Tumor and Stem Cell Biology

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Novel Dyskerin-Mediated Mechanism of p53 Inactivation through Defective mRNA Translation

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Abstract

In up to 60% of human cancers, *p53* gene mutations are responsible for direct inactivation of the tumor suppressor function of p53. Alternative mechanisms of p53 inactivation described thus far mainly affect its posttranslational regulation. In X-linked dyskeratosis congenita, a multisystemic syndrome characterized by increased cancer susceptibility, mutations of the *DKC1* gene encoding dyskerin cause a selective defect in the translation of a subgroup of internal ribosome entry site (IRES)–containing cellular mRNAs. In this study, we show that impairment of dyskerin function can cause p53 inactivation due to a defect in p53 mRNA translation. siRNA-mediated reduction of dyskerin levels caused a decrease of p53 mRNA translation, protein levels, and functional activity, both in human breast cancer cells and in primary mammary epithelial progenitor cells. These effects seemed to be independent of the known role of dyskerin in telomerase function, and they were associated with a specific impairment of translation initiation mediated by IRES elements present in p53 mRNA. In a series of human primary breast cancers retaining wild-type p53, we found that low levels of dyskerin expression were associated with reduced expression of p53-positive target genes. Our findings suggest that a dyskerin-mediated mechanism of p53 inactivation may occur in a subset of human tumors. *Cancer Res; 70(11); 4767–77. ©2010 AACR.*

Introduction

The tumor protein p53 plays a well-established role in protecting against cancer by controlling the cellular response against various types of acute stress (1). The levels of p53 are generally influenced by its posttranslational degradation via the proteasome. In unstressed cells, p53 is indeed present in a latent state and is maintained at low levels through MDM2-dependent targeted degradation (2, 3). The exposure to different cellular stresses blocks p53 degradation, thus giving rise to its accumulation (1). The effects of the cellular response to this p53 activation may be ascribed to the role of p53 as a transcriptional modulator that regulates the expression of a wide and heterogeneous group of responsive genes. In general, p53 activation inhibits cell growth through cell cycle arrest or through induction of either proliferative senescence or apoptosis, thus preventing tumor development (4). In up to 60% of human cancers, *p53* gene mutations directly inactivate p53 function (5). Mechanisms leading to indirect p53 inactivation acting mainly on p53 posttranslational degradation have also been described in tumors (2, 3, 6–9). In addition to the block of its degradation, p53 induction after stress also requires its neosynthesis, which entails the transcription (10) and the active translation of its mRNA (11). p53 mRNA translation seems to be regulated by proteins binding p53 mRNA (12–14) as well as by internal ribosome entry site (IRES) elements present in p53 mRNA (15), which are able to mediate translation initiation under stress.

The impairment in proper translational control has been recently associated with cancer development and progression (16). In the rare multisystemic syndrome X-linked dyskeratosis congenita (X-DC), mutations of the *DKC1* gene encoding dyskerin, a pseudouridine synthase that modifies rRNA (17), cause a defect in the translation of a subgroup of IRES-containing cellular mRNAs (18) and are associated with increased cancer susceptibility (19, 20). The lack of dyskerin function is associated with an increased susceptibility to breast carcinomas in the DKC1 hypomorphic mouse (21), and dyskerin expression and functions are also reduced in a subset of human breast carcinomas (22). In the present study, we assessed whether the lack of dyskerin may cause a p53 inactivation induced by a defect in IRES-mediated mRNA translation in human breast epithelial cells. Moreover, we investigated the relationship between DKC1 mRNA levels

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and the expression of p53 target genes in primary human breast carcinomas. Our results show that the decrease of dyskerin levels reduces p53 levels and activity through a defect in IRES-dependent p53 mRNA translation, and that in primary tumors, low levels of dyskerin expression are associated with reduced p53 activity, thus suggesting the existence of a translational mechanism involving dyskerin for the inactivation of p53 in human tumors.

Materials and Methods

Cells and reagents

Cells were obtained from the American Type Culture Collection and IZSLER. During the study, cell line identity was verified by DNA fingerprinting and day-by-day morphology checking. Subconfluent cells were treated with MG115 and MG132 (10 nmol/L; Calbiochem), ionizing radiation (6 Gy), doxorubicin (0.5, 1, and 4 μmol/L), and deferoxamine (100 μmol/L; Sigma-Aldrich). For survival assays, cells were either trypsinized and counted after trypan blue staining or formalin fixed and stained with 0.05% crystal violet for 10 minutes.

Mammospheres (MS) were obtained as previously described, downscaling the method for small tissue quantities (300–900 mg; ref. 23). Fresh surgical specimens, obtained from patients with ductal breast carcinoma who underwent quadrantectomy or mastectomy, were collected to generate MS. Normal and tumor samples were histologically characterized to ensure the proper classification of normal and tumor tissue. Particular care was paid to generate MS from specimens in which only normal or tumor tissues were detectable at histologic examination. Briefly, tissues were placed in sterile Epicult (StemCell Technologies), minced, and incubated for 6 to 12 hours in the presence of 1,000 units of collagenase/hyaluronidase enzyme mix (StemCell Technologies). Samples were centrifuged at 80 × *g* for 2 minutes. The pellet was digested with dispase and DNase for 3 minutes (StemCell Technologies) and then pelleted at 450 × *g* for 5 minutes. Pellets were resuspended, filtered through a 40-μm nylon mesh (BD), and plated into low attachment wells (Corning), filled with mammary epithelial growth medium (MEGM) and supplemented with 10 ng/mL epidermal growth factor, 10 ng/mL basic fibroblast growth factor, 10 μg/mL insulin, 1 μmol/L hydrocortisone, and aliquots of gentamycin and amphotericine (Cambrex). Primary MS began to form after 4 to 6 days and were processed at day 10. Experimental procedures were performed on secondary MS, generated by incubating primary MS in 1× trypsin-EDTA solution (Cambrex) for 3 minutes, followed by two washes in complete MEGM and filtration through a 40-μm nylon mesh. When indicated, MS were treated with 5 μmol/L doxorubicin for 24 hours.

RNA interference

DKC1- and p53-specific double-stranded siRNAs and appropriate controls were obtained from Invitrogen. DKC1 RNA interference (RNAi) was done with either single or three pooled siRNA oligonucleotides (Invitrogen). p53 RNAi was done using the specific Stealth validated RNAi DuoPak. siRNAs were transfected in adherent cells using Lipofectamine 2000 (Invitrogen) and in MS using *in vitro* JET-PEI reagent (Polyplus Transfection).

Western blot

SDS-PAGE and immunoblotting were carried out according to standard procedures. Antibodies were anti-dyskerin (Santa Cruz Biotechnology), anti-p53 (clone BP-53-12, Novocastra), anti-p21 (clone SX118, Dako), and anti–β-actin (Sigma-Aldrich).

