Published in final edited form as:
Mol Carcinog. 2011 May ; 50(5): 334–345. doi:10.1002/mc.20715.

Dyskerin is required for tumor cell growth through mechanisms that are independent of its role in telomerase and only partially related to its function in precursor rRNA processing

Faizan Alawi[*] and **Ping Lin**
Department of Pathology, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA, USA.

Abstract

Dyskerin is an essential nucleolar protein required for the biogenesis of ribonucleoproteins that incorporate H/ACA RNAs. Through binding to specific H/ACA RNAs, dyskerin exerts most of its influence in the cell. To that end, dyskerin is a core component of the telomerase complex and is required for normal telomere maintenance. Dyskerin is also required for post-transcriptional processing of precursor rRNA. Germline dyskerin mutations increase cancer susceptibility. Conversely, wild-type dyskerin is usually overexpressed and not mutated in sporadic cancers. However, the contributions of dyskerin to sporadic tumorigenesis are unknown. Described herein, we demonstrate that acute loss of dyskerin function by RNA interference significantly reduced steady-state levels of H/ACA RNAs, disrupted the morphology and inhibited anchorage-independent growth of telomerase-positive and telomerase-negative human cell lines. Unexpectedly, dyskerin depletion only transiently delayed rRNA maturation but with no appreciable effect on the levels of total 18S or 28S rRNA. Instead, while rRNA processing defects typically trigger p53-dependent G_1 arrest, dyskerin-depleted cells accumulated in G_2/M by a p53-independent mechanism, and this was associated with an accumulation of aberrant mitotic figures that were characterized by multi-polar spindles. Telomerase activity and the rate of rRNA processing are typically increased during neoplasia. However, our cumulative findings indicate that dyskerin contributes to tumor cell growth through mechanisms which do not require the presence of cellular telomerase activity, and which may be only partially dependent upon the protein's role in rRNA processing. These data also reinforce the notion that loss and gain of dyskerin function may play important roles in tumorigenesis.

Keywords

small nucleolar RNA; H/ACA ribonucleoprotein; dyskeratosis congenita; mitotic spindle; tumorigenesis; rRNA

BACKGROUND

Dyskerin is a highly conserved and vital nucleolar protein [1–3]. Together with several other factors, dyskerin is required for the biosynthesis of ribonucleoproteins (RNPs) that incorporate H/ACA RNAs [4,5]. These functional, non-coding RNAs (ncRNAs) are characterized by a 5'-hairpin-hinge-hairpin-tail-3' secondary structure (H) with the nucleotides A-C-A located three positions from the 3' end. There are at least 100 known human H/ACA RNAs, including telomerase RNA (TERC) and subsets of small nucleolar

[*]Corresponding author: Faizan Alawi DDS, Assistant Professor, Department of Pathology, School of Dental Medicine, University of Pennsylvania, 240 South 40th Street, Philadelphia, PA 19104, Office: 1-215-573-7638, Fax: 1-215-573-2050, falawi@upenn.edu.

RNAs (snoRNAs) and Cajal body RNAs [5,6]. However, only one H/ACA RNA is incorporated into each RNP and dyskerin binds to and stabilizes the RNA molecules throughout the course and completion of RNP maturation. The protein components of functionally mature H/ACA RNPs include dyskerin complexed with three other highly conserved proteins - NHP2, NOP10 and GAR1 [5].

Dyskerin is thought to exert most of its influence in the cell via H/ACA RNA binding. Through binding to TERC, dyskerin is needed for normal function of the telomerase RNP and for telomere maintenance [6]. In contrast, a majority of H/ACA snoRNAs participate in post-transcriptional processing of nascent ribosomal RNA (rRNA) [2,3,7]. Most act as guides in the pseudouridination of precursor rRNA, and dyskerin catalyzes the isomerization of specific uridine residues to pseudouridines. Similarly, through direct interaction with H/ACA Cajal body RNAs, dyskerin participates in pseudouridination of spliceosomal RNA [5]. In addition, a number of H/ACA RNAs have do not have any known pseudouridination targets or function [5]. Thus, at least some H/ACA RNAs, and by extension, dyskerin-associated H/ACA RNPs may regulate other cellular processes beyond those that the protein is typically associated with. Indeed, recent reports suggest a role for dyskerin in the translational control of specific mRNAs [8–11], maintenance of telomere integrity independently of telomere length regulation [12], and in regulation of a subset of microRNAs [13,14]. It is not known if these latter functions are entirely dependent upon dyskerin-H/ACA RNA interactions. Nonetheless, dyskerin appears to regulate a diverse array of important cellular functions.

Germline dyskerin mutations are associated with the X-linked variant of dyskeratosis congenita [1]. Actively proliferating cells and tissues that undergo rapid turnover are most severely affected in these individuals. This results in a wide-ranging phenotype that affects primarily the lymphoid, hematologic, epithelial and bone tissue compartments. Mucocutaneous manifestations, aplastic anemia, pulmonary fibrosis and osteoporosis are among the most characteristic clinical findings [1]. There is also a significantly increased risk for the development of cancer; carcinomas of the head and neck, skin and gastrointestinal tract, lymphomas and leukemias are the most common [15]. Recent reports have begun to provide clarity into the molecular mechanisms by which loss of dyskerin function might contribute to neoplasia [9–11]. In contrast, an emerging body of evidence suggests that gain of dyskerin function may be relevant in the pathogenesis of sporadic cancers.

We have previously shown that human dyskerin expression strongly correlates with active cell proliferation [16]. Dyskerin expression is also upregulated under experimental conditions that promote cell growth and proliferation, including via oncogenic stimulation [17]. To that end, wild-type dyskerin is overexpressed and not mutated in a variety of cancer types relative to patient-matched controls and high levels have been associated with cancer progression and poor prognosis [18–24]. However, the mechanisms by which wild-type dyskerin contributes to sporadic tumorigenesis remain obscure.

Telomerase activity is upregulated in the vast majority of human cancers [25]. This is primarily a result of increased expression of telomerase reverse transcriptase (TERT), which is the catalytic component of the telomerase complex [25]. However, we recently demonstrated that dyskerin levels do not correlate with TERT expression and are not dependent upon the presence of cellular telomerase activity in either human cell lines or in cancer tissues [16]. In the current report, we further these findings to show that not only is dyskerin required for the *in vitro* growth of telomerase-positive and telomerase-negative transformed cell lines, but also that the repertoire of cellular functions that may be influenced by dyskerin continues to expand.

METHODS

Cell culture

U2OS human osteosarcoma cells and HeLa cervical carcinoma cells were obtained from American Type Culture Collection (Manassas, VA). The oral squamous cell carcinoma cell line, UM-SCC-1 was a gift of Dr. Thomas Carey (University of Michigan). All cell lines were grown in D-MEM containing high glucose (4,500 μg/mL), 1 mmol/L of glutamine, 10% fetal bovine serum, with 100 IU/mL penicillin and 100 IU/mL streptomycin.

siRNA transfections

siGENOME SMARTPool siRNA duplexes targeting *DKC1* and *GAPDH*, and a negative control, Non-Targeting siRNA pool #1 were from Dharmacon (Lafayette, CO). To validate the effects of the *DKC1* siRNA, we repeated most experiments using a custom-designed anti-*DKC1* siGENOME SMARTPool targeting the 3'-untranslated region. Transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), as per the manufacturer's protocol. All transfections were performed at least in triplicate, and every experiment was performed two or more times.

Western blot analysis

Whole cell lysates were extracted as described previously [17]. Antibodies against dyskerin, β-actin, GAPDH, fibrillarin, nucleophosmin, p53, and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All Western blot analyses were performed at least in duplicate.

Cell proliferation, cell cycle distribution, and anchorage-independent growth assays

An equal number of siRNA-transfected cells were seeded in 96-well plates and cell proliferation was measured using the Cell Proliferation Reagent WST-1 (Roche Applied Science, Indianapolis, IN), as previously described [16]. For cell cycle distribution analysis, cells were fixed with cold 80% ethanol, resuspended in PBS, and stained with 10 μg/ml propidium iodide containing 1 mg/ml RNase A (Sigma-Aldrich). Analyses were performed on a Becton-Dickinson Facstar^{PLUS} flow cytometer (BD Biosciences, San Jose, CA).

To measure growth in soft agar, transfections were performed in triplicate with siCTRL or siDyskerin, respectively, and then harvested 24 hrs later. After counting, 6000 cells from each transfection well were suspended in 0.3% low-melting point agarose (Invitrogen) diluted in D-MEM. One third of the suspension was then laid over a bed of 0.6% agarose/D-MEM in each of three wells of a 6-well culture plate. The cells were allowed to grow for 5 days, after which they were analyzed by phase contrast microscopy using an Olympus IX50-S8F inverted microscope. All images were obtained using SPOTsoftware Version 4.6 (Diagnostic Instruments, Inc., Sterling Heights, MI).

