and dyskerin may be SUMO3 mediated and whether this interaction is responsible for mediating dyskerin nucleolar localization. To test this, we performed FLAG co-IPs from HEK293 cells expressing FLAG-tagged dyskerin fused to SUMO3 at the C terminus. Interestingly, fusing SUMO3 to the C terminus of the K467R variant (Fig. 5A) is able to rescue the robust GAR1 interaction defect of K467R (Fig. 4B), and wild-type dyskerin with a C-terminal SUMO3 fusion is also able to interact with GAR1 comparably to wild-type dyskerin alone (Fig. 5A). However, the subnuclear localization of both of these SUMO3 fusions does differ from that of wild-type dyskerin, as assessed by IF, consistent with a predicted requirement for SUMOylation reversibility for proper regulation of dyskerin subnuclear localization. Importantly, compared to fusion of SUMO3 to the N terminus of K446X or the nonfusion K467R variant, these C-terminal SUMO3 fusions display more colocalization with fibrillarin in the nucleolar compartment, though less frequent exclusive nucleolar localization of these fusions is observed compared to wild-type dyskerin (Fig. 3B and Fig. 5B). This suggests that the nucleolar localization of K467R, as well as interaction between GAR1 and dyskerin, may indeed be SUMO3 dependent. To further elucidate a potential SUMO3-mediated GAR1-dyskerin interaction and using the prediction software GPS-SUMO 4.0, we identified a single predicted SIM within GAR1 at residues 70 to

FIG 4 Legend (Continued)

represent experimental replicates of $n=3$. Statistically significant reductions in enrichment relative to wild type are indicated by an asterisk (*, $P < 0.01$). Error bars represent the SEM. (D) Dyskerin-H/ACA RNA interactions were assessed by IP of FLAG-tagged dyskerin followed by RNA extraction and qPCR. Relative to wild-type IP fractions, dyskerin variants with substantial localization defects (K467R and K446X) display significantly reduced enrichment of the E2, U64, and U85 H/ACA RNAs following IP. HEK293 cells lacking FLAG-tagged dyskerin (indicated as mock) were used as a negative control for RNA binding to the FLAG antibody and/or protein G-Sepharose. Mock cells were subject to the same IP protocol detailed for fractions containing FLAG-tagged dyskerin. These data represent experimental replicates of $n=3$. Statistically significant reductions in enrichment relative to the wild type are indicated by a hashtag (#, $P < 0.0001$). Error bars represent the SEM. (E) HEK293 cells without or with stable expression of FLAG-dyskerin (WT or N/NoLS variants) were depleted of endogenous dyskerin using siRNA and confirmed by immunoblotting against endogenous dyskerin. The same membrane was first probed using an antidyskerin antibody, followed by an antitubulin antibody as a loading control. Arrows indicate FLAG-tagged dyskerin, the asterisk indicates endogenous dyskerin, and the arrowhead indicates tubulin. Q-TRAP was performed using cell lysate. Statistically significant reductions in relative telomerase activity (RTA) compared to mock (untreated) HEK293 cells are indicated by an asterisk (*, $P < 0.0001$), and this was repeated in experimental triplicates. Error bars represent the SEM.

FIG 5 Efficient interaction between dyskerin and GAR1 is mediated by SUMO3. (A) Following transient transfection of FLAG-tagged dyskerin in HEK293 cells and coimmunoprecipitation (IP) from cell lysates using FLAG antibody, the interaction between endogenous GAR1 and wild-type (WT)-SUMO3 fusion, K467R-SUMO3 fusion, or WT dyskerin was assessed by immunoblotting. SUMO3 fusion variants run at the expected higher molecular weight than WT dyskerin alone due to the SUMO3 moiety. A nonspecific band revealed by the anti-GAR1 antibody at 25 kDa is indicated by an asterisk. (B) C-terminal SUMO3 fusions of the FLAG-dyskerin wild type and the K467R variant were transiently expressed in HEK293 cells, and localization was assessed in fixed cells by indirect immunofluorescence. Representative images of the most prevalent localization phenotype of FLAG-dyskerin (Cy3 is shown in red) and the nucleolar marker fibrillarin (FITC is shown in green) are shown. The nucleus is indicated by DAPI staining of nuclear DNA (in blue). Quantification of localization phenotype scoring as a percentage of FLAG-positive HEK293 cells is indicated (≥ 50 cells per condition were counted, in experiment replicate $n=3$), and scale bars indicate $10\mu\text{m}$. (C) A linear schematic of human GAR1, with glycine- and arginine-rich domains indicated (RGG-1 and RGG-2). The predicted SIM (70-VVLLG-74) is indicated, and residues expected to physically interface with dyskerin based on previous homologue structural studies in yeast are indicated in boldface and italicized. (D) Substitution of all five predicted SIM residues to alanine (GAR1 5A) impairs the interaction between GAR1 and dyskerin, as demonstrated by co-IP of 3 $\times$ FLAG-GAR1 and endogenous dyskerin from HEK293 cells transiently expressing 3 $\times$ FLAG-GAR1. Interaction between endogenous dyskerin and WT or 5A GAR1 was assessed by immunoblotting following FLAG IP from cell lysates. Immunoblotting targets are indicated to the right of each panel as "WB: α target." and a list of antibodies can be found in Materials and Methods. Each co-IP and immunoblotting experiment was performed in experimental replicates with a minimum of $n=2$; representative blots are shown.

