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DKC1 is a direct and conserved transcriptional target of c-MYC

Faizan Alawi *, Megan N. Lee

School of Dental Medicine, University of Pennsylvania, Department of Pathology, 240 South 40th Street, Philadelphia, PA 19104, USA

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Abstract

Recent studies have identified upregulation of the dyskeratosis congenita 1 (*DKC1*) gene in association with various sporadic cancers. Whole genome analyses have suggested that *DKC1* may be regulated by the c-MYC oncoprotein. c-MYC is among the most commonly deregulated proteins in human cancer. However, controversy remains as to whether *DKC1* is a direct or indirect target of c-MYC. Using human and rodent cell lines expressing conditionally active *c-MYC* transgenes, we show that c-MYC activation is associated with relatively acute induction of *DKC1* expression. Chromatin immunoprecipitation assays reveal c-MYC binding to two distinct, phylogenetically conserved regions within the *DKC1* promoter and intron one. We further demonstrate that c-MYC-mediated *Dkc1* transcription can occur in the absence of *de novo* protein synthesis. These data indicate that *DKC1* is a direct and conserved transcriptional target of c-MYC, and suggest a biologic basis for *DKC1* overexpression in neoplasia.

Keywords: Dyskerin; Ribosome biogenesis; Telomerase; c-MYC-estrogen receptor; Gene regulation; E-box

Dyskerin, the product of the dyskeratosis congenita 1 (*DKC1*) gene, is a nucleolar, RNA-binding protein that is highly conserved in eukaryotes [1]. Dyskerin is an integral component of the telomerase ribonucleoprotein complex, and is required for normal telomerase activity and telomere maintenance [2]. Dyskerin also plays an important role in rRNA processing, and is necessary for normal ribosome biogenesis [3]. Recently, a role in internal ribosome entry site (IRES)-mediated translation has also been identified [4].

Germline mutations in the *DKC1* gene give rise to the X-linked recessive form of dyskeratosis congenita (DC)—a rare, multisystem disorder associated with cancer susceptibility (OMIM #305000; [1]). However, *DKC1* is not mutated but rather frequently overexpressed in sporadic cancers [5–8]. Yet, the factors that stimulate upregulation of *DKC1* in neoplasia remain unknown.

c-MYC encodes a basic helix–loop–helix transcription factor that modulates a number of vital cellular processes, including telomerase activation and ribosome biogenesis; its deregulated expression has been implicated in the pathogenesis of various cancers [9]. c-MYC often activates multiple genes within common molecular pathways in order to potentiate its diverse cellular functions. c-MYC directly regulates the transcription of telomerase reverse transcriptase (*TERT*) and various genes involved in ribosomal RNA processing [10,11]. Since *DKC1* is a component of both of these c-MYC-regulated pathways, we hypothesized that *DKC1* may also be a direct transcriptional target of c-MYC.

Various high-throughput, whole genome approaches have identified a vast array of genes, including *DKC1*, whose expression may be modulated by c-MYC [12–14]. Yet, differences between studies have led to contradictory findings as to the true nature of the relationship between c-MYC and *DKC1*. In this report, we demonstrate that activation of c-MYC expression or function in several settings is associated with relatively acute induction of *DKC1* expression. We further show that *DKC1* activation is a direct, and not indirect, consequence of c-MYC transcriptional activity.

Materials and methods

Cell lines and c-MYC induction. P493-6 cells, which express a tetracycline (Tc)-Myc repressible gene [11,15], were cultured in Roswell Park

* Corresponding author. Fax: +1 215 573 2050.
E-mail address: falawi@path.dental.upenn.edu (F. Alawi).

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Memorial Institute-1640 medium (RPMI) with Glutamax (Invitrogen, Carlsbad, CA) supplemented with 10% Tet System Approved Fetal Bovine Serum (FBS; Clontech, Mountain View, CA). *K-ras*-transformed, *p53*-null mouse colonocytes [16], which express a chimeric, human c-MYC-estrogen receptor (MYC-ER) fusion protein and normal human diploid skin fibroblasts, strain 2091 (American Type Culture Collection), were cultured in Dulbecco's minimal essential medium (DMEM) with 10% FBS. All media formulations were supplemented with 100 U/ml of penicillin and 100 μg/ml of streptomycin sulfate.

To repress c-MYC in the P493-6 system, the cells were maintained for 72 h in complete RPMI containing 0.1 μg/ml Tc. To re-stimulate c-MYC, the cells were washed three times in PBS and then cultured in Tc-free RPMI containing 10% FBS or 0.1% FBS for the various time points indicated. The MYC-ER fusion protein was activated by adding 4-hydroxytamoxifen (4-OHT; Sigma–Aldrich, St. Louis, MO) to the growth media at a final concentration of 250 nM. To assess *Dkc1* transcription in the absence of protein synthesis, the cells were serum-starved for 48 h and then incubated with cycloheximide (CHX; Sigma–Aldrich) at 20 μg/ml for 30 min before addition of 4-OHT. The cells were then harvested at 24 h. All induction experiments were performed at least three times.

mRNA analysis and quantitation. Total cellular RNA was extracted using the RNeasy (Qiagen, Valencia, CA) technique, according to the manufacturer's recommendations. First-strand cDNA was prepared from 2 to 5 μg of total RNA using the Superscript First Strand Synthesis System (Invitrogen). For one or two-step, quantitative real-time RT-PCR analyses, Quantitect Primer Assays (Qiagen) for human, rat, and mouse *c-MYC*, *DKC1* (except for mouse, which was not available), *TERT*, vimentin (*VIM*), β-actin (*ACTB*), and ETS-related transcription factor 1 (*ELF1*) were used, respectively. A custom-designed primer set was used to detect mouse *Dkc1*; forward 5′-TTAGGACAACGACACCACCA-3′ and reverse 5′-CCCAGCTGGACATAATGCTT-3′. All reactions were performed in a LightCycler Instrument with Software Version 4.0 (Roche Molecular Systems, Alameda, CA); using the SYBR-Green PCR or RT-PCR kit (Qiagen), as per the manufacturer's recommendations. Relative gene expression levels were normalized to *ACTB* or *VIM* using the $2^{-\Delta\Delta CT}$ method. All reactions were performed at least three times.