SnoRNA extraction and analysis

Total RNA was isolated using the miRNeasy kit (Qiagen, Valencia, CA) and then enriched for small RNAs (< 200 nucleotides) using the RNeasy MinElute Cleanup kit (Qiagen). The miScript PCR system (Qiagen) was used for quantitative RT-PCR analysis on a 7500 Real Time PCR System (Applied Biosystems, Carlsbad, California). Pre-designed primers for SNORA66, SNORA73A, SNORA74A, SNORD13, SNORD25, SNORD43, RNU5, RNU6B, and miR-let-7g were obtained from Qiagen. Custom designed primers were used for SNORA34, SNORA36B and SNORA81. These include: SNORA34_F "CAGAGGCCTAAAGGACTGTC", SNORA34_R "AATGAAATAGCCATTCCCTACTGA", SNORA81_F

"TGCAGACACTAGGACCATGT", SNORA81_R
"CACCCCAGTCTTTACAATCTAGTG", SNORA36B_F
"TGTTAAGTTCAGTTCAGGGTAGC", and SNORA36B_R
"TTCTATCCAATCATTTTCCCTAGC". For quantitation, relative levels of these ncRNAs were normalized to RNU6B. The expression of this latter RNA remained stable under our experimental conditions.

Metabolic labeling of rRNA

SiRNA-treated cells were grown for thirty minutes in methionine-free DMEM (Invitrogen) containing dialyzed fetal bovine serum (Invitrogen). L-[methyl-^{3}H]-methionine (Perkin-Elmer, Waltham, MA) was then added to a final concentration of 50 μCi/mL. After thirty minutes, the cells were washed with PBS and then incubated for up to two hours in complete medium containing an excess of non-radioactive methionine. After agarose gel electrophoresis using the Northern Max-Gly kit (Ambion, Austin, TX), the gels were transferred to GeneScreen Plus Hybridization Transfer membranes (Perkin-Elmer), dried and then analyzed by autoradiography using Biomax MS film and Biomax TranScreen LE (Kodak, Rochester, NY). These latter exposures were performed at −80°C for at least three days.

Immunofluorescent labeling of mitotic cells

Asynchronous HeLa cells were cultured in 4-well Lab Tek chamber slides (Thermo Scientific, Rochester, NY) and transfected with siDyskerin or siCTRL in three of the four wells. Seventy-two hours later, the cells were fixed in 4% paraformaldehyde for 15 min at 37°C. After rinses with PBS and PBS-0.1% Tween-20 (PBST), the cells were blocked and permeabilized with 0.2% bovine serum albumin and 0.1% Triton X-100 in PBS for 60 min. Cells were incubated with anti-α-tubulin (1:100; Santa Cruz) for three hours at room temperature, and then with a Cy-3 labeled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). DAPI (4,6-diamidino-2-phenylindole) was used to stain the DNA. Coverslips were mounted onto the slides using GelMount (Biomeda Corporation, Foster City, CA) and allowed to dry overnight prior to viewing. Cells were analyzed using a Nikon Eclipse ES400 fluorescence microscope equipped with an Olympus DP70 Digital Camera System and associated imaging software (Optical Apparatus, Ardmore, PA). All images were processed with Adobe Photoshop CS (Abobe Systems, San Jose, CA) using the "auto-contrast" functions.

RESULTS

Dyskerin depletion perturbs cell cycle progression and proliferation

We began our studies by examining the effects of dyskerin depletion on the proliferation of telomerase-positive UM-SCC1 oral squamous cell carcinoma cells and HeLa cervical carcinoma cells, and telomerase-negative U2OS osteosarcoma cells. U2OS cells do not express either TERC or TERT; telomere maintenance occurs through homologous DNA recombination [26].

The cells were transiently transfected with siRNAs targeting *DKC1* (siDyskerin), *GAPDH* (siGAPDH) or a non-specific control (siCTRL). After 48 hours, dyskerin expression was markedly and specifically reduced in all cell lines relative to the controls (Figure 1A and Supp. Fig. S1). In parallel, flow cytometric analysis revealed a small but significant accumulation of siDyskerin cells in G_2/M (≈3.5% – 9%, depending on the experiment and cell line used) relative to the siCTRL and siGAPDH cells (Figure 1B and Supp. Fig. S1). This was reproducible in several repetitions of our experiments. Similar findings were also

seen using a custom-designed siRNA reagent that targeted the 3'-untranslated region of *DKC1* (not shown).

There was no effect on cell proliferation after 72 hours of dyskerin depletion. However, after six days of persistent dyskerin knockdown in U2OS cells, there was a small by significant decrease in proliferation ($p < 0.01$; Figures 1C and 6A). This was associated with a 50% reduction in the number of siDyskerin cells relative to siCTRL cells (Figure 1D). However, unexpectedly, at this time, there was still only a mild disruption in the cell cycle profile of the siDyskerin cells (Figure 1E). In contrast, even with prolonged dyskerin depletion, there was no effect on the proliferation of UM-SCC1 or HeLa cells (not shown).

Dyskerin is required for anchorage-independent growth of tumor cells

The effects of gene manipulation in monolayer cell cultures do not always parallel those observed when cells are grown in the absence of a solid surface to which they could adhere. Thus, we next tested the effects of dyskerin depletion on anchorage-independent growth. U2OS and HeLa cells were transfected with the dyskerin and CTRL siRNAs. Twenty-four hours later, the cells were plated in soft agar, and allowed to grow for for at least 5 days before microscopic analysis. As expected, U2OS and HeLa siCTRL cells readily proliferated in the agarose (Figure 2A). In contrast, the siDyskerin U2OS and HeLa cells showed very poor anchorage-independent growth. Few siDyskerin cells were identified, and where there were cells clustered together, the colonies were small. Anchorage-independent growth is an *in vitro* hallmark of most transformed cells and strongly correlates with tumorigenic growth potential *in vivo* [27]. Thus, downregulation of dyskerin inhibited the tumorigenicity of telomerase-positive and telomerase-negative cells.

Loss of dyskerin disrupts cellular morphology

As shown in Figure 2A, dyskerin knockdown resulted in unusual morphologic changes whereby a number of the cells exhibited thin, markedly elongated cellular processes. Knockdown of dyskerin also elicited similar morphologic changes in siDyskerin U2OS cells (but not UM-SCC1 or HeLa cells) that were grown in monolayer (Figure 2B). To confirm that the unusual morphology was a specific consequence of dyskerin knockdown, we repeated the U2OS siRNA transfections, except this time we used the siRNA that targeted the 3'-untranslated region of *DKC1*. On the 4th day after initial transfection, the cells were transfected with pcDNA3-Flag-Dyskerin or the vector alone. Twenty-four hours later, the cells were either harvested for protein or passaged, and then grown for an additional 24 hrs before fixation and microscopic analysis. As expected, re-introduction of dyskerin restored the proliferation and morphology of the siDyskerin cells (Figure 2C, D). Flag-dyskerin had no observable effect on the siCTRL and siGAPDH cells, and the vector only control did not reverse the effects of dyskerin depletion. This indicated that the observed effects were a specific consequence of reduced dyskerin function.

Dyskerin regulates steady-state levels of H/ACA snoRNAs

To investigate the mechanisms by which dyskerin contributed to tumor cell growth, we first tested the effects of dyskerin depletion on H/ACA RNAs. Within 48 hours after siRNA transfection, loss of dyskerin led to a significant and specific reduction in the levels of six randomly selected H/ACA snoRNAs. Although levels of these RNAs were variably affected, in almost all cases, the expression of each was reduced by 2- to 4-fold relative to the controls; this was independent of the cell line tested (Figure 3A, B). In contrast, levels of all non-H/ACA RNAs remained essentially unchanged; these ncRNAs are not bound or regulated by dyskerin [5,14]. We note that TERC expression was also reduced almost 2-fold in the telomerase-positive cells (Figure 3B). TERC is not expressed in U2OS cells [26] and hence was undetectable in these cells. Northern blot analysis was then used to validate a

subset of the qRT-PCR results. As expected, levels of SNORA36B and SNORA81 were markedly decreased after dyskerin depletion, while levels of non-HACA RNAs were unaffected (Supp. Fig. S2).

Dyskerin is required for pseudouridination of small nuclear spliceosomal RNAs [5]. Yet, in our experiments, the levels of RNU5A, RNU6B and RNU6 (see Supp. Fig. S2) were remarkably constant; RNU6B was thus used as a quantitative control for normalization. This suggests that loss of dyskerin function may not affect the steady-state levels of spliceosomal RNA. This reinforces the findings of a recent report which showed that loss of murine dyskerin also did not affect the levels of spliceosomal RNA [7].