74 (70-VLLG-74) proximal to the previously predicted dyskerin-GAR1 interface (Fig. 5C). In order to assess whether this predicted SIM could mediate the interaction between GAR1 and dyskerin, we substituted each residue in the predicted SIM to alanine in a 3×FLAG-tagged GAR1 construct (annotated as “5A”) and assessed the interaction of 3×FLAG-tagged GAR1 with endogenous dyskerin. Wild-type 3×FLAG-tagged GAR1 is able to interact with endogenous dyskerin, as assessed by FLAG co-IP from HEK293 cells expressing 3×FLAG-GAR1; however, GAR1 5A displays a reduced interaction with endogenous dyskerin (Fig. 5D). These data demonstrate that GAR1 contains a SIM which mediates the efficient interaction between dyskerin and GAR1 in a SUMO3-dependent manner, relying on the SUMO3 site K467 in the C-terminal N/NoLS of dyskerin, and that this interaction with GAR1 governs the localization of dyskerin in the nucleolus.

DISCUSSION

Dyskerin and the H/ACA RNP complex play essential roles in H/ACA RNA biogenesis, posttranscriptional modification of rRNA and snRNA, and human telomerase assembly and activity. The ability of dyskerin to carry out its various functions relies heavily on its nuclear and subnuclear compartmentalization, where it assembles with H/ACA RNA and localizes to sites of function, including the nucleolus, where pseudouridine synthesis occurs on rRNA. In this study, we further demonstrate the interconnectedness of dyskerin localization, H/ACA RNP assembly, and complex function. More specifically, we demonstrate that efficient nuclear localization of dyskerin, driven by the K-rich C-terminal N/NoLS, and mature H/ACA complex assembly and nucleolar localization mediated by K467 in this N/NoLS are crucial for dyskerin assembly with H/ACA RNA, including hTR. Furthermore, we demonstrate that the localization of dyskerin can be mediated by SUMOylation sites in the C-terminal N/NoLS.

A previous study of dyskerin nuclear localization characterized two N/NoLS regions, one in the N terminus (amino acids 11 to 20) and one in the C terminus (amino acids 446 to 514) (29). This foundational study reported that removing or mutating the N-terminal region alone did not disrupt localization of dyskerin, whereas removal of the C-terminal region alone drastically impeded nuclear localization, and combinatorial removal of both regions abolished nuclear localization altogether. As such, we focused on this C-terminal N/NoLS region as the primary driver of dyskerin nuclear localization. Strikingly, we found that efficient localization of dyskerin to the nucleus, while impaired by truncation of the C-terminal N/NoLS at K446, can be driven by mimicking SUMOylation through fusing dyskerin to a SUMO3 moiety. This suggests that the loss of SUMOylation sites from this truncation variant may be responsible for inefficient dyskerin nuclear import and/or retention. Given the multitude of dyskerin SUMOylation sites identified by MS which fall outside the C-terminal N/NoLS of dyskerin, it is also likely that dyskerin SUMOylation takes place within the nucleus for some sites following nuclear import. Establishing which SUMOylation sites in particular govern nuclear localization requires further investigation, since pinpointing the MS-identified SUMOylation sites in this region responsible for nuclear localization was not evident by removal or substitution of K467, K468, K498, or K507. However, this mutational analysis instead revealed that K467 plays an important regulatory role in subnuclear localization of dyskerin to the nucleoli. Importantly, in our study and in a previous work by Heiss et al. (29), full truncation of the C-terminal region does not prevent nucleolar localization of dyskerin *per se*. As such, we postulate that the C-terminal N/NoLS may govern several aspects of stepwise dyskerin localization (nuclear import, nucleoplasmic assembly with H/ACA RNA, and nucleolar miscibility) through regulated conformational changes. More specifically, we speculate that a conformational change of this C-terminal region governed by SUMOylation at K467 may be responsible for licensing dyskerin nucleolar localization. However, the absence of this C-terminal region as a whole allows for dyskerin nucleolar localization in the absence of K467 SUMOylation, albeit in the context of inefficient nuclear localization, because no

