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Short Communication

A functional connection between dyskerin and energy metabolism

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ABSTRACT

The human *DKC1* gene encodes dyskerin, an evolutionarily conserved nuclear protein whose overexpression represents a common trait of many types of aggressive sporadic cancers. As a crucial component of the nuclear H/ACA snoRNP complexes, dyskerin is involved in a variety of essential processes, including telomere maintenance, splicing efficiency, ribosome biogenesis, snoRNAs stabilization and stress response. Although multiple minor dyskerin splicing isoforms have been identified, their functions remain to be defined. Considering that low-abundance splice variants could contribute to the wide functional repertoire attributed to dyskerin, possibly having more specialized tasks or playing significant roles in changing cell status, we investigated in more detail the biological roles of a truncated dyskerin isoform that lacks the C-terminal nuclear localization signal and shows a prevalent cytoplasmic localization. Here we show that this dyskerin variant can boost energy metabolism and improve respiration, ultimately conferring a ROS adaptive response and a growth advantage to cells. These results reveal an unexpected involvement of *DKC1* in energy metabolism, highlighting a previously underscored role in the regulation of metabolic cell homeostasis.

1. Introduction

The human *DKC1* gene encodes dyskerin, a highly conserved nuclear protein. Within the nucleus, dyskerin participates in the small nucleolar ribonucleoprotein complexes (snoRNPs), where it binds to H/ACA small nucleolar RNAs (snoRNAs) and acts as a snoRNA-guided pseudouridine synthase, directing the enzymatic conversion of specific uridines to pseudouridines on target RNAs (reviewed by [1]). Dyskerin also participates in the telomerase active complex, contributing to safeguarding telomere integrity [2]. Considering this wide repertoire of essential functions, it is not surprising that *DKC1* loss-of-function causes X-linked dyskeratosis congenita and its severe variant Hoyeraal-Hreidarsson syndrome, both characterized by a plethora of disparate symptoms and affecting highly renewing tissues [3–6]. While a large number of studies have deeply investigated the consequences triggered by *DKC1* downregulation (reviewed by [5]), to date, little is known about the effects of *DKC1* overexpression, despite being well established that it represents a hallmark of many types of sporadic cancers [7–17]. In addition, *DKC1* overexpression is associated with resistance to cancer-treating agents and tumor aggressiveness, and is thus considered a marker of poor prognosis [9,14–18]. It is worth noting that *DKC1* encodes multiple minor splice isoforms [19,20] whose functions remain poorly understood. In particular, a truncated dyskerin variant that retains intron 12, shows a peculiar cytoplasmic localization and stimulates cell proliferation [19], raising the possibility that it is involved in additional, previously undetermined, biological functions. Consistent with this view, this specific splice variant has recently been related to lipid metabolism [21]. Here we further explored the impact of this dyskerin isoform on cell physiology, and demonstrated that it exhibits new, uncanonical functions; having the ability to promote a metabolic shift that enhances mitochondrial functionality, producing a globally positive impact on oxidative metabolism and conferring a ROS adaptive response and a growth advantage to cells.

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2. Materials and methods

2.1. Cell culture, rotenone and dimethyl malonate treatments

Stably transfected HeLa clones (3XF-Mock, carrying p3XFLAG-CMV-10 empty vector; 3XF-Iso3 expressing the FLAG-tagged Isoform 3) used in these experiments were previously described [19] and cultured in high glucose (4.5 g/l) DMEM medium. For rotenone treatment, cells were exposed overnight to 0.25 μM rotenone (R8875, Sigma-Aldrich, Saint Louis MO) and analyzed by Flow cytometry as described below. For dimethyl malonate (136441, Sigma) treatment, cells were exposed to 100 μM dimethyl malonate for 12 h, and viable cells were counted following 0.4% Trypan Blue (Thermo Fisher Scientific, Waltham, MA) staining. Quiescent cells were obtained by starvation, upon 18 h culture in serum-free medium.

2.2. MTT assay

Reduction of (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) (M2128, Sigma) to formazan salt is dependent on NAD(P)H-dependent cellular oxidoreductases [22] and reflects cell proliferation and metabolic activities. To measure MTT reduction by colorimetric assay, 2.5 * 10^3–1 * 10^4 cells were seeded, in triplicate, in flat bottom 96 wells plates and incubated overnight to allow complete attachment. The following day, cells were washed and incubated for three hours in 100 μl DMEM without phenol red (D2429, Sigma) supplemented with 0.45 mg/ml MTT; the medium was then replaced by 100 μl of 0.1 M HCl in isopropanol and cells were incubated 30 min for lysis. Resuspension of insoluble formazan and following steps were according to MTT manufacturer's protocol. Optical densities were recorded by a Sinergy H4 spectrophotometer (BioTek, Winooski, VT).

2.3. Oxygen consumption measurements

Trypsinized cells were resuspended in PBS at 5 * 10^6cells/ml; 10^6 cells were added to 3 ml of fresh DMEM and oxygen consumption rate was recorded by a Clark-type electrode (Yellow Springs Instruments Co., Yellow Springs, OH).