Dyskerin depletion only transiently disrupts rRNA maturation in U2OS cells

The majority of H/ACA snoRNAs are needed for precursor rRNA processing, and pseudouridination is required for normal rRNA maturation [5]. Except for SNORA73A, each of the other H/ACA snoRNAs described above has a known uridine target in 45S precursor rRNA, with the subsequent pseudouridine incorporated into either mature 18S or 28S rRNA. Yet, surprisingly, after 6 days of persistent dyskerin depletion, there was no appreciable effect on the relative abundances of mature 28S and 18S rRNA before and after dyskerin depletion, or between the siDyskerin and siCTRL cells (Figure 4A). However, a pulse-chase rRNA labeling experiment did reveal a disruption in precursor rRNA processing in the siDyskerin U2OS cells, and to a lesser extent in UM-SCC1 cells (Figure 4B and Supp. Figure S3). In U2OS siCTRL cells labeled with L-[methyl-^{3}H]-methionine, levels of the 45S rRNA precursor were significantly decreased over the duration of the experiment. This was associated with a concomitant accumulation of newly synthesized 28S rRNA, thereby indicative of appropriate processing. In contrast, between 30–60 minutes after the chase, persistence of the large 45S rRNA precursor was clearly evident in the siDyskerin cells. After 30 minutes, relative levels of 45S rRNA (normalized to 5S rRNA) were 1.3–1.5 fold higher in the siDyskerin cells relative to amount of 45S rRNA in the siCTRL cells (Figure 4C). By 60 minutes, relative levels of 45S rRNA were 1.7–2 fold higher compared to the control. Concomitantly, after the first 30 minutes in the siDyskerin cells, levels of newly formed 28S rRNA were reduced 40–60% (depending on the siRNA used) relative to the siCTRL cells, and by 15–35% after 60 minutes (Figure 4D). Yet, after 120 minutes, processing of 45S rRNA and levels of 28S and 18S rRNA appeared equivalent in the siCTRL and siDyskerin cells. Similar findings were observed in three independent experiments. Thus, loss of dyskerin transiently perturbed rRNA processing in U2OS cells, but not to an extent that there was an appreciable effect on the levels of total mature rRNA.

Atypical multi-polar mitoses are increased following dyskerin depletion

Telomerase activity peaks during S phase and is repressed during G_1 and G_2/M [28]. In mammalian cells, the bulk of rRNA processing occurs during G_1 and S phases, diminishes during G_2 and completely ceases early in mitosis, only to be restored upon mitotic exit [29]. In light of our findings and the fact that dyskerin is a nucleolar protein, our observation that loss of dyskerin delayed transit through G_2/M arrest could not be readily explained. However, emerging evidence suggests that dyskerin may also be an evolutionarily conserved component of the mitotic spindle [30–34]. Moreover, we have observed at least partial co-localization of human dyskerin with α-tubulin during metaphase and anaphase, respectively; α-tubulin is a core component of the mitotic spindle (P. Lin, F. Alawi, in preparation). Dyskerin was recently identified within purified HeLa cell mitotic spindle extracts [30,31]. To that end, dyskerin depletion in these cells led to a small but remarkably consistent and highly significant increase (≈7%; $p = 0.0001$) in the proportion of multi-polar mitotic figures relative to the siCTRL cells (Figure 5A, B). Most were tri-polar, however quadri-, penta- and even rare hexa-polar spindles were identified. There were no significant

differences between the siCTRL and mock-transfected cells. This novel finding indicates a possible rationale for the observed increase in G_2/M cells following dyskerin knockdown, and importantly, suggests a new addition to the repertoire of cellular functions that may be influenced by this multi-functional protein.

Loss of dyskerin elicits growth arrest independently of p53

Human cells that harbor dyskerin mutations proliferate poorly *in vitro* and show a high rate of apoptosis [6,35,36]. However, the decrease in proliferation of siDyskerin U2OS cells did not result from activation of the apoptosis signaling cascade (not shown). Similarly, there was no objective evidence of cellular senescence, as determined by a lack of senescence-associated β-galactosidase staining (not shown). These observations are similar to those described by Sieron et al [21] who reported that dyskerin depletion reduced the proliferation of prostate cancer cells without eliciting either apoptosis or senescence.

At least one murine dyskerin-mutant has been shown to impair cell proliferation via a p53-dependent mechanism [12]. Defects in telomere maintenance, impaired rRNA processing and disruptions in normal mitosis are all known triggers of p53 [9,12,37]. U2OS cells express wild-type p53 [38] while the protein is inactivated in HeLa [39] and mutated in UM-SCC1 cells [40]. However, dyskerin depletion did not lead to upregulation of either p53 mRNA or protein in U2OS cells (Figure 6A, B).

Activation of p53 may also occur through phosphorylation; serine 15 is phosphorylated following DNA damage [12]. However, a p53 antibody that specifically recognizes phosphorylated serine 15 did not identify any reactivity in the siDyskerin lysates (not shown). We cannot exclude the possibility that p53 activation may have occurred through phosphorylation at other residues. Yet, we note that loss of dyskerin also had no effect on the expression of p53 transcriptional targets, including $p21^{Waf/Cip1}$ and $p27^{Kip1}$ (Figure 6B); these latter factors are also implicated in cell cycle control [10,11]. Thus, the exact mechanisms by which loss of dyskerin reduced cell proliferation remain unclear.

DISCUSSION

Several lines of evidence indicate that wild-type dyskerin may be important in the pathogenesis of sporadic cancer [17–22]. Conversely, germline dyskerin mutations increase cancer susceptibility [2,9,10,15]. Emerging evidence suggests that the mechanisms by which loss of dyskerin contributes to tumorigenesis may not be related to the role of the protein in either telomerase or in rRNA processing [9–11]. Our cumulative findings suggest wild-type dyskerin may also contribute to tumor cell growth via alternative mechanisms.

Murine dyskerin is required for cell proliferation via a mechanism that is dependent upon its interaction with a functional telomerase complex [12]. In the absence of either Terc or Tert, dyskerin-mutant primary murine cells proliferate at the same rate as dyskerin-wild-type cells. However, we recently demonstrated that dyskerin levels do not correlate with TERT expression or telomerase activity in either human cell lines or in cancer tissues [16]. Sieron et al [21] recently showed that loss of dyskerin function impaired the proliferation of telomerase-positive human prostate cancer cells. We now show that loss of dyskerin function can also limit the proliferation of telomerase-negative cells; U2OS cells do not express either TERT or TERC [26]. We have also shown for the first time that dyskerin is required for anchorage-independent growth of telomerase-negative and telomerase-positive cells. Thus, while dyskerin is a core component of the telomerase complex [6], our findings strengthen the notion that wild-type dyskerin plays an important role in human tumor cell growth and that this function is not dependent upon the presence of cellular telomerase activity.

Sieron et al [21] also reported unusual morphologic changes similar to the ones we observed after dyskerin depletion. Although it wasn't directly investigated, the authors theorized that the anti-proliferative and morphologic effects of dyskerin knockdown were likely related to a defect in protein synthesis resulting from diminished rRNA processing. However, our findings suggest that the role of dyskerin in tumor cell growth may be only partially related to its function in rRNA processing.

Despite a significant and persistent decrease in H/ACA snoRNA expression, there was only a transient delay in the maturation of newly synthesized rRNA following dyskerin depletion. Due to an increased metabolic demand, the rate of rRNA processing is necessarily amplified in tumor cells [41]. Thus, we cannot exclude the possibility that even a transient disruption in rRNA maturation may be sufficient to slow the growth of tumor cells. Moreover, since rRNAs are incorporated into ribosomes, it is possible that an abnormality in ribosome function resulting from improperly modified rRNAs could adversely affect protein synthesis and impair cell proliferation. Yet, while targeted dyskerin depletion disrupts rRNA pseudouridination in human breast cancer cells [42], Montanaro et al [11] recently demonstrated that there is no effect on global protein synthesis. During the course of our studies, Gu et al [43] demonstrated that while the relative abundances of 18S and 28S rRNAs are not diminished in dyskerin-mutant human cells relative to wild-type cells, the mature rRNAs do exhibit a subtle shift in electrophoretic mobility under very specific experimental conditions. Therefore, we cannot completely exclude the possibility that dyskerin depletion may have had an aberrant effect on rRNA maturation, albeit undetectable under our own experimental conditions. However, defects in rRNA processing typically trigger p53-dependent G_1 arrest [37]. In contrast, the dyskerin-depleted cells consistently accumulated in G_2/M. At least one mutant form of murine dyskerin triggers p53 activation; rRNA processing and cell proliferation are impaired in these cells [7]. Yet, loss of dyskerin function either through mutation or depletion may actually lead to a reduction in p53 protein expression [9,11]. In our experiments, loss of dyskerin function in U2OS cells had no effect on expression of either p53 mRNA or protein or its direct transcriptional targets. Taken together, this suggests that the role of dyskerin in sporadic tumorigenesis is not dependent on the presence of p53 and may not be entirely dependent on its role in rRNA processing. The factors that link dyskerin to cell cycle regulation remain poorly defined and will require elucidation.

We note that in murine cells, germline dyskerin mutations do not significantly delay G_2/M progression [7,9]. This discrepancy could be a result of differences in cell type or in the experimental conditions, or even due to possible functional differences between murine and human dyskerin. However, if similarly small cell cycle differences were evident in dyskerin-mutant cells, their significance may have been overlooked.

In an attempt to rationalize the G_2/M accumulation, our experiments have led to discovery of a possible novel role for dyskerin in mitosis. During mitosis, nucleolar disintegration is accompanied by relocation of several rRNA processing factors to the perichromosal region and the mitotic spindle [30,31,44]. In addition to playing key roles in precursor rRNA processing, at least some of these factors are required for normal mitotic spindle formation. Recent high-throughput, mass spectrometry-based studies identified human dyskerin and its *Drosophila* and *Xenopus* orthologs contained within purified mitotic spindle extracts derived from cells of each of the respective species [30–33]. In addition, yeast dyskerin was originally characterized as a microtubule binding protein [34]. We have now demonstrated for the first time that loss of dyskerin led to the accumulation of atypical mitoses with multi-polar spindles. Future studies will be needed to determine the mechanisms by which dyskerin contributes to mitotic spindle integrity. Whether or not this function is dependent upon dyskerin-H/ACA RNA interactions will also require more detailed investigation.