conformational change is required for the K446X truncation variant. This would be consistent with reports that full-length dyskerin and dyskerin homologues are difficult to purify *in vitro* due to insolubility issues, which can be resolved by the removal of this C-terminal region (32, 57), and that are also in agreement with a lack of reported structure of this functionally required tail due to its apparent intrinsic low complexity (6, 58, 59). It also seems likely that a conformational change in dyskerin may be responsible for regulating the exchange of GAR1 for NAF1 upon H/ACA complex maturation, although this requires further investigation.

Our observations provide novel insight into potential mechanisms regulating the temporal mutually exclusive interaction of dyskerin with NAF1 or GAR1, since this mechanism has not been fully elucidated to date. Indeed, though both the K467R variant and K446X variant display defects in H/ACA RNA interactions, unlike the K467R variant, K446X is competent for nucleolar localization and interacting with GAR1. This suggests that the K446X variant forms a functional protein-based H/ACA complex but is unable to co-IP H/ACA RNAs relative to wild-type dyskerin due to the prominent cytoplasmic localization of K446X. In contrast, the K467R variant has a striking GAR1 interaction defect and is excluded from the nucleolus completely. Therefore, though both K446X and K467R variants are defective at interacting with H/ACA RNAs, we propose that the explanation for this defect is different for these two mislocalization variants. These observations deviate from the current hypothesis in the field of a handover model wherein dyskerin interacts with H/ACA RNAs as part of the precomplex involving NAF1, which is then replaced by GAR1 to form a mature protein and H/ACA RNA complex solely after the dyskerin-RNA interaction is established (32). A protein-only H/ACA complex involving dyskerin, NOP10, NHP2, and GAR1 has been observed *in vitro* in the absence of an H/ACA RNA, and functional H/ACA RNPs can assemble in cytosolic extract if exogenous H/ACA RNA is added to the cytosolic extract (60). This precedent, along with the observations reported here, suggests that mature protein-only H/ACA complexes can assemble and remain assembled. However, the assembly of such complexes *in vivo* under normal conditions seems unlikely given that H/ACA RNAs do not typically undergo nucleocytoplasmic shuttling during biogenesis (61) and given the functional consequences of defective H/ACA complex components evidenced by mutations that cause the premature aging disease DC (17).

Meanwhile, subnuclear localization of dyskerin-SUMO3 fusion proteins, either variant or wild type, was observed to differ from wild-type dyskerin alone. We postulate that the constitutive nature of this SUMOylation mimic disrupts nucleolar localization due to the inability of deSUMOylating proteases to reverse this imitated posttranslational modification. This proposal is based on not only the abundance of nucleolar SUMO targets and SUMOylation machinery involvement in nucleolar integrity (62–66) but also on the nucleolar localization of SUMO-specific proteases (SEN3 and SEN5) involved in deconjugation of SUMO2/3 from target proteins (67). SEN3 in particular has been demonstrated to interact with the nucleolar resident protein nucleophosmin (NPM1), the 60S maturation factors PELP1, TEX10, WDR18, and Las1L and is capable of deSUMOylating NPM1, PELP1, and Las1L (68–70). Consistent with the hypothesis that SUMO removal may regulate nucleolar localization of SUMO target proteins, depletion of SEN3, and thus reduction of nucleolar deSUMOylation, has been reported to lead to nucleolar release of the PELP1-TEX10-WDR18 complex (70). Furthermore, in yeast the nucleolar SUMO-specific protease Ulp2 has been demonstrated to reverse SUMOylation of rDNA-bound SUMO targets, and engineered increased SUMOylation by depletion of Ulp2 leads to a reduction of several nucleolar proteins bound to rDNA (71, 72). Intriguingly, NPM1 is responsible for localization of SEN3 to the nucleolus (73). NPM1 is a resident protein of the outermost nucleolar component where ribosomal subunit maturation takes place, the granular component, and as such would make a good candidate for gatekeeping localization of nucleolar proteins in a SUMO removal-dependent manner. This remains uninvestigated but would also fit into models of phase-mediated nucleolar compartmentalization (33, 74), as discussed in greater detail below.