Real-time and semiquantitative PCR

Total RNA was extracted from frozen samples using Trizol reagent (Invitrogen) and reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR analysis was done in a Gene Amp 7000 Sequence Detection System (Applied Biosystems) using the TaqMan approach. For each sample, three replicates were analyzed. Sets of primers and fluorogenic probes specific for DKC1, p53, p21, PUMA, and MDM2 mRNAs were purchased from Applied Biosystems, whereas hTR-specific primers and probe were synthesized as described (22). The relative amounts of the studied target genes were calculated using the expression of human β-glucuronidase (Applied Biosystems) and 18S RNA (for polysomal analysis) as an endogenous control. The final results were determined as follows: N target = $2^{-(\Delta \mathrm{Ct\ sample}\ -\ \Delta \mathrm{Ct\ calibrator})}$, where ΔCt values of the sample and calibrator were determined by subtracting the Ct value of the endogenous control gene from the Ct value of each target gene. Telomerase activity was measured by real-time PCR using the Quantitative Telomerase Detection Kit (Allied Biotech, Inc.). Each evaluation was done in triplicate using 1 μg of protein extract. For the other mRNAs studied by semiquantitative reverse transcription-PCR (RT-PCR), the following primers and annealing temperatures were used, respectively: p21 mRNA, 5′-TGGGGATGTCCGTCAGAACC, 5′ TGGAGTGGTAGAAATCTCTCATGCT, 59°C; BNIP3, 5′-CGTTCCAGCCTCGGTTTCTATTTA, 5-CGCCTTCCA-ATATAGATCCCCAAT, 51°C; PUMA, 5′-CAGACTGT-GAATCCTGTGCT, 5′-ACAGTATCTTACAGGCTGCC, 62°C. Cycling conditions were predenaturation step at 95°C for 2 minutes; 28 cycles of denaturation at 95°C for 1 minute, annealing at the appropriate temperature for 1 minute, extension at 72°C for 1 minute; final extension at 72°C for 7 minutes.

Analysis of protein synthesis in cells

Protein synthesis was measured as the rate of incorporation of labeled leucine during a 30-minute incubation of the subconfluent cell monolayers in complete medium containing 50 mg/L leucine and trace amounts of [^{3}H]leucine as previously described (24).

Isolation of polyribosomal mRNA

Subconfluent cells were washed in PBS at 4°C. The cellular pellet was lysed in 2 volumes of 10 mmol/L

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Tris-HCl (pH 7.4), 10 mmol/L NaCl, 3 mmol/L $MgCl_2$ 0.5% NP40 for 10 minutes at 4°C. The lysates were then centrifuged at 14,000 × *g* for 10 minutes at 4°C and the supernatant was used for the isolation of polyribosomes. Lysates were stratified onto a 15% to 50% sucrose gradient in 30 mmol/L HEPES/KOH (pH 7.5), 80 mmol/L KCl, 1.8 mmol/L Mg-acetate, and centrifuged at 4°C for 15 hours at 40,000 × *g*. From gradients, 1-mL fractions were collected and their absorbance was read at 260 nm. Polyribosomal fractions were pooled and centrifuged at 100,000 × *g* for 15 hours at 4°C. RNA was extracted from pellets using Trizol reagent.

DNA and mRNA transfection

For the evaluation of p53 transcriptional activity, cells were transfected with 300 ng of DNA per sample of the PathDetect p53 *cis*-reporter gene plasmid (Stratagene). As transfection efficiency control, 100 ng of pRL-CMV (Promega) per sample were cotransfected in each case. After 24 hours, cells were harvested for luciferase assay. For the RNA transfection, capped mRNA was transcribed from linearized pR-CrPV-IRES-F (gift from Dr. Davide Ruggero, University of California, San Francisco, San Francisco, CA; ref. 18) and pR-p53-IRES-F plasmids (gift from Dr. Barsanjit Mazumder, Cleveland State University, Cleveland, OH; ref. 25) using the mMessage mMachine T7 kit (Ambion) and cells were transfected with 1 μg of RNA per sample using DMRIEC (Invitrogen). After 8-hour transfection, cells were harvested and analyzed with dual-luciferase kit (Promega).

Patient materials

One hundred nineteen breast carcinomas were selected from a series of consecutive patients who had undergone surgical resection for primary breast carcinoma at the Surgical Department of the University of Bologna between 1994 and 2006, on the sole basis of frozen tissue availability. Nuclear immunostaining of p53 (clone BP53-12.1, Biogenex) and p21 (clone EA10, Oncogene Science) was assessed by image cytometry and expressed as the percentage of labeled nuclear area over the total neoplastic nuclear area (labeling index). For assessing p53 status, we considered that the *p53* gene was mutated when more than 10% of the tumor cell nuclei were stained and p21 labeling index was less than 10% (26). In a subset of cases (34 of 119), p53 status was also assessed by direct sequencing. Tumors were grouped (high, medium, and low expression) according to DKC1 mRNA levels measured by real-time RT-PCR using the previously described survival-associated cutoffs (22).

Statistical analysis

The χ^2 or Mann-Whitney *U* test, when appropriate, was used for the comparisons among groups. Agreement between scores was assessed by the κ statistics. All statistics were obtained using the SPSS statistical software package (SPSS, Inc.). *P* values <0.05 were regarded as statistically significant.

Results

Lack of dyskerin hinders p53 mRNA translation in human breast cancer cells

We selectively reduced DKC1 mRNA levels by transient RNAi-mediated knockdown (KD) in p53 wild-type MCF-7 breast cancer cells. This approach led to a strong reduction in DKC1 mRNA and protein lasting for at least 96 hours (Fig. 1A). The reduction of dyskerin levels did not cause a global decrease in protein synthesis, as evaluated by [^{3}H]leucine incorporation, as well as in mRNA translation, evaluated by measuring the activity of firefly luciferase after transfection of an *in vitro* transcribed capped mRNA (Fig. 1B). Whereas DKC1 KD had no significant effects on global p53 mRNA levels (Fig. 1C, left), it strongly decreased the recruitment of p53 mRNA to polysomal fractions (Fig. 1C, right). As previously described for DKC1 mutations (17), DKC1 KD also influenced the translation of the IRES-containing XIAP mRNA, whereas it did not affect the translation of the housekeeping β-actin mRNA (Fig. 1C). XIAP mRNA levels are known to be constant and XIAP regulation seems to occur specifically at the translational level (27). Surprisingly, our results show that after DKC1 KD, there is a 1.7-fold increase in XIAP mRNA. We may hypothesize that, because DKC1 KD strongly decreases XIAP translation, a feedback network might increase XIAP mRNA transcription or stability.

We then investigated whether the observed translational defect of p53 mRNA affects p53 level. Our results indicate that DKC1 KD strongly downregulated the p53 levels at the steady state (Fig. 1D, left). We confirmed these results by using three different siRNAs for DKC1 KD also (Supplementary Fig. S1). To evaluate if the decrease of dyskerin affects p53 levels independently from its proteasome-mediated degradation, we compared p53 levels in control and DKC1 KD MCF-7 cells, exposed to the specific proteasome chemical inhibitors MG115 and MG132. Also under these conditions, DKC1 KD strongly reduced p53 levels, showing that the reduction of dyskerin levels downregulates p53levels independently of proteasome-mediated degradation (Fig. 1D, right).