Nonetheless, our findings suggest that the repertoire of cellular processes that may be influenced by dyskerin continues to expand.

How loss and gain of dyskerin function may be associated with tumorigenesis

It is intriguing to speculate about the mechanisms by which loss and gain of dyskerin function may contribute to tumorigenesis. Normal mitotic spindle assembly and function are essential for the preservation of genomic integrity [22]. To that end, loss- and gain-of-function of various mitotic spindle proteins may play a role in cancer development and/or progression [22,30,31]. Intriguingly, *DKC1* was identified as one of only seventy genes that, collectively, constitute a gene expression profile that strongly correlates with the development of aneuploidy and is associated with poor clinical prognosis in a variety of human cancers [22]. A majority of the genes identified in this molecular signature are known to play important roles in mitotic spindle assembly, chromosome segregation and/or cytokinesis. We note that transient overexpression of dyskerin had no appreciable effect on wild-type U2OS, UM-SCC1 or HeLa cells, and we have not been able to obtain stable dyskerin-expressing transfectants (not shown). Nonetheless, it is highly intriguing to speculate that an alteration of physiologic dyskerin function, irrespective of the mechanism, may perturb mitosis and contribute to tumorigenesis. This will require more detailed investigation.

In addition to the effects on H/ACA snoRNAs, we recently demonstrated that loss of dyskerin function leads to a reduction in the levels of a subset of miRNAs that are encoded within H/ACA snoRNAs [13]. MiRNAs are a class of ncRNAs that directly regulate post-transcriptional gene expression [14]. At least some miRNAs have oncogenic potential while others have been ascribed tumor suppressive functions [45]. To our knowledge, the miRNAs that are regulated by dyskerin have not yet been functionally validated. Nonetheless, it is conceivable that dysregulation of miRNA-mediated translational control, either through supraphysiologic or diminished dyskerin function could potentially impact an array of factors that may be important for cancer development and/or progression [13]. This will also need further study.

Finally, we previously showed that dyskerin is a direct transcriptional target of the c-MYC oncoprotein [17]. c-MYC is a transcription factor implicated in the pathogenesis of several cancers; high c-MYC levels are often associated with poor prognosis [17,46]. The specific contributions of dyskerin to c-MYC-mediated tumorigenesis are not known. However, recent findings indicate that other protein components of mature H/ACA RNPs, including NHP2 and GAR1, are also transcriptionally regulated by c-MYC [47,48]. The status of GAR1 in neoplasia is unknown. However, like dyskerin, NHP2 expression is significantly upregulated in sporadic cancers and high levels may be associated with poor clinical prognosis [46,48]. Conversely, germline NHP2 and NOP10 mutations give rise to autosomal recessive forms of dyskeratosis congenita, and cancer susceptibility is also a feature of these genetic forms of the disease [15,49,50].

Pathogenic mutations in dyskerin, NHP2 and NOP10 impair the assembly of H/ACA RNPs [4,51]. Alternatively, it has been suggested that high levels of dyskerin may also disrupt the biogenesis of H/ACA RNPs [35]. To that end, loss- and gain-of-function of the precursor H/ACA RNP assembly factor, SHQ1, impairs H/ACA RNP maturation [52]. Since the functions of several H/ACA RNAs have not yet been identified [5], it is possible that disruption of H/ACA RNP biogenesis by any mechanism may affect an array of important cellular processes, and could potentiate cancer development and/or progression. Hence, the findings from our current study may open up new and novel avenues of investigation into the mechanisms by which dyskerin and H/ACA RNPs contribute not only to sporadic

tumorigenesis, but also in the pathogenesis and phenotype of X-linked dyskeratosis congenita.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ABBREVIATIONS USED

TERC	telomerase RNA
snoRNA	small nucleolar RNA
RNP	ribonucleoprotein
miRNA	microRNA

Acknowledgments

We are grateful to Dr. Steven Artandi for pcDNA3-Flag-dyskerin. We also thank Dr. Subu Yerramilli (Qiagen) for assistance with the design of H/ACA snoRNA custom primers, and Dr. Sunday Akintoye for help with the cellular photography.

FUNDING

This work was supported in part by National Institutes of Health [grant number DE018416]; and an American Cancer Society Institutional Research Grant [IRG-78-002-30].

REFERENCES

1. Heiss NS, Knight SW, Vulliamy TJ, et al. X-linked dyskeratosis congenita is caused by mutations in a highly conserved gene with putative nucleolar functions. Nat Genet. 1998; 19:32–38. [PubMed: 9590285]
2. Ruggero D, Grisendi S, Piazza F, et al. Dyskeratosis congenita and cancer in mice deficient in ribosomal RNA modification. Science. 2003; 299:259–262. [PubMed: 12522253]
3. Mochizuki Y, He J, Kulkarni S, Bessler M, Mason PJ. Mouse dyskerin mutations affect accumulation of telomerase RNA and small nucleolar RNA, telomerase activity, and ribosomal RNA processing. Proc Natl Acad Sci U S A. 2004; 101:10756–10761. [PubMed: 15240872]
4. Grozdanov PN, Fernandez-Fuentes N, Fiser A, Meier UT. Pathogenic NAP57 mutations decrease ribonucleoprotein assembly in dyskeratosis congenita. Hum Mol Genet. 2009; 18:4546–4551. [PubMed: 19734544]
5. Meier UT. How a single protein complex accommodates many different H/ACA RNAs. Trends Biochem Sci. 2006; 31:311–315. [PubMed: 16647858]
6. Mitchell JR, Wood E, Collins K. A telomerase component is defective in the human disease dyskeratosis congenita. Nature. 1999; 402:551–555. [PubMed: 10591218]
7. Ge J, Rudnick DA, He J, et al. Dyskerin ablation in mouse liver inhibits rRNA processing and cell division. Mol Cell Biol. 2010; 30:413–422. [PubMed: 19917719]
8. Yoon A, Peng G, Brandenburger Y, et al. Impaired control of IRES-mediated translation in X-linked dyskeratosis congenita. Science. 2006; 312:902–906. [PubMed: 16690864]
9. Bellodi C, Kopmar N, Ruggero D. Deregulation of oncogene-induced senescence and p53 translational control in X-linked dyskeratosis congenita. EMBO J. 2010; 29:1865–1876. [PubMed: 20453831]
10. Bellodi C, Krasnykh O, Haynes N, et al. Loss of function of the tumor suppressor DKC1 perturbs p27 translation control and contributes to pituitary tumorigenesis. Cancer Res. 2010; 70:6026–6035. [PubMed: 20587522]

11. Montanaro L, Calienni M, Bertoni S, et al. Novel Dyskerin-Mediated Mechanism of p53 Inactivation through Defective mRNA Translation. Cancer Res. 2010; 70:4767–4777. [PubMed: 20501855]
12. Gu BW, Bessler M, Mason PJ. A pathogenic dyskerin mutation impairs proliferation and activates a DNA damage response independent of telomere length in mice. Proc Natl Acad Sci U S A. 2008; 105:10173–10178. [PubMed: 18626023]
13. Alawi F, Lin P. Loss of dyskerin reduces the accumulation of a subset of H/ACA snoRNA-derived miRNA. Cell Cycle. 2010; 9
14. Scott MS, Avolio F, Ono M, Lamond AI, Barton GJ. Human miRNA precursors with box H/ACA snoRNA features. PLoS Comput Biol. 2009; 5 e1000507.
15. Alter BP, Giri N, Savage SA, Rosenberg PS. Cancer in dyskeratosis congenita. Blood. 2009; 113:6549–6557. [PubMed: 19282459]
16. Alawi F, Lin P, Ziober B, Patel R. Correlation of dyskerin expression with active proliferation independent of telomerase. Head Neck. 2010 In press.
17. Alawi F, Lee MN. DKC1 is a direct and conserved transcriptional target of c-MYC. Biochem Biophys Res Commun. 2007; 362:893–898. [PubMed: 17822678]
18. Schaner ME, Ross DT, Ciaravino G, et al. Gene expression patterns in ovarian carcinomas. Mol Biol Cell. 2003; 14:4376–4386. [PubMed: 12960427]
19. Westermann F, Henrich KO, Wei JS, et al. High Skp2 expression characterizes high-risk neuroblastomas independent of MYCN status. Clin Cancer Res. 2007; 13:4695–4703. [PubMed: 17652624]
20. Turano M, Angrisani A, De RM, Izzo P, Furia M. Real-time PCR quantification of human DKC1 expression in colorectal cancer. Acta Oncol. 2008; 47:1598–1599. [PubMed: 18607840]
21. Sieron P, Hader C, Hatina J, et al. DKC1 overexpression associated with prostate cancer progression. Br J Cancer. 2009; 101:1410–1416. [PubMed: 19755982]
22. Carter SL, Eklund AC, Kohane IS, Harris LN, Szallasi Z. A signature of chromosomal instability inferred from gene expression profiles predicts clinical outcome in multiple human cancers. Nat Genet. 2006; 38:1043–1048. [PubMed: 16921376]
23. Soung YH, Lee JW, Kim SY, et al. Absence of DKC1 exon 3 mutation in common human cancers. Acta Oncol. 2006; 45:342–343. [PubMed: 16644581]
24. Poncet D, Belleville A, t'kint de RC, et al. Changes in the expression of telomere maintenance genes suggest global telomere dysfunction in B-chronic lymphocytic leukemia. Blood. 2008; 111:2388–2391. [PubMed: 18077792]
25. Shay JW, Keith WN. Targeting telomerase for cancer therapeutics. Br J Cancer. 2008; 98:677–683. [PubMed: 18231105]
26. Tilman G, Loriot A, Van BA, et al. Subtelomeric DNA hypomethylation is not required for telomeric sister chromatid exchanges in ALT cells. Oncogene. 2009; 28:1682–1693. [PubMed: 19252523]
27. Callow MG, Clairvoyant F, Zhu S, et al. Requirement for PAK4 in the anchorage-independent growth of human cancer cell lines. J Biol Chem. 2002; 277:550–558. [PubMed: 11668177]
28. Tomlinson RL, Ziegler TD, Supakorndej T, Terns RM, Terns MP. Cell cycle-regulated trafficking of human telomerase to telomeres. Mol Biol Cell. 2006; 17:955–965. [PubMed: 16339074]
29. Sirri V, Roussel P, Hernandez-Verdun D. In vivo release of mitotic silencing of ribosomal gene transcription does not give rise to precursor ribosomal RNA processing. J Cell Biol. 2000; 148:259–270. [PubMed: 10648559]
30. Dephoure N, Zhou C, Villen J, et al. A quantitative atlas of mitotic phosphorylation. Proc Natl Acad Sci U S A. 2008; 105:10762–10767. [PubMed: 18669648]
31. Sauer G, Korner R, Hanisch A, Ries A, Nigg EA, Sillje HH. Proteome analysis of the human mitotic spindle. Mol Cell Proteomics. 2005; 4:35–43. [PubMed: 15561729]
32. Somma MP, Ceprani F, Bucciarelli E, et al. Identification of Drosophila mitotic genes by combining co-expression analysis and RNA interference. PLoS Genet. 2008; 4 e1000126.
33. Yokoyama H, Gruss OJ, Rybina S, et al. Cdk11 is a RanGTP-dependent microtubule stabilization factor that regulates spindle assembly rate. J Cell Biol. 2008; 180:867–875. [PubMed: 18316407]