It is important to note that there have been no reports of DC patients harboring mutations in the gene encoding GAR1, and most X-DC mutations in *dkc1* fall in the N-terminal or PUA regions of dyskerin, upstream of the C-terminal tail we examine here (17). However, two X-DC mutations have been reported in the C-terminal tail: a point mutation that causes a single amino acid substitution (G486R) and a frameshift mutation that truncates the second portion of the C terminus, beginning at D493. It is not known whether these mutations affect the localization of dyskerin. Given that these mutations would affect the dyskerin protein downstream of the A481X truncation, and the observation here that the A481X variant is competent for nuclear and nucleolar localization as well as mature H/ACA RNP complex assembly, it is tempting to speculate that these X-DC mutations are unrelated to dyskerin localization, but this would require further examination.

Interestingly, though the K/E-rich C-terminal tail of dyskerin is evolutionarily conserved and similar tails have been reported to contain SUMOylation sites for other nucleolar proteins such as NOP58 of the C/D RNP complex (75), this SUMO-dependent regulation of nucleolar localization observed for dyskerin is not likely a generalizable mechanism. For instance, the SUMO sites in the K/E-rich tail of NOP58 were previously demonstrated to be dispensable for nuclear and nucleolar localization of NOP58. These sites instead are required for regulating the localization of C/D box snoRNAs (76). Similarly, it is possible that the dyskerin N/NoLS variants assessed here with defects in H/ACA RNA interaction could lead to mislocalization of H/ACA RNAs. There is a precedent for defective dyskerin-hTR interaction causing mislocalization of hTR; Shukla et al. observed that disrupting this interaction through depletion of dyskerin or mutating hTR leads to reduced hTR levels and mislocalized cytoplasmic hTR foci termed CyTER (77). Whether this is also caused by dyskerin mislocalization requires further investigation.

Here, we also report that the efficient interaction between dyskerin and GAR1 is mediated through a newly characterized SIM in GAR1 (amino acids 70-VLLG-74). SIMs are typically short hydrophobic stretches of residues that can form an extended β -strand backbone, which then noncovalently interacts with SUMO moieties to foster stronger or more frequent SUMO-mediated protein-protein interactions (38). It is important to note that this predicted motif is not well conserved in lower eukaryotes or archaea (59). We found that substituting all five of these GAR1 residues to alanine impairs the interaction of GAR1 with endogenous dyskerin, indicating that an efficient interaction between GAR1 and dyskerin relies on this SIM, which is proximal to but does not overlap any of the residues structurally identified previously to mediate the interaction between these two proteins in yeast and archaea (59, 78). Anecdotally, this SUMO-mediated interaction between GAR1 and dyskerin may also offer some explanation for the reported difficulty of *in vitro* reconstitution of H/ACA complexes using full-length proteins, and indeed the GAR1-dyskerin interface that has been identified structurally using homologues from other organisms does not account for the C-terminal N/NoLS of human dyskerin since this region was absent from the dyskerin homologues used for crystallization (58, 59, 79, 80). These structural data also indicate that the GAR1-dyskerin interaction does take place in the absence of SUMOylation and without the dyskerin C-terminal N/NoLS *in vitro*, indicating that while this GAR1 SIM contributes to the efficient interaction between dyskerin and GAR1 in a cellular context, this SIM is not required *per se*. We also observed that substituting the dyskerin SUMO3 site K467 to arginine abolishes the interaction between GAR1 and dyskerin and that this GAR1 interaction defect of the K467R variant can be rescued by fusing K467R to SUMO3. It is not known whether K467 directly interfaces with GAR1 due to the absence of data on this C-terminal region of dyskerin from structural studies. However, the observation that fusion of K467R to a SUMO3 moiety can recover the ability of this variant to interact with GAR1 strongly implies that SUMOylation of K467 mediates the efficient interaction between GAR1 and dyskerin.