Chromatin immunoprecipitation (ChIP) assay. Normal human fibroblasts were plated on 150-mm culture dishes, incubated for 24 h, and then quiesced by maintaining the cells in 0.1% FBS for 48 h. The cells were re-stimulated with 10% FBS for 0 or 2 h and then fixed in 1% formaldehyde for 10 min. Chromatin was sheared to an average estimated size of 300–1000 bp by sonication (5–8 times with 10 s pulses, 40% output using a Virsonic Model 274506) and immunoprecipitated using the EZ ChIP Chromatin Immunoprecipitation Kit (Upstate, Lake Placid, NY) as per the manufacturer's protocol. A polyclonal anti-c-MYC antibody (sc-764X, Santa Cruz Biotechnology, Santa Cruz, CA) was used to immunoprecipitate cross-linked chromatin fragments. Precipitation with normal mouse IgG was used as a negative control. DNA fragments were detected by PCR using primer sets specific for *DKC1* genomic DNA (Supplementary Table 1). All PCR products were resolved on 2% agarose gels and stained with ethidium bromide.

Western blot analysis. Proteins were extracted using M-PER (Pierce Biotechnology, Rockford, IL) as per the manufacturer's protocol. Cellular extracts (30–50 μg) were boiled, then loaded onto a 10% SDS–PAGE gel, and transferred to a membrane. The filters were probed with antibodies to DKC1 (clone H-300, Santa Cruz), c-MYC (clone 9E10, Labvision), and actin (clone C-11, Santa Cruz).

Results

Expression of c-MYC is associated with upregulation of DKC1 in human cells

We initially correlated expression of the two genes in human tissues and cell lines using data available in the publicly accessible Gene Expression Atlas [17]. We queried the entire human dataset for *DKC1* and *c-MYC*. We then log transformed the average relative expression levels of both genes from one of the datasets (Supplementary Table 2). These values are proportional to mRNA content in each of the respective samples evaluated. A linear regression analysis of the log transformed values determined that *DKC1* and *c-MYC* expression significantly correlate in 78 different normal and neoplastic tissues and cell lines ($r^2 = 0.6397$, $p < 0.0001$; Supplementary Figs. S1, S2).

To further correlate expression of *DKC1* with c-MYC in human cells, we used the P493-6 B cell line that expresses a Tc-repressible *c-MYC* transgene [15]. Following a 72-h incubation with 0.1 μg/ml of Tc, *c-Myc* mRNA and protein expression are almost completely absent (Fig. 1A, top panel and data not shown; also see Refs. [15,18]). *DKC1* mRNA levels were decreased greater than sevenfold in the c-Myc-repressed cells compared to the normal cells (Fig. 1A, bottom panel). Following activation of c-MYC in normal serum conditions (10% FBS), *DKC1* mRNA expression increased twofold after only 2 h, and peaked at 24 h (Fig. 1B). This suggests that *DKC1* transcription is an immediate-early event in these cells. Dyskerin levels were also upregulated but appreciable changes were only evident after 24 h of activation (Fig. 1C) with an additional modest increase up to 48 h post-stimulation (not shown). *TERT*, a known direct target of c-MYC [10], was also induced while *VIM* and *ACTB* (non-targets) remained unchanged. *DKC1* was also activated following c-MYC induction in serum-starved P493-6 cells (data not shown). Under conditions of serum deprivation, the P493-6 cells grow in size, but do not proliferate [15,18]. Thus, *DKC1* expression is upregulated following activation of c-MYC in proliferating and non-proliferating B cells.

c-MYC binds to DKC1 regulatory sequences in vivo

As a heterodimer with its obligate binding partner MAX, c-MYC initiates transcription by binding to canonical, 5′-CACGTG-3′, and/or several non-canonical E-box domains, including CATGTG, CACATG, CATGCG, CACGCG, and CACGAG [9,19]. Transcription is usually effected by binding to E-boxes within the promoter regions and/or intron 1 of the respective target genes [20].

A detailed analysis of the human *DKC1* genomic locus revealed several putative E-box sites distributed across the entire sequence, including 10 within the promoter–exon–intron one region (Fig. 2A). To determine if endogenous c-MYC directly binds any of these E-boxes *in vivo*, a chromatin immunoprecipitation (ChIP) assay was performed. For this analysis, c-MYC expression was induced by serum stimulation of quiescent, serum-starved, normal human diploid fibroblasts. c-MYC binding to its target genes can be effectively and reproducibly identified by ChIP after only 2 h of serum stimulation [20,21].

Using a series of *DKC1*-specific primer sets (Supplementary Table 1), c-MYC binding to two distinct sequences was strongly induced following serum stimulation