34. Jiang W, Middleton K, Yoon HJ, Fouquet C, Carbon J. An essential yeast protein, CBF5p, binds in vitro to centromeres and microtubules. Mol Cell Biol. 1993; 13:4884–4893. [PubMed: 8336724]
35. Wong JM, Collins K. Telomerase RNA level limits telomere maintenance in X-linked dyskeratosis congenita. Genes Dev. 2006; 20:2848–2858. [PubMed: 17015423]
36. Montanaro L, Chilla A, Trere D, et al. Increased mortality rate and not impaired ribosomal biogenesis is responsible for proliferative defect in dyskeratosis congenita cell lines. J Invest Dermatol. 2002; 118:193–198. [PubMed: 11851894]
37. Rubbi CP, Milner J. Disruption of the nucleolus mediates stabilization of p53 in response to DNA damage and other stresses. EMBO J. 2003; 22:6068–6077. [PubMed: 14609953]
38. Montanaro L, Mazzini G, Barbieri S, et al. Different effects of ribosome biogenesis inhibition on cell proliferation in retinoblastoma protein- and p53-deficient and proficient human osteosarcoma cell lines. Cell Prolif. 2007; 40:532–549. [PubMed: 17635520]
39. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell. 1990; 63:1129–1136. [PubMed: 2175676]
40. Kanazawa T, Kommareddi PK, Iwashita T, et al. Galanin receptor subtype 2 suppresses cell proliferation and induces apoptosis in p53 mutant head and neck cancer cells. Clin Cancer Res. 2009; 15:2222–2230. [PubMed: 19276245]
41. Ruggero D, Pandolfi PP. Does the ribosome translate cancer? Nat Rev Cancer. 2003; 3:179–192. [PubMed: 12612653]
42. Montanaro L, Brigotti M, Clohessy J, et al. Dyskerin expression influences the level of ribosomal RNA pseudo-uridylation and telomerase RNA component in human breast cancer. J Pathol. 2006; 210:10–18. [PubMed: 16841302]
43. Gu BW, Zhao C, Fan JM, Dai Q, Bessler M, Mason PJ. Anomalous electrophoretic migration of newly synthesized ribosomal RNAs and their precursors from cells with DKC1 mutations. FEBS Lett. 2009; 583:3086–3090. [PubMed: 19729012]
44. Boisvert FM, van KS, Navascues J, Lamond AI. The multifunctional nucleolus. Nat Rev Mol Cell Biol. 2007; 8:574–585. [PubMed: 17519961]
45. Gartel AL, Kandel ES. miRNAs: Little known mediators of oncogenesis. Semin Cancer Biol. 2008; 18:103–110. [PubMed: 18295504]
46. Wu CH, Sahoo D, Arvanitis C, Bradon N, Dill DL, Felsher DW. Combined analysis of murine and human microarrays and ChIP analysis reveals genes associated with the ability of MYC to maintain tumorigenesis. PLoS Genet. 2008; 4 e1000090.
47. Schlosser I, Holzel M, Murnseer M, Burtscher H, Weidle UH, Eick D. A role for c-Myc in the regulation of ribosomal RNA processing. Nucleic Acids Res. 2003; 31:6148–6156. [PubMed: 14576301]
48. Slizhikova DK, Vinogradova TV, Sverdlov ED. [The NOLA2 and RPS3A genes as highly informative markers for human squamous cell lung cancer]. Bioorg Khim. 2005; 31:195–199. [PubMed: 15889794]
49. Vulliamy T, Beswick R, Kirwan M, et al. Mutations in the telomerase component NHP2 cause the premature ageing syndrome dyskeratosis congenita. Proc Natl Acad Sci U S A. 2008; 105:8073–8078. [PubMed: 18523010]
50. Walne AJ, Vulliamy T, Marrone A, et al. Genetic heterogeneity in autosomal recessive dyskeratosis congenita with one subtype due to mutations in the telomerase-associated protein NOP10. Hum Mol Genet. 2007; 16:1619–1629. [PubMed: 17507419]
51. Trahan C, Martel C, Dragon F. Effects of dyskeratosis congenita mutations in dyskerin, NHP2 and NOP10 on assembly of H/ACA pre-RNPs. Hum Mol Genet. 2010; 19:825–836. [PubMed: 20008900]
52. Grozdanov PN, Roy S, Kittur N, Meier UT. SHQ1 is required prior o NAF1 for assembly of H/ACA small nucleolar and telomerase RNPs. RNA. 2009; 15:1188–1197. [PubMed: 19383767]

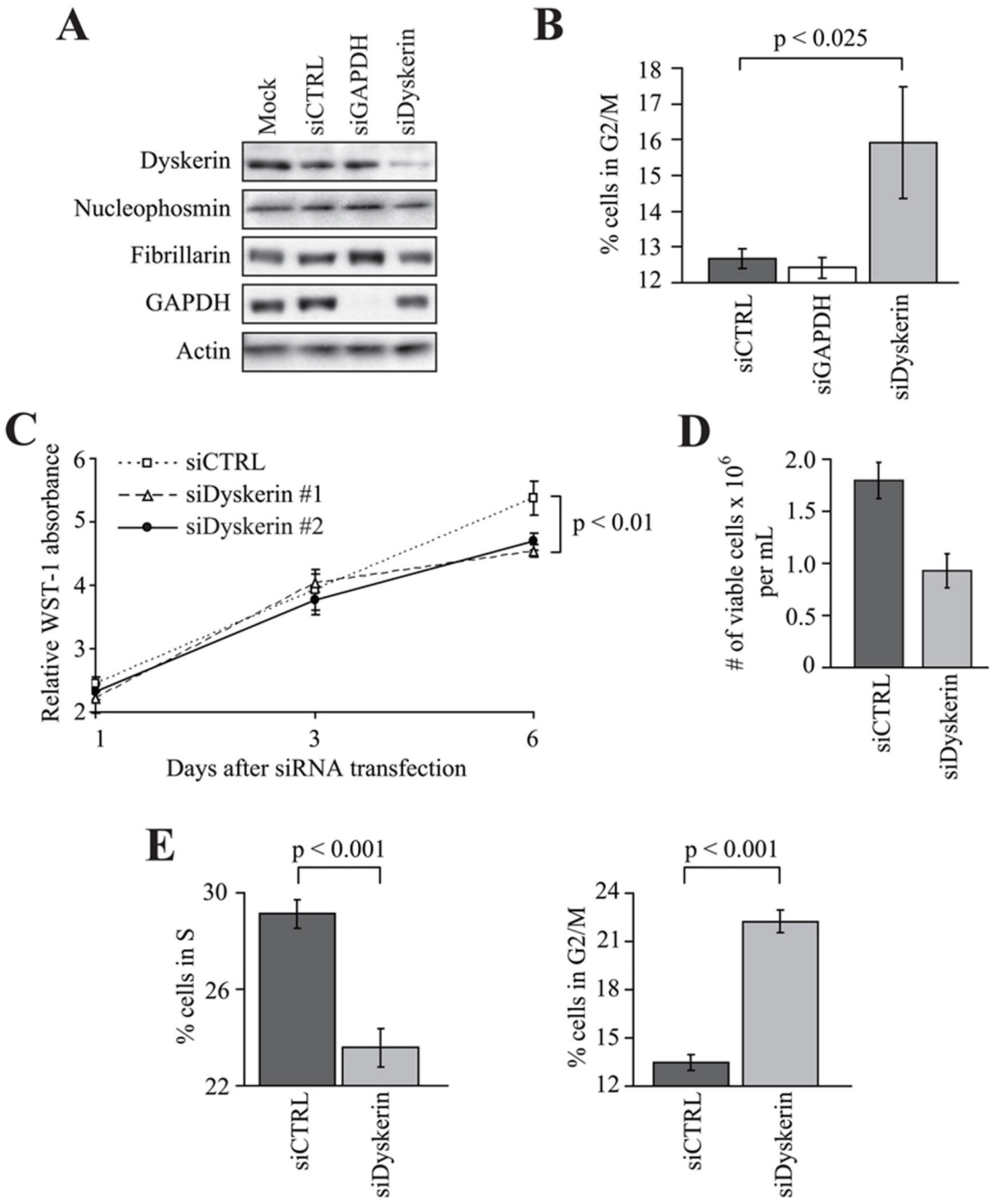

Figure 1. Loss of dyskerin function reduces the proliferation of telomerase-negative U2OS cells