Dyskerin reduction impairs p53 function in human breast cancer cells

To evaluate the functional consequences on p53 of the translational defect observed, first we assessed if DKC1 KD leads to a reduction in p53 transcriptional activity. To this purpose, a reporter assay in which firefly luciferase is placed under the control of a repeated p53-specific response element was used. In DKC1 KD cells, we observed a strong reduction of p53 transcriptional activity, similar to that obtained through the transfection of p53-specific siRNAs (Fig. 2A). Next, to acquire more information on the effect of dyskerin reduction and p53 function, we exposed DKC1 KD MCF-7 cells to different stresses activating the p53-dependent response. After exposure to ionizing radiations, DKC1 KD reduced the p53 accumulation and the expression of the p53 target gene product p21 (ref. 28; Fig. 2B). DKC1 KD also induced a strong reduction of p53 accumulation after

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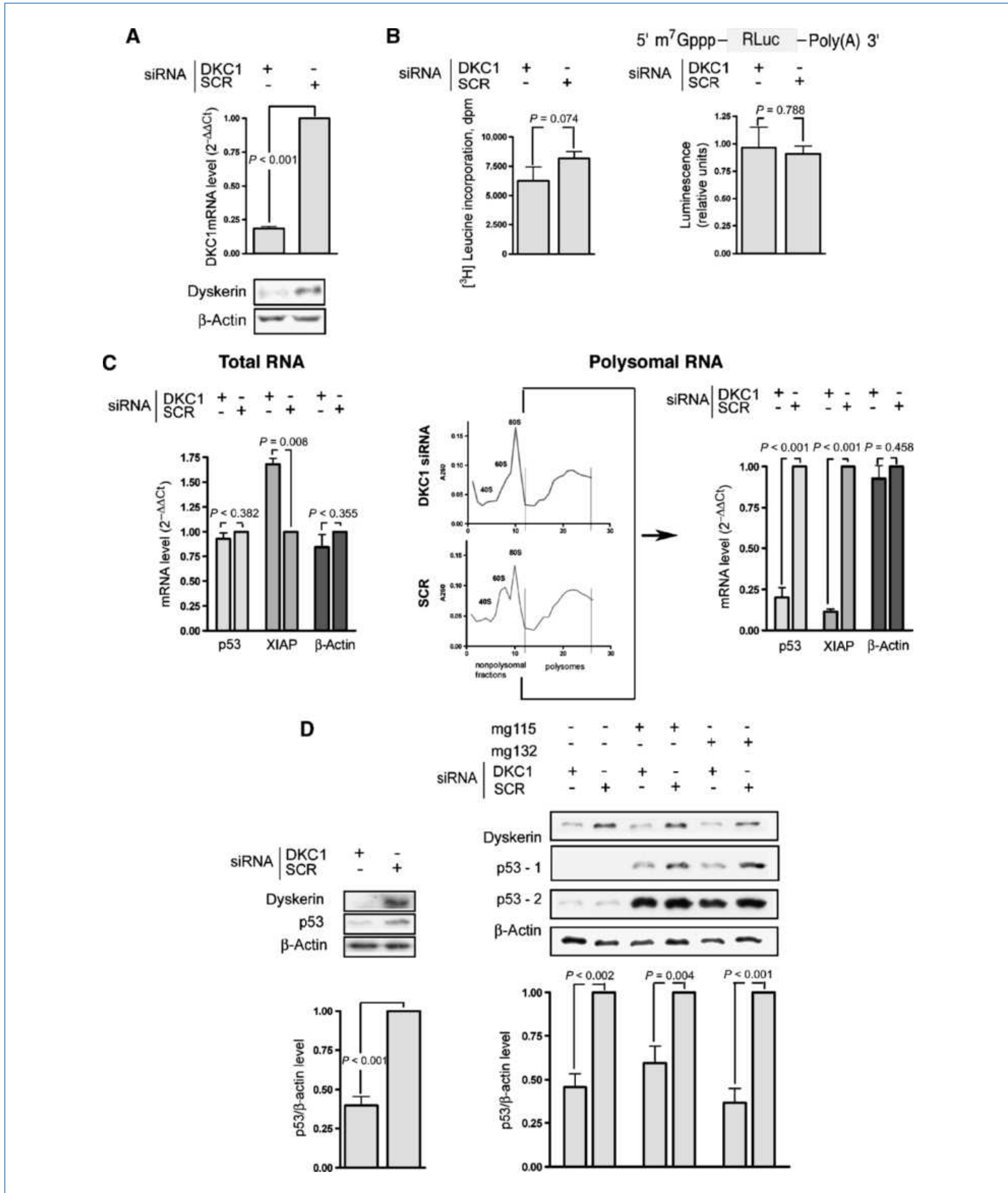

Figure 1. Dyskerin KD hinders p53 mRNA translation in breast cancer cells. A, dyskerin expression by real-time RT-PCR and Western blot analysis in MCF-7 cells after transfection with DKC1-specific or control (SCR) siRNAs. B, [^{3}H]Leucine incorporation and Renilla luciferase (Rluc) activity after transfection of an *in vitro* transcribed capped mRNA in DKC1 KD and control MCF-7 cells. C, total (left) and polysome-associated (right) p53, XIAP, and β-actin mRNA levels assessed by real-time PCR after DKC1 KD (representative polysomal profiles are shown). D, immunoblots showing steady-state p53 levels in DKC1 KD and control MCF-7 cells (left). Immunoblots showing p53 levels in DKC1 KD and control MCF-7 cells treated for 3 h with MG115 or MG132 (right). Different exposure times are shown for p53. siRNA transfection was done 96 h before cell harvesting. Histograms represent means and SDs from three independent experiments.

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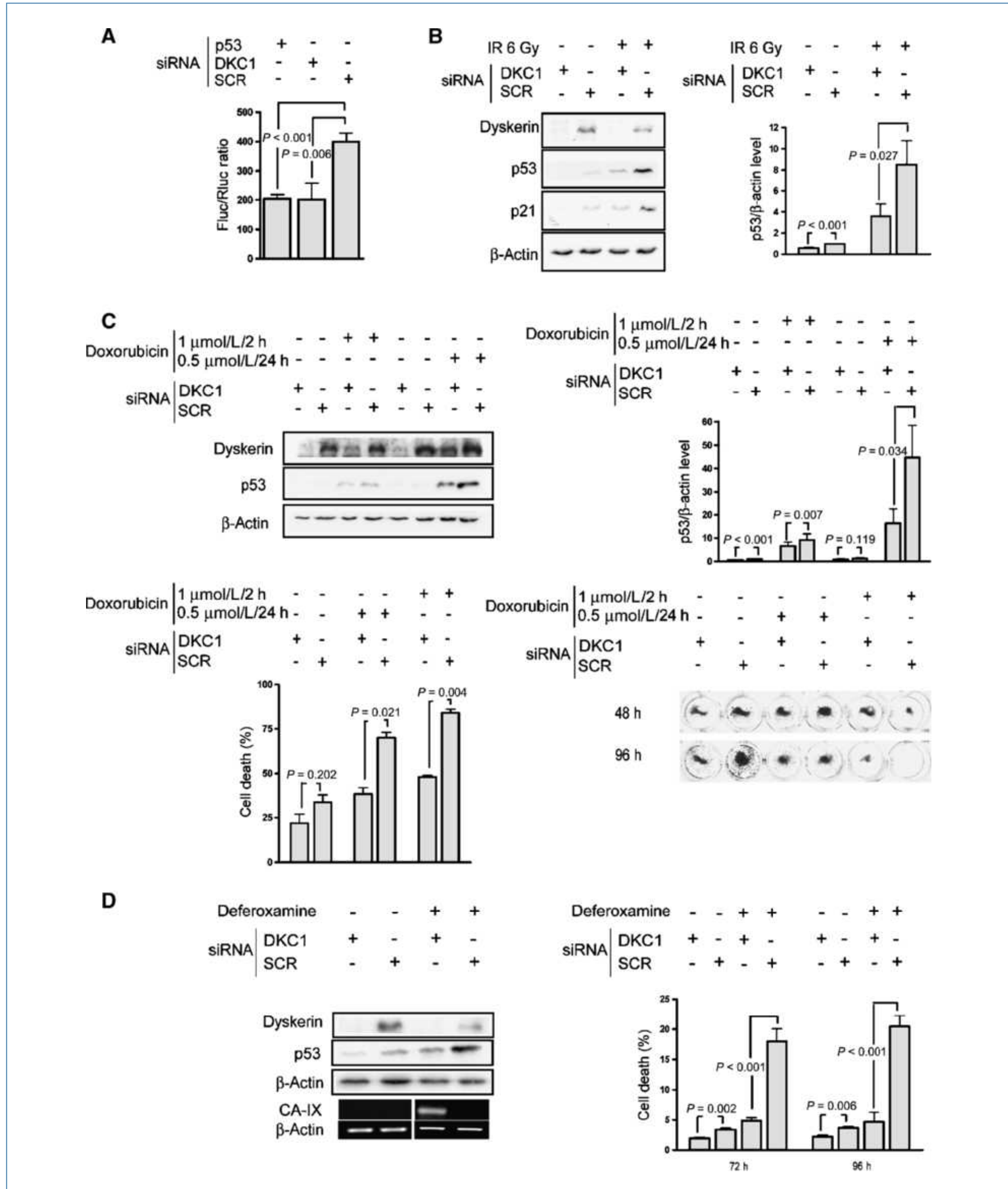