Finally, we postulate that the SUMO-mediated interaction between GAR1 and dyskerin is required for dyskerin localization to the nucleolus. Along with the data we present here, this hypothesis is rooted in recent analyses of the nucleolar resident protein

fibrillarin. Fibrillarin is a small nucleolar counterpart to dyskerin responsible for the 2'-O-methylation posttranscriptional modification of rRNA in the DFC, guided by C/D box snoRNA rather than H/ACA box snoRNA; for each of these there are hundreds in a cell (81–85). Several studies have demonstrated that localization of fibrillarin to the DFC is mediated by an intrinsically disordered GAR domain, as well as by interactions with nascent pre-rRNA as the RNA is sorted radially from its site of transcription through the three nucleolar components, of which the DFC is the center (33, 34). These studies and others have shown that the nucleolus represents a complex membrane-free compartment with three distinctly liquid-liquid phase separated components, which as a whole are phase separated from the surrounding nucleoplasm (33, 74, 86–88). This context is important to bear in mind when considering dynamic localization of resident nucleolar proteins in and out of these separated phases. The regulated miscibility of fibrillarin with the DFC relies on its GAR domain and protein-RNA interactions. As such, we propose that dyskerin miscibility with the DFC relies on its interaction with GAR1, not only through acting as a GAR domain for dyskerin and the entire H/ACA complex in *trans* but also by providing high H/ACA complex-to-guide RNA affinity which facilitates accurate H/ACA complex placement on target RNA, like rRNA in the nucleolus (89, 90). This hypothesis is supported by our observations that (i) the K467R dyskerin variant is unable to interact with GAR1 and the H/ACA box RNAs, (ii) this K467R variant is unable to colocalize with fibrillarin in the nucleolus, and (iii) improving the interaction between the K467R variant and GAR1 by fusing K467R to SUMO3 also allows for partial colocalization of the K467R variant with fibrillarin in the nucleolus. Furthermore, the ability of the nucleolar-miscible K446X truncation to fully assemble with H/ACA pre-RNP and mature RNP components, including interacting with GAR1, also lends support to this hypothesis. However, we cannot exclude the possibility that the exogenous overexpression of FLAG-tagged K446X dyskerin masks a more subtle GAR1-dyskerin interaction defect than that observed for the K467R variant. We also speculate that the lack of GAR domains in the archaeal homologues of GAR1 and fibrillarin provides evolutionary support for the notion that GAR domains mediate membrane-free compartmentalization of these complexes in eukaryotes, since archaea lack nuclear compartmentalization altogether and would have no need for GAR domain-mediated nucleolar miscibility of the otherwise evolutionarily conserved H/ACA or C/D RNP complexes (91). Further confirmation of the phase dynamics of human dyskerin with or without GAR1 is needed to elucidate this hypothesis.

MATERIALS AND METHODS

Plasmids, cell culture, and transfections. The plasmid pcDNA3.1-FLAG-dyskerin^{WT} from the lab of François Dragon was used to generate point mutations or truncations via site-directed mutagenesis, as previously described (16, 46). Specifically, primers were designed to generate K467R, K468R, K467/468R, A481X, and K446X. For expression of 3×FLAG-GAR1 in human cells, the pcDNA3.1 3×F-GAR1 plasmid was purchased from Addgene (catalog no. 126873), and the predicted SIM 70-VLLG-74 was substituted to 70-AAAAA-74 by site directed mutagenesis. The construct pcDNA3.1-6×His-SUMO3 was obtained from Frédéric Antoine Mallette (Université de Montréal). The plasmid encoding eGFP-tagged dyskerin (pmEGFP-C1-DKC1) was a gift from the lab of Ling-Ling Chen (Shanghai Institute of Biochemistry and Cell Biology) (34). All constructs underwent Sanger DNA sequencing at Génome Québec CES.

Human embryonic kidney (HEK293) cells were maintained in Dulbecco modified Eagle medium (DMEM; Wisent) supplemented with 10% fetal bovine serum (FBS; Wisent), and antibiotic-antimycotic (Gibco) at 37°C and 5% CO₂. Polyclonal FLAG-dyskerin stable cells were maintained under selective pressure in G418 (750 µg/ml). Transfection of pmEGFP-C1-DKC1, pcDNA3.1-FLAG-dyskerin constructs, pcDNA3.1-6×His-SUMO3, and/or pcDNA3.1 3×FLAG-GAR1 was performed using Lipofectamine 2000 transfection reagent (Invitrogen) according to the reagent protocol. Prior to transfection, the medium was changed to DMEM with 10% FBS and lacking antibiotic-antimycotic, and 5 h after transfection the medium was replaced with DMEM containing both FBS and antibiotic-antimycotic.