A, U2OS cells were transiently transfected with siRNA directed against dyskerin (siDyskerin) or GAPDH (siGAPDH). A non-target control (siCTRL) was used as a negative control. By 3 days after initial transfection, dyskerin expression was reduced by more than 80% relative to the controls, while GAPDH expression was essentially undetectable. There was no effect on the expression of other rRNA processing factors, including fibrillarin and nucleophosmin. Similar results were obtained using a coding region siRNA (siDyskerin#1; shown here) and a custom-designed *DKC1* siGENOME SMARTPool (siDyskerin #2; not shown). A western blot demonstrating dyskerin depletion after transfection with siDyskerin #2 is shown in Fig. 6A. **B,** Cells were labeled with propridium iodide and analyzed by flow

cytometry. There was a significant increase in the proportion of dyskerin-depleted cells in G_2/M ($p < 0.025$). **C,** U2OS cells showed a significant reduction ($p < 0.01$) in their proliferation after six days of persistent dyskerin depletion, irrespective of the siRNA used. Relative cell proliferation was measured using the WST-1 colorimetric assay. **D,** Total number of viable siDyskerin cells was reduced by almost 50% relative to the siCTRL cells. There was no difference in the number of dead cells between the two conditions (not shown). **E,** After six days of persistent dyskerin depletion, flow cytometric analysis revealed only a small but highly significant increase in the proportion of siDyskerin cells in G_2/M. Only the siDyskerin #2 results are shown here. Error bars denote the standard deviations derived from parallel transfections performed in triplicate wells. The experiments were conducted twice for the cell proliferation assays and three times for the cell cycle analyses. One representative experiment is shown here.

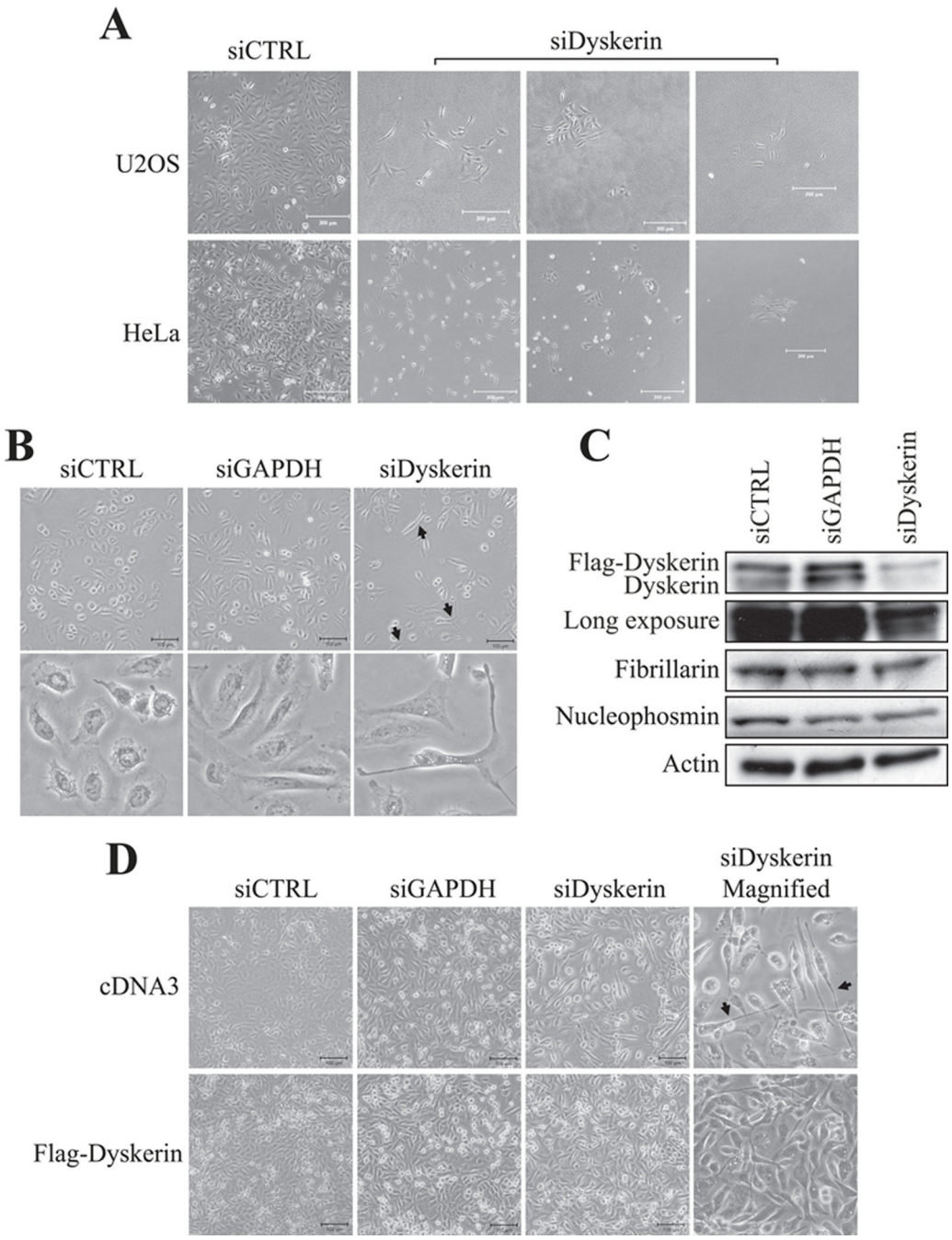

Figure 2. Suppression of dyskerin function inhibits anchorage-independent growth and alters cell morphology

A, U2OS and HeLa cells were transfected and 24 hours later, seeded into soft agar. After 5 days of growth, the cells were analyzed by phase contrast microscopy. While the siCTRL cells readily grew under these conditions, siDyskerin cells proliferated very poorly in the absence of a solid matrix. Bars = 300 μM. **B,** U2OS cells were transfected with siDyskerin #1. After the 5th day, cells were harvested and re-seeded into 100 cm^2 culture dishes. Twenty-four hours later they were fixed and analyzed. Loss of dyskerin resulted in fewer adherent cells relative to the controls and, of the cells that remained, several showed thin, elongated cytoplasmic processes (arrows). A representative focus from each panel is

enlarged to show detail. **C,** Cells were transfected with the siRNA targeting the 3'-untranslated region of *DKC1* (siDyskerin #2). On the 4th day, the cells were transfected with pcDNA3-Flag-dyskerin. Immunoblotting with anti-dyskerin antibody showed expression of the exogenous Flag-dyskerin transgene while endogenous dyskerin was effectively depleted. As a negative control, the cells were also transfected with pcDNA3 only (not shown). Transfections were performed in duplicate with similar results. **D,** In the absence of dyskerin, the cells exhibited abnormal morphology (arrows). In contrast, restoration of dyskerin function increased the number and restored the morphology of U2OS cells that were depleted of the endogenous protein. A representative focus from the siDyskerin panel is enlarged to show detail. Bars = 100 μM.