Figure 2. Reduction of dyskerin levels downregulates p53-mediated response in breast cancer cells. A, p53 transcriptional activity after DKC1, p53, and control (SCR) siRNA transfection, assessed by reporter assay. B, immunoblots showing p53 and p21 levels in DKC1 KD and control cells 8 h after ionizing radiation exposure. C, immunoblots showing p53 levels in DKC1 KD and control cells after doxorubicin treatment (top). Cell death (by trypan blue exclusion test; bottom left) and growth (after crystal violet staining; bottom right) analyses under doxorubicin treatment are also shown. D, immunoblots and RT-PCR analysis showing p53 and CA-IX mRNA levels, respectively, in DKC1 KD and control cells for 48 h with deferoxamine. Cell death analysis under DFX treatment is also shown. Experiments were done in MCF-7 cells. siRNA transfection was done 96 h before cell harvesting. Histograms represent means and SDs from three independent experiments.

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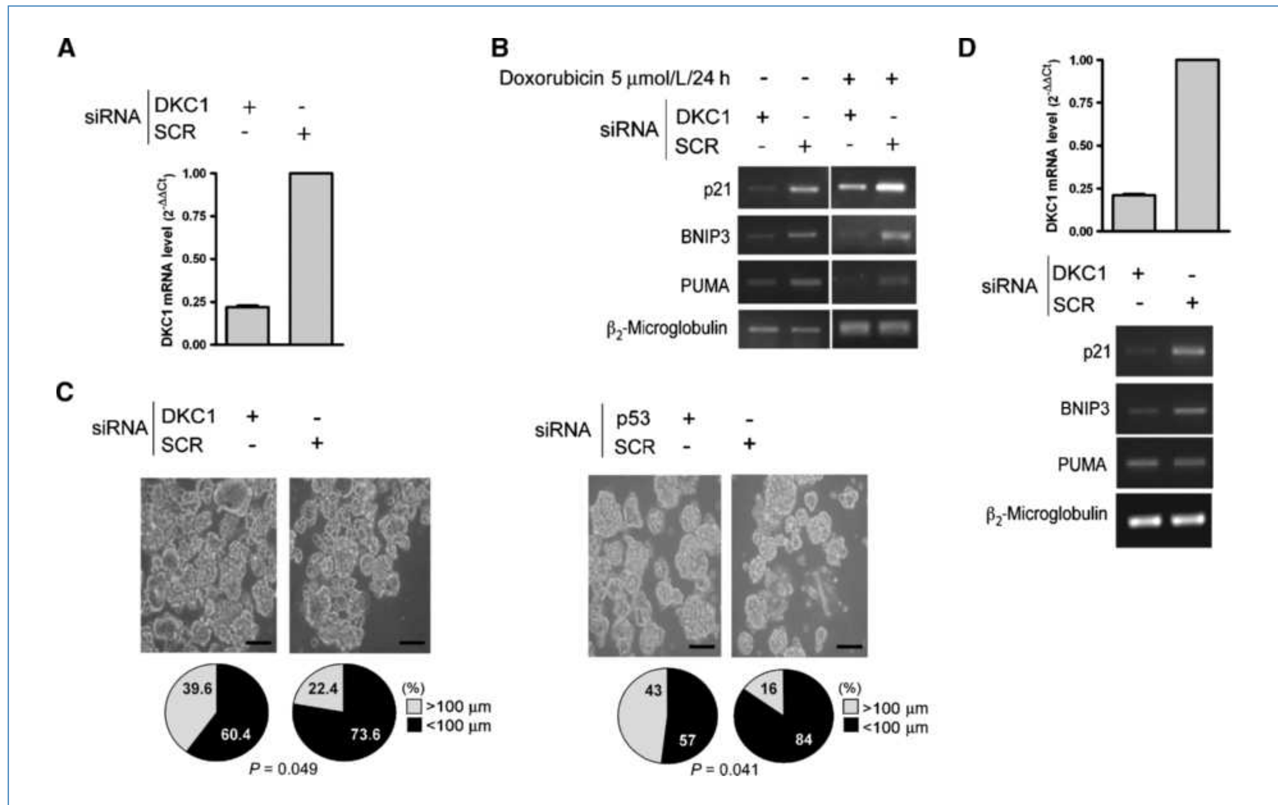

Figure 3. The reduction of dyskerin levels downregulates p53 activity in human primary mammary gland progenitors. A, DKC1 mRNA levels by real-time RT-PCR in human normal mammary gland tissue after 96 h in DKC1 KD and control (SCR) cells. B, semiquantitative RT-PCR analysis showing expression of p21, PUMA, and BNIP3 in primary human MS from normal tissue after DKC1 KD at the basal level and after doxorubicin treatment. C, size of MS from human normal mammary gland tissue 96 h after DKC1 and p53 KD. Phase-contrast microscopy micrographs and MS size distribution are shown. Bar, 100 μm. D, DKC1 mRNA levels by real-time RT-PCR and expression of p21, PUMA, and BNIP3 by semiquantitative RT-PCR analysis in primary human MS from tumoral tissue 96 h after DKC1 KD.

treatment with the genotoxic agent doxorubicin (Fig. 2C). This effect was associated with an increased cellular resistance to p53-dependent doxorubicin-induced death and with a growth advantage under doxorubicin treatment (Fig. 2C). Moreover, DKC1 KD strongly inhibited the p53 accumulation induced by the hypoxia-mimetic agent deferoxamine (Fig. 2D). In these conditions, the expression of the hypoxia-induced, p53 negatively regulated carbonic anhydrase IX gene (29) was strongly upregulated, indicating a reduction in p53-mediated transcriptional repression. After DKC1 KD, cells were also more resistant to deferoxamine-mediated cell death, an observation consistent with the known prosurvival effect of p53 reduction and carbonic anhydrase IX overexpression under hypoxia-like conditions (refs. 30, 31; Fig. 2D). Significantly, we confirmed that the reduction of dyskerin levels also affects p53 accumulation in an additional p53 wild-type breast cancer cell line such as ZR-75-1 (ref. 32; Supplementary Fig. S2).