Transfection of siRNA was performed with Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's user protocol. siRNA targeting the 3' UTR (24 nM, 72-h treatment, *sidkc1*) was used for depletion of endogenous dyskerin (CCUCAAAGCUUGUGUACAGdTdT). The siRNA sequence targeting the 3' UTR was previously described (92). A mock transfection (no siRNA) and transfection of a scramble siRNA (GAGCGAGGGCGACUUAACCUUAGG) were used as negative controls in each experiment. siRNAs were ordered through Thermo Fisher Scientific.

SUMO interaction motif prediction. The GPS-SUMO 4.0 prediction tool was used to predict possible SUMO-interacting motifs in GAR1. The coding amino acid sequence for isoform 1 of GAR1 was obtained in FASTA format through UniProt (identifier [Q9NY12-1](#)). The "SUMO Interaction Threshold" was

set to “medium.” The SUMO Interaction prediction score obtained for residues 70-VLLG-74 was 31.605, with a cutoff of 29.92 and a *P* value of 0.112.

Nickel affinity purification of SUMOylated FLAG-dyskerin. For analysis of SUMOylated FLAG-dyskerin by immunoblotting, HEK293 cells expressing 6×His-SUMO3 and/or FLAG-dyskerin (wild type, K446X, or SUMO3-K446X) were lysed under denaturing conditions. Briefly, cells were washed with 1× phosphate-buffered saline (PBS) and collected by scraping. One-fifth of cells per condition were kept for input and lysed in 2× Laemmli buffer, followed by boiling. The remainder of the cell pellet was lysed in 6 M Gu-HCl buffer (10 mM Tris-HCl [pH 8], 6 M Gu-HCl, 10 mM imidazole, and 0.1 M NaH₂PO₄, adjusted to pH 8 with NaOH) at room temperature by passage through a 21G11/4 syringe (5×), followed by passage through an insulin syringe (3×). Cell lysate was cleared by centrifugation at 13,000 rpm for 20 min at 4°C. The supernatant was incubated with NiNTA resin (prewashed 2× with 1× PBS and 1× with Gu-HCl buffer) on a rotator at room temperature overnight. Resin was then washed 1× with Gu-HCl buffer, 1× with wash buffer 1 (10 mM Tris-HCl [pH 8], 8 M urea, 10 mM imidazole, and 0.1 M Na H₂PO₄, adjusted to pH 8 with NaOH), and 2× with wash buffer 2 (10 mM Tris-HCl [pH 8], 8 M urea, 10 mM imidazole, 0.1 M NaH₂PO₄, and 0.1% [vol/vol] Triton X-100, adjusted to pH 6.3 with NaOH). For elution, resin was incubated in elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 500 mM imidazole, adjusted to pH 8) for 3 h on a rotator at 4°C. The eluate was collected by centrifugation, and the resin was discarded.

Immunofluorescence. To assess localization of FLAG-dyskerin to the nucleolus, HEK293 cells expressing FLAG-dyskerin constructs were fixed with 4% formaldehyde-PBS for 10 min at room temperature. The fixing solution was removed, and coverslips were briefly rinsed with PBS, followed by permeabilization of cells with 0.1% Triton X-100-PBS for 5 min at 4°C. Permeabilized cells were then washed with PBS before blocking in 5% BSA-PBS for 1 h at room temperature. Cells were probed for FLAG-dyskerin with rabbit anti-FLAG (Sigma-Aldrich, F7425, 1:500) in PBG (1% cold fish water gelatin, 0.5% bovine serum albumin [BSA], in PBS) overnight at 4°C in a humidity chamber. When assessing localization of only exogenous FLAG-tagged dyskerin, this was followed by probing with mouse antifibrillarin (monoclonal antibody 72B9 obtained from Kenneth Michael Pollard, 1:30) as a nucleolar marker, in PBG at 37°C for 1 h. Coverslips were washed with PBS and immunostained in PBG with secondary antibodies conjugated to fluorescein isothiocyanate (FITC; donkey anti-mouse IgG; Jackson ImmunoResearch Lab, Inc., 1:125) or Cy3 (donkey anti-rabbit IgG; Jackson ImmunoResearch Lab, Inc., 1:125). Coverslips were washed with PBS and mounted in Vectashield with DAPI (Vector Laboratories). Cells with FLAG-dyskerin signal were manually scored based on localization phenotype as a percentage of the number of cells with FLAG signal detected; ≥50 cells were counted in experimental triplicates for scoring of localization of each FLAG-tagged dyskerin construct. Images were captured using an Axio Imager 2 microscope (63×; Carl Zeiss, Jena, Germany). Nucleolar localization was determined by colocalization with fibrillarin clusters, nucleoplasmic localization was determined by colocalization with DAPI, and cytoplasmic localization was determined by concentrated signal outside and surrounding DAPI.