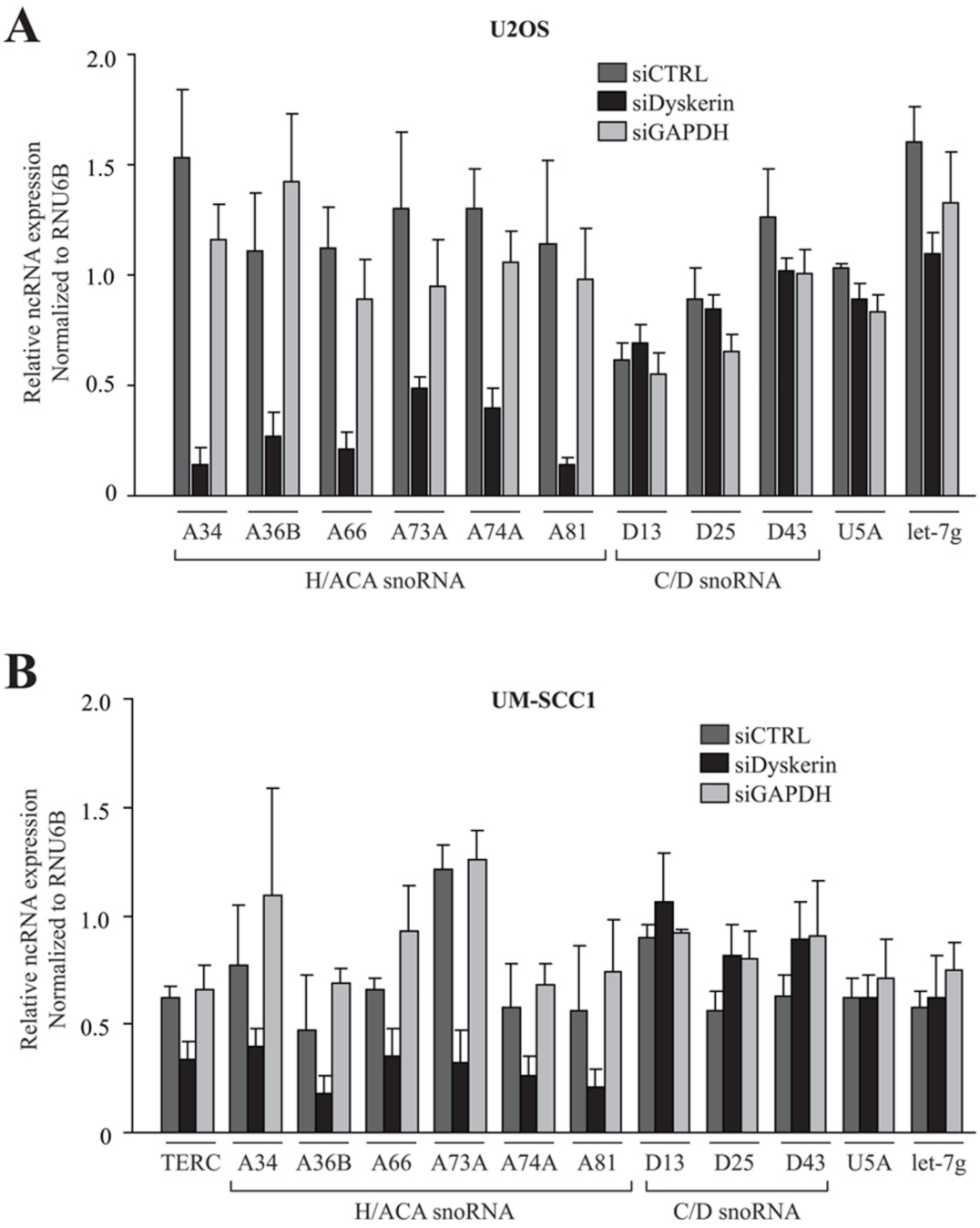

Figure 3. Loss of dyskerin selectively reduces the accumulation of H/ACA RNAs

A, U2OS and **B,** UM-SCC1 cells were harvested on the 3rd day after siRNA transfection. Total RNA was extracted and then enriched into one fraction containing small RNA (<200 nucleotides) and another containing large RNA. There was a greater than 2-fold decrease in the expression of H/ACA snoRNAs, relative to the siCTRL and siGAPDH controls, following dyskerin depletion in both cell lines. In contrast, there was essentially no effect on the accumulation of other small RNAs that are not known to be bound to or stabilized by dyskerin. **B,** TERC expression was also reduced by more than 2-fold in UM-SCC1 cells. As expected, TERC expression was not detectable in U2OS cells. Except for TERC (*), the levels of each transcript was measured relative to that of RNU6B, and then normalized to

the ratio of the respective transcripts in the wild-type, untreated cells. We note that similar results were obtained when transcript levels were normalized to RNU5A expression (not shown). *Since TERC is 451 nucleotides in length, this transcript was enriched in the large RNA fraction. Thus, for TERC analysis, quantitation was performed relative to β-actin expression. The solid bars represent the mean values derived from qRT-PCR analyses of triplicate transfections conducted in one representative experiment; error bars denote standard deviations. The experiment was repeated twice with similar results.

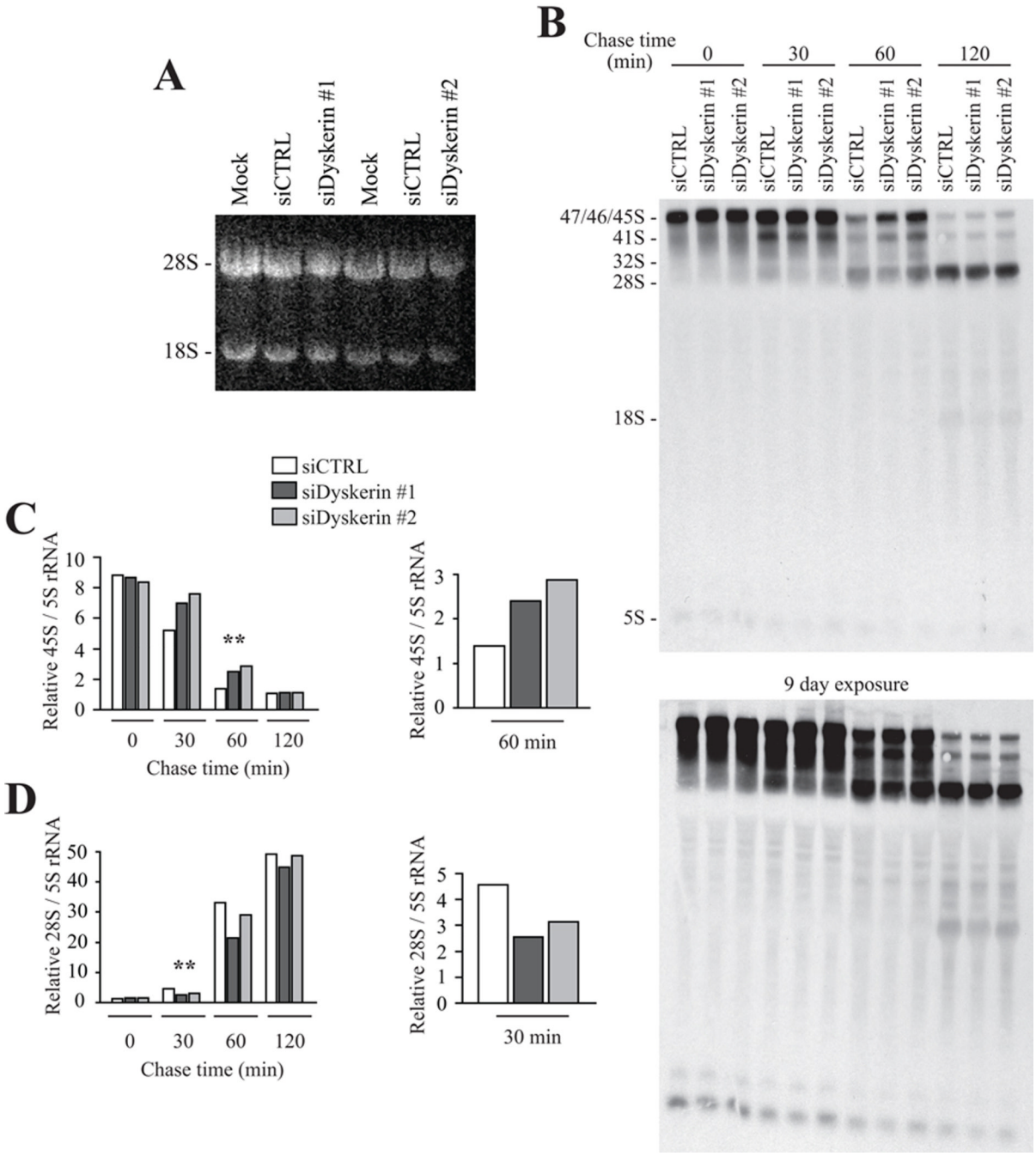

Figure 4. Dyskerin depletion transiently impairs precursor rRNA processing

U2OS cells were transfected with the appropriate siRNAs and analyzed six days later. **A,** There was no appreciable effect on the amounts of either mature 18S or 28S rRNA following dyskerin depletion. The gel was stained with ethidium bromide and the bands were visualized on an Image Station 4000 (Kodak, Rochester, NY). **B,** The cells were metabolically labeled with L-[methyl-^{3}H]-methionine as described in Methods and Materials. After 30 minutes, the 45S rRNA precursor persisted in the siDyskerin cells relative to the siCTRL cells. This was associated with a decrease in the amount of newly synthesized 28S rRNA. Following 60 minutes, persistence of 45S rRNA is clearly evident in the siDyskerin cells, while differences in the levels of 28S rRNA are not as dramatic. By

120 minutes, the rate of rRNA processing was essentially the same in the siCTRL and siDyskerin cells. Similar results were observed with both dyskerin siRNAs. The bottom panel shows the same blot exposed for nine days. **C,** Relative quantitation of 45S rRNA normalized to 5S rRNA over the duration of the pulse-chase experiment. The values at the 60 minute time point (**) are shown in the right panel. **D,** Quantitation of 28S rRNA normalized to the levels of 5S rRNA. The relative accumulation of 28S rRNA at the 30 minute timepoint (**) is shown on the right. All quantitations were performed using NIH Image J. One representative experiment is shown.