2.4. Immunofluorescence analysis and MitoTracker Green staining

Immunofluorescence microscopy analysis was performed on confluent cells as previously described [19]. Confocal micrographs were taken by either the Zeiss LSM 700 microscope (Zeiss, Oberkochen, Germany), or by the multiphoton Leica TCSSP5 MP (Leica, Solms, Germany), using HC PL IRAPO 40x or 63x water objectives and analyzed by ImageJ software tools [23]. For LUT quantitative analysis, confocal images of 3XF-Iso3 and 3XF-Mock cells were captured using identical filters, laser power and gain settings. The intensity of the fluorescent signals was calculated from total sum of planes. Obtained values were normalized in respect to cell areas (MitoTracker Green and PRDX-2, and reported as Intensity/cell area ratio) or in respect to F-actin signal (TOM20, and reported as TOM20 intensity/actin intensity ratio). To measure mitochondrial mass, live cells were stained with 100 nM MitoTracker Green (M7514, Thermo) for 30 min and confocal pictures were acquired and analyzed as described above. Antibodies used are listed in Appendix A, Supplemental Table 1.

2.5. qPCR and qRT-PCR analysis

DNA and RNA were extracted using TRI Reagent (T9424, Sigma) according to the manufacturer's instructions and quantified by NanoDrop 9000 (Thermo). qRT-PCR experiments aimed at determining PGC1-α and PPRC1 expression in quiescent cells were performed as described in [19]; the HPRT1 and GSS housekeeping genes were used for normalization. For qPCRs, 25 ng total DNA were used. Cycling profile for amplification of 16 S mt-rRNA gene consisted of; one step at 95 °C for 10 min; 40 two-step cycles at 95 °C for 10 s, and at 60 °C for 60 s. In order to avoid underestimation of the mitochondrial DNA content due to eventual degradation, the integrity of total DNA was checked by gel electrophoresis and the length of the amplified fragment was designed to be 136 bp. The following cycling profile was used for amplification of the TSH receptor gene; one step 95 °C for 10 min, 40 three-step cycles at 95 °C for 45 s, at 52 °C for 30 s, and at 72 °C for 35 s. All oligonucleotides were selected using Primer3 software [24] and synthesized on demand by Sigma; their sequences are listed in Appendix A, Supplemental Table 2. For PGC1-α expression analysis, oligonucleotides were derived from regions common to all known isoforms.

2.6. Flow cytometry analysis

For TMRE staining, cells were washed three times with PBS, trypsinized and resuspended in staining solution [156 mM NaCl, 3 mM KCl, 2 mM $MgSO_4$, 1.25 mM KH_2PO_4, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES and 10 nM TMRE (T669, Thermo)]. After incubation for 20 min at 37 °C in the dark, the staining solution was discarded and cells were resuspended in PBS. For H2DCFDA staining, cells were washed three times with PBS, stained with 50 μM H2DCFDA (D399, Thermo) in culture media for 30 min at 37 °C in the dark, washed, trypsinized and resuspended in PBS. For MitoSOX Red staining, cells were washed three times with PBS, trypsinized and resuspended in culture media containing 5 μM MitoSOX Red (M36008, Thermo) dissolved in DMSO. After staining for 10 min at 37 °C in the dark, cells were washed and resuspended in PBS; centrifugation steps were performed in 15 ml conical tubes at 600 × *g*. Propidium iodide staining, with or without prior overnight exposure to 0.25 μM rotenone (R8875, Sigma), was performed as described in [25]. After appropriate staining, cell data acquisition was performed by FACSCalibur or BD Accuri C6 flow cytometers (Becton Dickinson, Franklin Lakes NJ). FlowJo vX. 0.7 (FlowJo LLC, Ashland OR) was used for analysis. Autofluorescent cells were excluded by red (H2DCFDA) or green (TMRE and MitoSOX Red) signal following definition of forward Scattering (FSC) and Side Scattering (SSC) parameters, used to identify cells and exclude debris. Flow cytometry raw data were deposited in flowrepository under the accessions: FR-FCM-ZY2Q for TMRE, FR-FCM-ZY2N for H2DCFDA; FR-FCM-ZY2X for MitoSOX and FR-FCM-ZY2P for Propidium iodide.

2.7. Evaluation of NAD(P)H and FAD autofluorescence in live cells

In vivo FAD and NAD(P)H signals were measured according to [26] and recorded by setting the Leica TCSSP5 MP "Laser (MP, MP) (Power)" at "1747.00 W (720 nm)%", using a HC PL IRAPO 40x water objective to avoid geometric aberrations. According to the protocol [26], regions of interest (ROI) were selected on the basis of high mitochondrial density and fluorescence measured by LAS-Lite 4.2 program (Leica Microsystems CMS GmbH, Mannheim, Germany); numerical analysis was performed by Excel software (Microsoft, Redmond, WA).

2.8. Western blotting analysis

Unless otherwise stated, proteins were extracted from confluent cells and analyzed by western blotting as previously described [19]. The Page ruler (26616, Thermo) was used as protein ladder; β-tubulin was used as internal loading control. Membrane pictures were taken by a ChemiDoc XRS + System (Bio-Rad, Hercules CA), bands densities analyzed with Image Lab Software (Bio-Rad) and numerical analysis performed by Excel software (Microsoft). Antibodies used are listed in Appendix A, Supplemental Table 1.