Colocalization of FLAG-tagged dyskerin with coilin or SMN was assessed in HEK293 cells expressing FLAG-dyskerin constructs. Cells were rinsed with PBS, fixed with 4% formaldehyde-PBS for 10 min at room temperature, washed with PBS, and permeabilized with 0.5% Triton X-100–PBS for 10 min at room temperature. Permeabilized cells were then washed with PBS before blocking in PBG for 30 min at room temperature. To assess localization of FLAG-dyskerin to Cajal bodies, cells were incubated with rabbit anti-FLAG (Sigma-Aldrich, F7425, 1:500) and mouse anticoin (from Michael Terns [93], 1:10,000) in PBG overnight at 4°C. To assess localization of FLAG-dyskerin to nuclear gems, the cells were incubated with rabbit anti-FLAG and mouse anti-SMN (BD Biosciences, BD610646, 1:500) in PBG overnight at 4°C. Coverslips were washed with PBG and immunostained with secondary antibodies conjugated to FITC (donkey anti-mouse IgG; Jackson ImmunoResearch Lab, Inc., 1:125) or Cy3 (donkey anti-rabbit IgG; Jackson ImmunoResearch Lab, Inc., 1:125). Coverslips were then washed with PBG, rinsed briefly with PBS, and mounted in Vectashield with DAPI (Vector Laboratories). For FLAG/coilin costaining, images were captured using an Axio Imager 2 microscope (63×; Carl Zeiss, Jena, Germany). For FLAG/SMN costaining, images were acquired using Infinity Capture software and camera (40×; Leica DM2000 fluorescence microscope; Lumenera, Nepean, Ontario, Canada). Only nuclei with both FLAG and coilin/SMN signal were scored. Nuclei that had ≥1 FLAG focus overlapping partially or completely with coilin/SMN foci were considered positive for colocalization.

Immunoprecipitation. Protein-protein interactions were assessed by immunoprecipitating FLAG-dyskerin wild type or N/NoLS variants from HEK293 cells and immunoblotting for endogenous dyskerin-interacting proteins; by immunoprecipitating 3×FLAG-GAR1 wild type or 5A and immunoblotting for endogenous dyskerin. Monoclonal M2 mouse anti-FLAG antibody (Sigma-Aldrich, F3165) and protein G-Sepharose (GE Healthcare) preblocked in 1% BSA-PBS were used to immunoprecipitate (IP) FLAG-tagged and 3×FLAG-tagged proteins. The protocol used to assess protein-protein interactions was the same used to analyze the interaction between FLAG-dyskerin and hTR and was modified based on a protocol that has been previously described for another hTR-interacting protein (94), as well as used for FLAG-tagged dyskerin (16). Briefly, cells were first lysed in low-salt buffer (25 mM HEPES-KCl [pH 7.9], 5 mM KCl, 0.5 mM MgCl₂, 0.5% NP-40, 1× protease inhibitor cocktail [Roche], 20 mM *N*-ethylmaleimide, and 40 U/ml RNaseOUT) for 10 min on ice. Lysates were cleared by centrifugation at 5,000 rpm for 5 min at 4°C, supernatants were kept on ice, and pellets underwent a second lysis in high-salt buffer (25 mM HEPES-KCl [pH 7.9], 350 mM NaCl, 10% [wt/vol] sucrose, 0.01% NP-40, 1× protease inhibitor cocktail [Roche], 20 mM *N*-ethylmaleimide, and 40 U/ml RNaseOUT) with 30 s of vortexing, followed by 30 min on a rotator at 4°C. Both low-salt and high-salt lysates were then cleared by centrifugation at 13,000 rpm for 30 min at 4°C, the supernatants were pooled, and the total lysate was precleared at 4°C on a rotator for 30 min using protein G-Sepharose that was prewashed with 1× PBS. Bradford analysis was used to

calculate total protein concentration prior to IP. Lysates were incubated with anti-FLAG antibody for 2 h at 4°C on a rotator before preblocked protein G-Sepharose was added, followed by an additional 1 h of incubation at 4°C on a rotator. IPs were washed 4× with 1 ml of modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 10 mM MgCl₂, 1% NP-40, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 0.1× protease inhibitor cocktail [Roche], and 20 mM *N*-ethylmaleimide). For protein-protein interactions, elution from protein G-Sepharose was performed using Laemmli buffer and boiling. For protein-RNA interactions, elution was performed with TRIzol reagent (Invitrogen), followed by chloroform extraction and reverse transcription. Inputs (10% of lysate volume used for IP) were collected after preclearing with protein G-Sepharose and prior to IP and then treated with either Laemmli buffer and boiled or with TRIzol reagent.