Dyskerin reduction impairs p53 function in primary mammary gland progenitors

To verify if the observed impairment of p53 function may also occur in human nonneoplastic primary breast epithelial cells, we evaluated the effect of DKC1 KD incultures of progenitor cells derived from normal mammary gland tissue expanded as MS (23, 33). siRNA sequences were effectively delivered in MS, producing a strong DKC1 KD (Fig. 3A). Although the material obtained from this source was too scarce for assessing p53 levels by Western blot analysis, we were able to document that DKC1 KD reduced the expression of direct p53 targets such as p21 (28), PUMA (34), and BNIP3 (35) both at the steady state and after exposure to doxorubicin (Fig. 3B), thus indicating a decrease of p53 activity. Moreover, dyskerin reduction led to an increase in the size of the MS, as it occurs in consequence of p53 KD (Fig. 3C). Also in a similar experiment performed in MS obtained from a human primary breast carcinoma (36), DKC1 KD lowered the levels of p21 and BNIP3 mRNAs (Fig. 3D). Besides providing information on primary breast epithelial cells of human origin, these results on MS are particularly relevant in light of the observation that carcinogenesis in the mammary gland might result from transformation of progenitor cells by the deregulation of self-renewal pathways in which p53 is largely involved (37, 38).

Dyskerin reduction acts on p53 independently of its role in telomerase

One well-characterized result of dyskerin dysfunction is the impairment of telomerase complex functionality due

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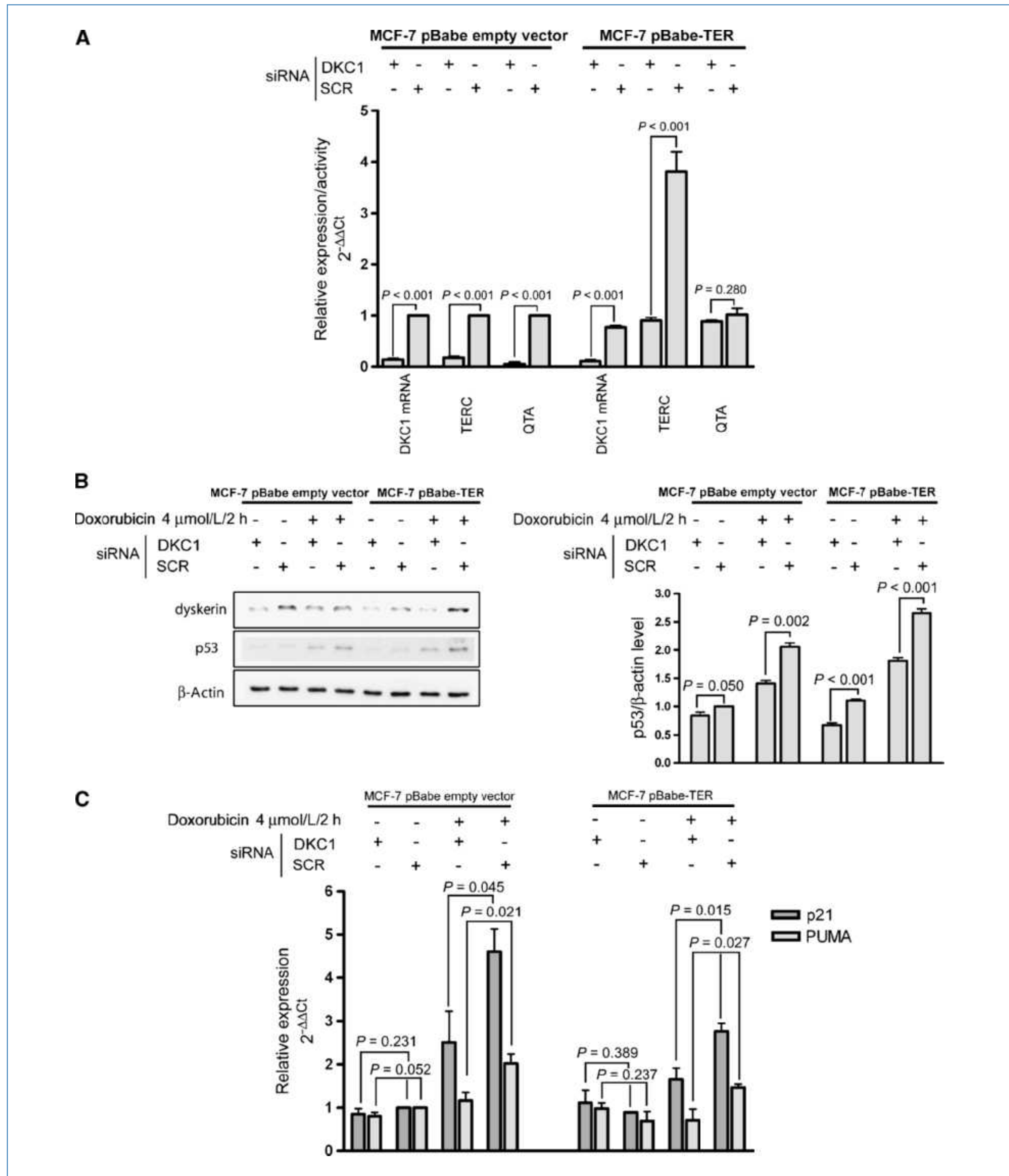

Figure 4. Reduction of dyskerin levels acts on p53 independently of its effects on telomerase activity. A, DKC1 mRNA expression, TERC levels measured by real-time PCR, and quantitative telomerase activity by a telomere repeat amplification protocol measured by real-time PCR in pBabe-TER MCF-7 cells and in control pBabe empty vector MCF-7 cells after DKC1 and control (SCR) siRNA transfection. B, representative immunoblots showing dyskerin and p53 levels in TERC-overexpressing pBabe-TER and in pBabe empty vector MCF-7 control cells after DKC1 and control (SCR) siRNA transfection at the steady state and after doxorubicin treatment. C, expression of p21 and PUMA mRNAs by real-time RT-PCR in pBabe-TER and in control pBabe empty vector MCF-7 cells after DKC1 and control (SCR) siRNA transfection at the steady state and after doxorubicin treatment. siRNA transfections were done 96 h before cell harvesting. All histograms show fold changes relative to the values observed in the control pBabe empty vector MCF-7 cells. Histograms represent means and SDs from three independent experiments.