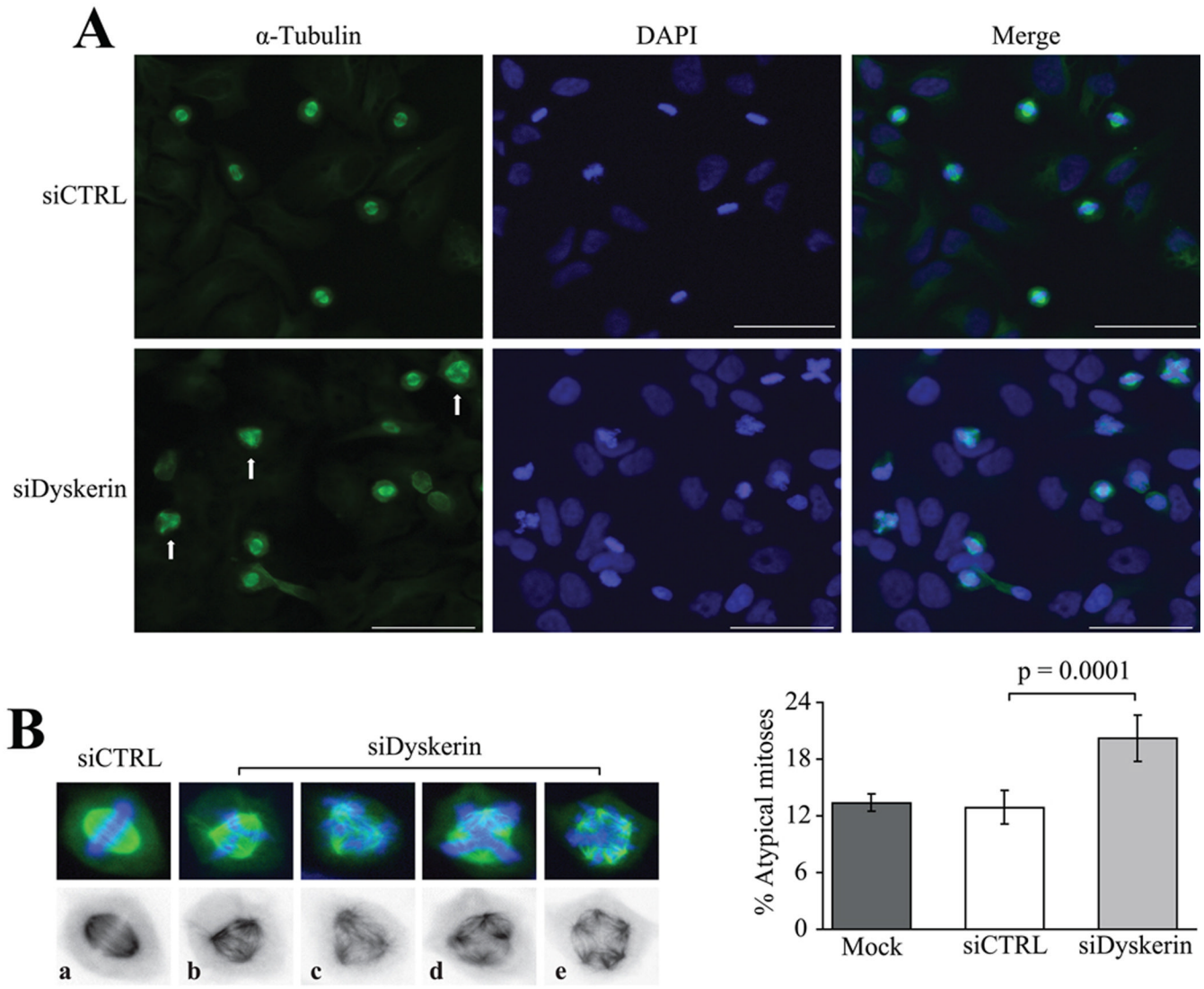

Figure 5. Loss of dyskerin leads to the accumulation of multi-polar mitotic spindles

HeLa cells were cultured in 4-well chamber slides and transfected with siDyskerin or siCTRL in three of the four wells. Seventy-two hours later, the cells were fixed, and then immunolabeled with α-tubulin and DAPI. The experiments were then repeated to yield six unique sets of dyskerin-depleted immunolabeled cells, six sets of siCTRL cells, and four sets of mock-transfected HeLa cells. At least 350 consecutive mitoses (metaphase and anaphase only) were then counted from each of the wells, and the number of atypical mitoses was measured as a percentage of the total number of metaphase and anaphase cells counted. **A,** Representative field demonstrating three mitotic figures with multi-polar spindles in the siDyskerin cells (arrows). Six normal-appearing mitoses are seen in the siCTRL panel. Bar = 100 μM. **B,** For illustrative purposes: (a) normal bi-polar spindle (metaphase), (b) tri-polar (metaphase), (c) tri-polar (anaphase), (d) quadri-polar (metaphase), and (e) hexa-polar (metaphase). Overall, more than 19% of siDyskerin metaphases and/or anaphase cells exhibited multi-polar spindles. In contrast, only 12–13% of the siCTRL and mock-transfected mitoses, respectively, were atypical; p = 0.0001. The solid bars represent the average number of atypical mitoses calculated for each group; error bars denote standard deviations.

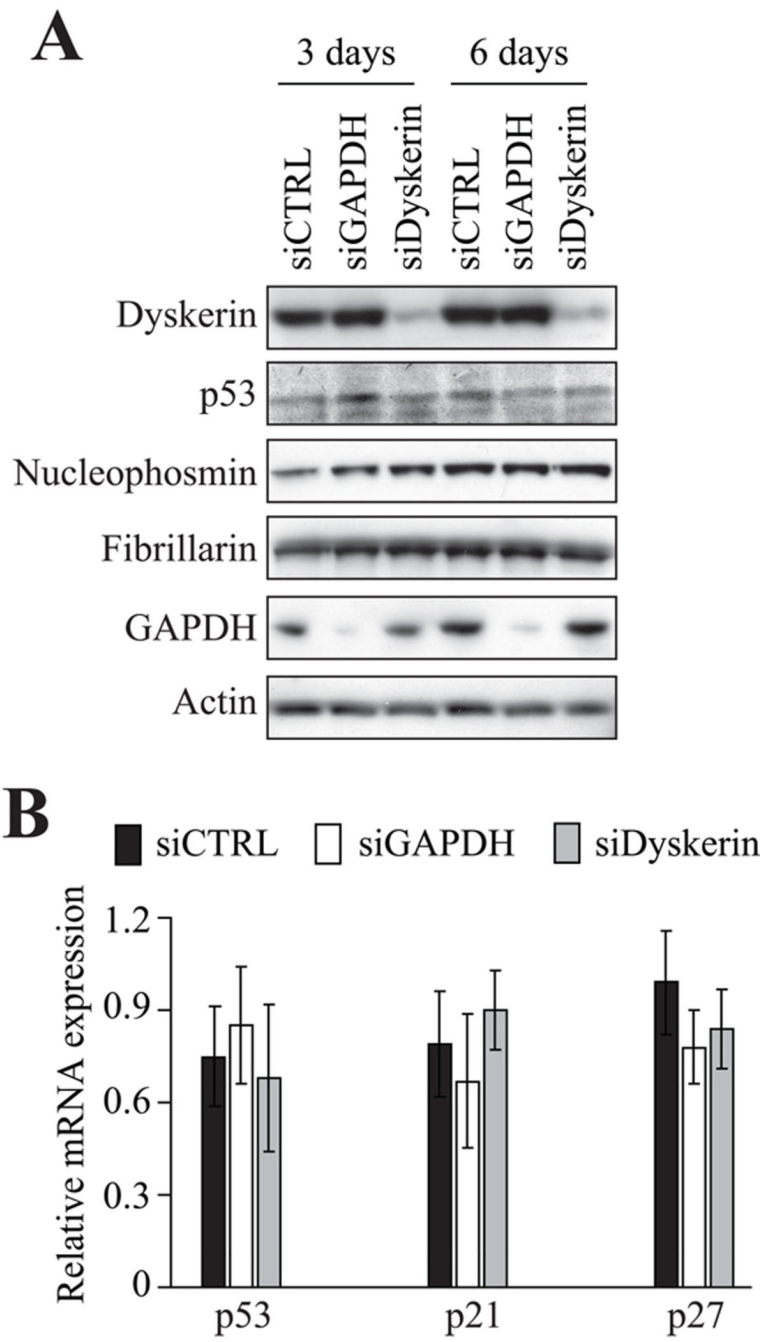

Figure 6. Downregulation of dyskerin has no effect on p53 expression

A, Dyskerin expression remained low for at least 6 days following siRNA transfection. However, p53 levels remained constant for the duration of the experiment. There was also no effect on other rRNA processing factors, including fibrillarin and nucleophosmin. **B,** There was no change in mRNA expression of p53 or of its transcriptional targets p21 or p27. Quantitation was performed relative to β-actin expression. The solid bars represent the mean values derived from qRT-PCR analyses of triplicate transfections conducted in one representative experiment; error bars denote standard deviations. The experiment was repeated three times with similar results.