Immunoblotting and antibodies. Analysis of protein expression and IP experiments was performed by resolving proteins by SDS-PAGE, transfer to polyvinylidene difluoride, and immunoblotting. The primary antibodies used for immunoblotting were anti-FLAG (Proteintech, 20543-1-AP, 1:4,000) or anti-FLAG (Proteintech, 66008-3-Ig, 1:4,000), anti-NAF1 (Abcam, ab157106, 1:1,000), anti-NHP2 (Proteintech, 15128-1-AP, 1:5,000), anti-NOP10 (Abcam, ab134902, 1:500), antidyskerin (Santa Cruz, sc-373956, H-3, 1:1,500), anti-GAR1 (Proteintech, 11711-1-AP, 1:1,000), anti-His (Santa Cruz, sc-8036, H-3, 1:500), and anti- α tubulin (Sigma, T5168, 1:5,000).

RNA extraction and RT-qPCR. RNA was extracted using TRIzol reagent (Invitrogen), according to the reagent protocol. Reverse transcription was performed with SuperScript II reverse transcriptase (Invitrogen) according to the user protocol, with hexameric random primers. PerfeCTa SYBR green FastMix with Low ROX (Quanta) was used for all qPCR analyses, in a 7500FAST real-time PCR system (ABI) as previously described (46). The comparative $\Delta\Delta C_T$ method was used to compare RNA enrichment between samples. For analysis of protein-RNA interactions, 5 μ l of RNA from input and 5 μ l of RNA from IP fractions were reverse transcribed into cDNA and subjected to qPCR using specific primers for target RNAs as previously described (16). The $\Delta\Delta C_T$ was calculated between the mean C_T of the IP and the mean C_T of the input for each sample.

Q-TRAP. Quantitative analysis of telomerase activity was done using the Q-TRAP protocol previously described (95). Briefly, HEK293 cells with or without expression of FLAG-dyskerin constructs were treated with scramble siRNA or siRNA to deplete endogenous dyskerin for 72 h prior to harvesting by scraping and lysis in NP-40 lysis buffer. A standard curve was generated with a serial dilution of mock-treated lysate (HEK293 cells untreated with siRNA and not expressing FLAG-dyskerin) for each experimental replicate ($n = 3$), with 1, 0.2, 0.04, 0.008, or 0.0016 μ g of total protein. For comparison of telomerase activity between conditions, 0.2 μ g of total protein was used for each sample.

Statistical analyses and multiple sequence alignment. All statistical analyses were performed using GraphPad Prism 7. One-way analysis of variance (ANOVA; $P < 0.01$) was used to compare hTR enrichment when assessing interaction between FLAG-dyskerin and hTR and for comparison of relative telomerase activity (RTA) in Q-TRAP experiments. For analysis of hTR interaction, the enrichment of hTR in each N/NoLS variant IP fraction was compared to the enrichment of hTR in the FLAG-dyskerin wild-type IP fraction. To analyze the telomerase activity, the RTA percentage of each condition was separately compared to the mock-treated (untreated HEK293 cells) RTA percentage. Each experiment was performed in triplicate, and error bars represent the standard errors of the mean (SEM) between experimental replicates. Dunnett's test was used to correct for multiple comparisons. Two-way ANOVA tests ($P < 0.0001$) were used to compare RNA enrichment when assessing interaction between FLAG-dyskerin and H/ACA RNAs. Analysis was performed in the same manner as for assessing hTR interaction. This was performed in triplicate, and error bars represent the SEM between experimental replicates. Tukey's *post hoc* test was used.

Clustal Omega (96, 97) was used to generate multiple sequence alignments of the full-length human dyskerin protein and the full-length homologues in mouse, rat, budding yeast, and fission yeast. Amino acid sequences for each protein were obtained in FASTA format from UniProt (identifiers [O60832](#) for human, [Q9ESX5](#) for mouse, [P40615](#) for rat, [P33322](#) for budding yeast, and [O14007](#) for fission yeast).

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D.E.M., C.A., and P.T. designed research. D.E.M., P.L.-L., J.Q., F.P.M., and E.B. performed experiments. D.E.M. and C.A. wrote the manuscript. All authors contributed to reviewing and editing of the manuscript.

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