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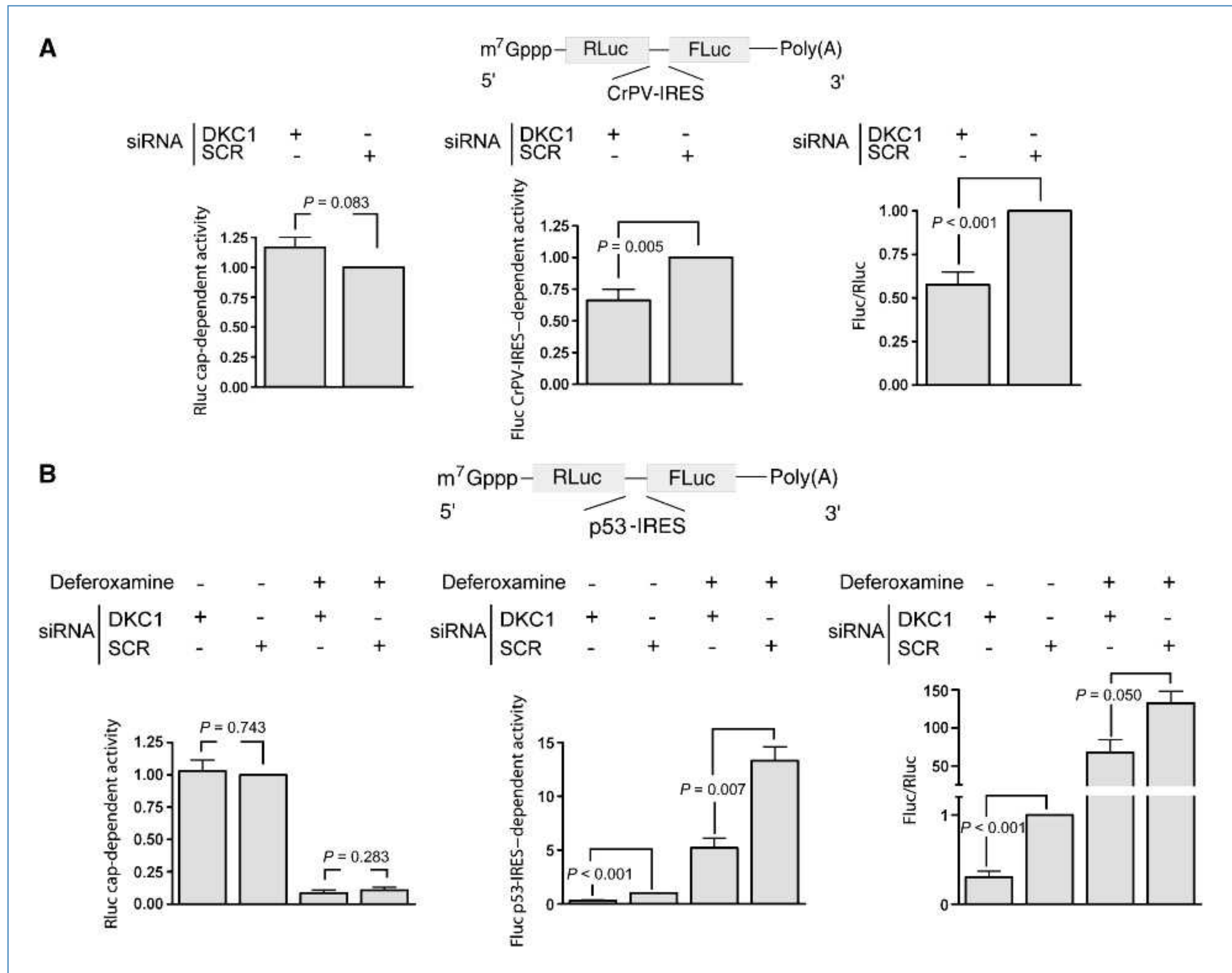

Figure 5. Reduction of dyskerin levels impairs p53-IRES–mediated translation. A, IRES-mediated translation assessed by measuring the FLuc and RLuc activity in MCF-7 cells after DKC1 and control (SCR) siRNA transfection and 8 h after transfection with a bicistronic mRNA transcribed from pRL CrPV-IRES. B, p53-IRES–mediated translation after DKC1 KD and 8 h after transfection with a bicistronic mRNA transcribed from pRL p53-IRES in MCF-7 cells with or without 72-h deferoxamine treatment. siRNA transfection was done 96 h before cell harvesting. Histograms represent means and SDs from three independent experiments.

to telomerase RNA component (TERC) destabilization (39). This was observed also in MCF-7 cells after DKC1 KD (Fig. 4A). In cells derived from X-DC patients, the overexpression of TERC is sufficient to rescue the telomerase defect associated with *DKC1* gene mutation (40). To rule out the possibility that the effect of DKC1 KD on p53 may be in any way linked to the impairment of telomerase function, we generated, by retroviral transduction, MCF-7 cells stably overexpressing TERC (MCF-7 pBabe-TER; ref. 41). In these cells, due to the high rate of TERC transcription, DKC1 KD did not cause a significant impairment in telomerase activity (Fig. 4A) but strongly lowered p53 levels both at the steady state and after doxorubicin treatment, similarly to what occurred in the empty vector–infected control cells (MCF-7 pBabe empty vector; Fig. 4B). Also, DKC1 KD reduced the expression levels of p53 target genes such as p21 and PUMA after doxorubicin treatment both in MCF-7 pBabe empty vector cells and in MCF-7 pBabe-TER, showing that p53 activity is not influenced by the effect of dyskerin reduction on telomerase activity (Fig. 4C). These results indicate that the reduction in dyskerin levels affects p53 levels and function independently of its role in telomerase activity.

Dyskerin reduction induces defective p53-IRES–mediated translation

We then investigated the mechanism leading to the alteration in p53 translation. The translation of a group of IRES-containing mRNAs is defective in cells bearing dysfunctional dyskerin (18). We evaluated how IRES-mediated translation is regulated in our model by measuring the activity of a firefly luciferase encoded by a downstream cistron under the control of an IRES sequence in a bicistronic mRNA. As occurs in X-DC (18), DKC1 KD in MCF-7 cells induced a defective translation

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mediated by viral IRES sequences, such as the cricket paralysis virus (CrPV)-IRES (Fig. 5A). Using a similar bicistronic mRNA approach, we then evaluated whether the translation mediated by an IRES sequence described in p53 mRNA can be influenced by dyskerin levels. DKC1 KD strongly impaired the p53-IRES–mediated translation both at the steady state and under deferoxamine (Fig. 5B). Remarkably, such hypoxia-like conditions elicited a strong reduction in the global levels of protein synthesis and cap-dependent translation (Fig. 5B and Supplementary Fig. S3A), similarly to what occurs in the presence of low oxygen tension (42). On the other hand, p53 accumulation required active mRNA translation (Supplementary Fig. S3B). According to this, p53-IRES–mediated translation substantially increased under deferoxamine treatment (Fig. 5B). In principle, such an increase should strongly contribute to maintaining p53 synthesis when cap-dependent translation is repressed. The observed impairment of p53-IRES–mediated translation should then be considered particularly relevant for p53 function under stress conditions. The obtained results were confirmed in MCF-7 cells using three different siRNA sequences and in other breast cancer–derived cell lines such as ZR-75-1 and MDA-MB-468 (Supplementary Fig. S4).

Dyskerin expression and p53 function in human breast cancer

We next aimed at investigating whether a similar mechanism could be present also in primary breast tumors. To this end, we measured DKC1 mRNA expression in a series of primary breast carcinomas. Supplementary Table S1 summarizes the biopathologic features of the studied series. To test the relationship between dyskerin expression and p53 activity in this series, we have first defined the p53 status by immunohistochemical analysis. The putatively p53-mutated tumors were identified by both the presence of p53 and the absence of p21 in the same specimen (26). We also assessed the agreement of the immunohistochemical method of p53 mutation detection with direct sequencing in a subgroup of cases using the κ statistics. A κ value of 1.00 indicates perfect agreement, and 0 indicates a level of agreement by chance only. The obtained κ value was 0.72, indicating a good concordance. Then, in the whole subgroup of immunohistochemically defined p53 wild-type tumors, we measured by real-time RT-PCR the expression levels of p21 and MDM2, two p53 target genes. The expression of these two genes resulted to be significantly associated with DKC1 mRNA levels (Fig. 6A), indicating a reduction of p53 activity in tumors expressing low dyskerin mRNA levels, grouped according to the previously described cutoff values of DKC1 mRNA expression (22). In the same series, we also studied the relationship between p53 status and dyskerin expression. We found that tumors with low dyskerin expression were more frequently characterized by a p53 wild-type status than tumors with high dyskerin expression (Fig. 6B), thus supporting the hypothesis that decreased dyskerin expression may represent an alternative mechanism of p53 inactivation, which, in some cases, replaces the p53 mutation mechanism.

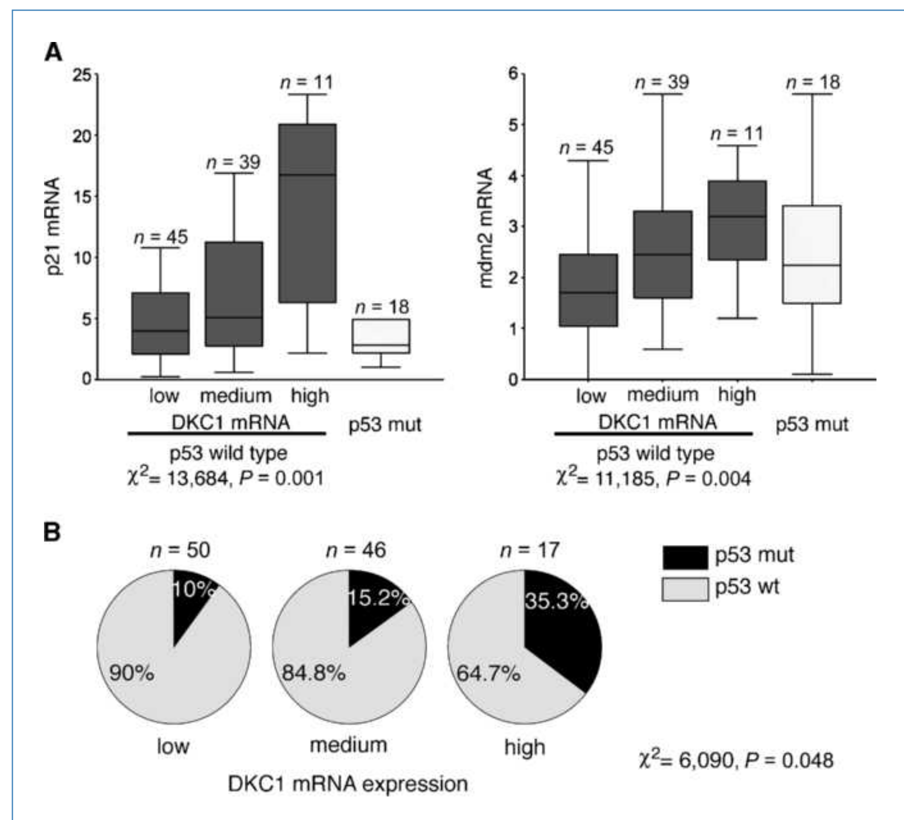

Figure 6. DKC1 mRNA expression levels and p53 function and status in human breast carcinomas. A, p21 and MDM2 mRNA expression by real-time PCR in p53 wild-type breast carcinomas grouped according to dyskerin mRNA expression levels. Gray box plots show mRNA values within the three DKC1 mRNA groups. The median value is depicted by horizontal columns, the interquartile range by boxes, and the minimum and maximum values by vertical columns. The expression in p53-mutated tumors is also shown for reference (white box plots). B, frequencies of p53-mutated primary breast carcinomas grouped according to DKC1 mRNA.

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Discussion

Our results show a novel dyskerin-dependent mechanism of p53 inactivation acting on p53 mRNA translation. The reduction of dyskerin levels impairs p53 function independently of its well-known role in telomerase activity and causes a strong defect in p53-IRES–mediated translation initiation. Data obtained on primary breast cancer specimens suggest that the described dyskerin-dependent mechanism of p53 inactivation may be active in a subset of human breast carcinomas.

It has been previously shown that the efficient translation of p53 mRNA is necessary for proper p53-mediated response (11). In this study, for the first time, we report that the lack of dyskerin impinges p53 mRNA translation and efficient p53-mediated response. This result provides a rationale for explaining the increased tumor susceptibility in X-DC. In fact, this phenotype can result from the contribution of the altered translation of p53 mRNA as well as of other IRES-containing mRNAs that code for tumor suppressors, such as p27 mRNA, as already described (18). In a recent report, an enhanced p53 response was identified in cells from mice bearing a specific truncating dyskerin mutation also found in the X-DC family (43). Our findings in the breast epithelial cellular types studied are not in line with such an observation. We may attempt to explain this discrepancy with the different approaches used to affect dyskerin function. In fact, in the previous study, the particular properties of the DKC1 mutation used may affect dyskerin function differently than the KD approach used in the present report. Our results also indicate that the defect in p53 translation and function is independent of the relevant role played by dyskerin in telomerase function. Given the well-established role of dyskerin in the site-specific rRNA uridine modification (44), this finding supports the proposed model in which an intrinsic ribosomal defect might affect the translation of specific cellular mRNAs (45). Our results also indicate that the reduction of dyskerin levels induced a strong defect in p53-IRES–mediated translation. Studies using different experimental approaches indicate that several cellular mRNAs coding for tumor suppressors contain an IRES element (15, 46, 47) and that the loss of proper IRES-mediated translation can contribute to tumorigenesis (18, 48). The observation that the decrease of IRES translation is associated with a strong reduction in the activity of p53 supports the involvement of defective IRES-mediated translation in tumorigenesis.

In the DKC1 hypomorphic mice, the defective uridine modification in rRNA has been associated with a high susceptibility to tumors, including breast carcinomas (21). We previously showed that dyskerin expression is extremely variable in human sporadic tumors of various histologic origins (22). In particular, in breast carcinomas, tumors characterized by very low dyskerin levels also exhibit defective rRNA pseudouridylation (22). In the present study, the observations obtained in human primary breast carcinomas indicate that p53 function is also reduced in the presence of low dyskerin levels. It is also worth recalling that mutation of the *p53* gene occurs in only about 20% of breast carcinomas, whereas in other solid tumors the mutation rates are about 70% (49, 50). Because the proper function of dyskerin is necessary for efficient p53 mRNA translation, it is tempting to speculate that, in breast carcinomas not bearing direct *p53* gene mutations, the alteration in dyskerin function could be one of the mechanisms resulting in a decrease of p53 activity, thus contributing to the neoplastic phenotype.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Vousden KH, Lane DP. p53 in health and disease. Nat Rev Mol Cell Biol 2007;8:275–83.
2. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. Nature 1997;387:296–9.
3. Kubbutat MH, Jones SN, Vousden KH. Regulation of p53 stability by Mdm2. Nature 1997;387:299–303.
4. Vousden KH, Lu X. Live or let die: the cell's response to p53. Nat Rev Cancer 2002;2:594–604.
5. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. Science 1991;253:49–53.
6. Linares LK, Hengstermann A, Ciechanover A, Müller S, Scheffner M. HdmX stimulates Hdm2-mediated ubiquitination and degradation of p53. Proc Natl Acad Sci U S A 2003;100:12009–14.
7. Danovi D, Meulmeester E, Pasini D, et al. Amplification of Mdmx (or Mdm4) directly contributes to tumor formation by inhibiting p53 tumor suppressor activity. Mol Cell Biol 2004;24:5835–43.
8. Sherr CJ. Tumor surveillance via the ARF-p53 pathway. Genes Dev 1998;12:2984–91.
9. Colaluca IN, Tosoni D, Nuciforo P, et al. NUMB controls p53 tumour suppressor activity. Nature 2008;451:76–80.
10. Hollstein M, Hainaut P. Massively regulated genes: the example of TP53. J Pathol 2010;220:164–73.
11. Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW. Participation of p53 protein in the cellular response to DNA damage. Cancer Res 1991;51:6304–11.
12. Mosner J, Mummenbrauer T, Bauer C, Sczakiel G, Grosse F, Deppert W. Negative feedback regulation of wild-type p53 biosynthesis. EMBO J 1995;14:4442–9.

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13. Takagi M, Absalon MJ, McLure KG, Kastan MB. Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. Cell 2005;123:49–63.
14. Grover R, Raym PS, Das S. Polypyrimidine tract binding protein regulates IRES-mediated translation of p53 isoforms. Cell Cycle 2008;7:2189–98.
15. Yang DQ, Halaby MJ, Zhan Y. The identification of an internal ribosomal entry site in the 5′-untranslated region of p53 mRNA provides a novel mechanism for the regulation of its translation following DNA damage. Oncogene 2006;25:4613–9.
16. Schneider RJ, Sonenberg N. Translational control in cancer development and progression. In: Translational control in biology and medicine. In: Mathews MB, Sonenberg N, Hershey JWB, editors. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2007.
17. Meier UT. The many facets of H/ACA ribonucleoproteins. Chromosoma 2005;114:1–14.
18. Yoon A, Peng G, Brandenburger Y, et al. Impaired control of IRES-mediated translation in X-linked dyskeratosis congenita. Science 2006;312:902–6.
19. 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;19:32–8.
20. Dokal I. Dyskeratosis congenita in all its forms. Br J Haematol 2000; 110:768–79.
21. Ruggero D, Grisendi S, Piazza F, et al. Dyskeratosis congenita and cancer in mice deficient in ribosomal RNA modification. Science 2003;299:259–62.
22. Montanaro L, Brigotti M, Clohessy J, et al. Dyskerin expression influences the level ofribosomal RNA pseudo-uridylation and telomerase RNA component in human breast cancer. J Pathol 2006;210:10–8.
23. Dontu G, Abdallah WM, Foley JM, et al. *In vitro* propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 2003;17:1253–70.
24. Petronini PG, Tramacere M, Mazzini A, Piedimonte G, Silvotti L, Borghetti AF. Hyperosmolarity-induced stress proteins in chick embryo fibroblasts. Exp Cell Res 1987;172:450–62.
25. Chaudhuri S, Vyas K, Kapasi P, et al. Human ribosomal protein L13a is dispensable for canonical ribosome function but indispensable for efficient rRNA methylation. RNA 2007;13:2224–37.
26. Nenutil R, Smardova J, Pavlova S, et al. Discriminating functional and non-functional p53 in human tumours by p53 and MDM2 immunohistochemistry. J Pathol 2005;207:251–9.
27. Holcik M. Translational upregulation of the X-linked inhibitor of apoptosis. Ann N Y Acad Sci 2003;1010:249–58.
28. El-Deiry WS, Harper JW, O'Connor PM, et al. WAF1/CIP1 is induced in p53-mediated G_1 arrest and apoptosis. Cancer Res 1994;54: 1169–74.
29. Kaluzová M, Kaluz S, Lerman MI, Stanbridge EJ. DNA damage is a prerequisite for p53-mediated proteasomal degradation of HIF-1α in hypoxic cells and downregulation of the hypoxia marker carbonic anhydrase IX. Mol Cell Biol 2004;24:5757–66.
30. Hammond EM, Giaccia AJ. The role of p53 in hypoxia-induced apoptosis. Biochem Biophys Res Commun 2005;331:718–25.
31. Sansone P, Storci G, Tavolari S, et al. IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. J Clin Invest 2007;117:3988–4002.
32. Wasielewski M, Elstrodt F, Klijn JG, Berns EM, Schutte M. Thirteen new p53 gene mutants identified among 41 human breast cancer cell lines. Breast Cancer Res Treat 2006;99:97–101.
33. Mani SA, Guo W, Liao MJ, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 2008;133:704–15.
34. Yu J, Wang Z, Kinzler KW, Vogelstein B, Zhang L. PUMA mediates the apoptotic response to p53 in colorectal cancer cells. Proc Natl Acad Sci U S A 2003;100:1931–6.
35. Yan J, Yun H, Yang Y, Jing B, Feng C, Song-bin F. Upregulation of BNIP3 promotes apoptosis of lung cancer cells that were induced by p53. Biochem Biophys Res Commun 2006;346:501–7.
36. Storci G, Sansone P, Trere D, et al. The basal-like breast carcinoma phenotype is regulated by SLUG gene expression. J Pathol 2008; 214:25–38.
37. Liu S, Dontu G, Wicha MS. Mammary stem cells, self-renewal pathways, and carcinogenesis. Breast Cancer Res 2005;7:86–95.
38. Korkaya H, Wicha MS. Selective targeting of cancer stem cells: a new concept in cancer therapeutics. BioDrugs 2007;21:299–310.
39. Mitchell JR, Wood E, Collins K. A telomerase component is defective in the human disease dyskeratosis congenita. Nature 1999; 402:551–5.
40. Wong JM, Collins K. Telomerase RNA level limits telomere maintenance in X-linked dyskeratosis congenita. Genes Dev 2006;20: 2848–58.
41. Montanaro L, Calienni M, Ceccarelli C, et al. Relationship between dyskerin expression and telomerase activity in human breast cancer. Cell Oncol 2008;30:483–90.
42. Braunstein S, Karpisheva K, Pola C, et al. A hypoxia-controlled cap-dependent to cap-independent translation switch in breast cancer. Mol Cell 2007;28:501–12.
43. 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;105:10173–8.
44. Wang C, Query CC, Meyer UT. Immunopurified small nucleolar ribonucleoprotein particles pseudouridylate rRNA independently of their association with phosphorylated Nopp140. Mol Cell Biol 2002;22:8457–66.
45. Montanaro L, Trere D, Derenzini M. Nucleolus, ribosomes, and cancer. Am J Pathol 2008;173:301–10.
46. Kullmann M, Göpfert U, Siewe B, Hengst L. ELAV/Hu proteins inhibit p27 translation via an IRES element in the p27 5′UTR. Genes Dev 2002;16:3087–99.
47. Wilker EW, van Vugt MA, Artim SA, et al. 14-3-3σ controls mitotic translation to facilitate cytokinesis. Nature 2007;446:329–32.
48. Barna M, Pusic A, Zollo O, et al. Suppression of Myc oncogenic activity by ribosomal protein haploinsufficiency. Nature 2008; 456:971–5.
49. Gasco M, Shami S, Crook T. The p53 pathway in breast cancer. Breast Cancer Res 2002;4:70–6.
50. Lacroix M, Toillon RA, Leclercq G. p53 and breast cancer, an update. Endocr Relat Cancer 2006;13:293–325.

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