Short Communication

A functional connection between dyskerin and energy metabolism

Alberto Angrisani, Nunzia Matrone, Valentina Belli, Rosario Vicidomini, Nunzia Di Maio, Mimmo Turano, Filippo Scialò, Paolo Antonio Netti, Antonio Porcellini, Maria Furia

1. Introduction

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.

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.

References:

1. Correspondence to: Department of Biology, University of Naples “Federico II”, Complesso Universitario Monte Sant’Angelo, via Cinthia, 80126 Napoli, Italy.
E-mail addresses: alberto.angrisani@unina.it (A. Angrisani), mfuria@unina.it (M. Furia).
2. Present address: Dipartimento Medico-Chirurgico di Internistica Clinica e Sperimentale “F. Magrassi e A. Lanzara. Laboratorio Oncologia Molecolare, Università della Campania Luigi Vanvitelli, Via S. Pansini 5, 80131, Napoli, Italy.
3. Present address: NICHD (National Institute of Child Health and Human Development)-Section on Metabolic Regulation NIH-35 Convent Dr, Bethesda, MD 20814, USA.

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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 colormetric assay, 2.5 × 10^5–1 × 10^6 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 0 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 Synergy H4 spectrophotometer (BioTek, Winooski, VT).

2.3. Oxygen consumption measurements

Trypan blue stained cells were resuspended in PBS at 5 × 10^6 cells/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, filters, laser power and gain settings. The intensity of the fluorescence measured by LAS-Lite 4.2 program (Leica Microsystems CMS GmbH, Mannheim, Germany); numerical analysis raw data were deposited in Flow Jo software (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.
Fig. 1. Overexpression of dyskerin Isoform3 boosts respiratory rate and mitochondrial membrane potential. (A) Schematic organization of full-length dyskerin (Isoform1) and dyskerin Isoform 3, lacking the C-terminal NLS. Colored boxes indicate structural domains: DKCLD, associated N-terminal domain of dyskerin-like proteins of unknown function; TRUB_N, pseudouridine synthase catalytic domain; PUA, pseudouridine synthase and archaeosine transglycosylase RNA binding domain; orange boxes, lysine-arginine rich NLS sequences. (B) 3XF-Iso3 and control cells (2.5 * 10^3 – 1*1 0^4) were seeded in triplicate, incubated overnight to allow attachment and the following day subjected to MTT assay to measure cell proliferation and metabolic activities. The amount of precipitated formazan was quantified by absorbance and expressed as optical density. (C) Oxygen consumption rate of 3XF-Iso3 and control cells was measured by Clark’s electrode. Note that respiration, expressed as nmol O2/min * 10^6 cells, was nearly doubled in 3XF-Iso3 cells. (D) Mitochondrial/nuclear DNA content was quantified by qPCR; the mitochondrial/nuclear DNA ratio is reported. In this experiment, the 16S rRNA coding region was amplified and normalized with respect to the TSH receptor (TSHR) single-copy nuclear gene. (E) On the left: LUT quantitative analysis of confocal images of 3XF-Iso3 and 3XF-Mock viable cells, captured under the same conditions, upon staining with MitoTracker Green (in gray), which is insensitive to mitochondrial ΔΨ and allows a direct visualization of the mitochondrial network. Sum of total confocal planes is shown; magnification: 40×; scale bars: 10 µm. The white-dashed squares are enlarged in the insets. On the right: to estimate the mitochondrial mass, the intensity of the MitoTracker Green fluorescent signal was calculated from the same confocal images; values obtained from the sum of total planes were normalized in respect to cell areas and expressed as Intensity/cell area ratio by ImageJ tools. Data derived from the analysis of n = 90 cells. (F) qRT-PCR analysis of PGC-1α and PPRC1 expression in 3XF-Iso3 and 3XF-Mock quiescent cells; GSS and HPRT1 were used as normalizing reference genes. (G) Histogram representative of PGC-1α expression in 3XF-Iso3 and 3XF-Mock quiescent cells, as derived from western blotting densitometric quantification normalized with respect to β-tubulin (original data in Supplemental Fig. 1A). (H) Mitochondrial ΔΨ determined by flow cytometry analysis of TMRE stained cells. The TMRE dye permeates and is sequestered in active mitochondria, so that the amount of sequestered dye is directly dependent on mitochondrial ΔΨ. On the left, data derived from three different experiments; on the right, one representative experiment. Gate 1 represents the population identified as “cells”; gate 2 the “stained” population. In the right histogram, fluorescence intensity is plotted vs. cells count. All experiments were performed in triplicate; in B–D, F–H data are expressed as the mean ± SD.
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**A. Rothenone treatment**

- **3XF-Mock**
  - FLA: Proidium iodide
  - FL2A: Pteridium iodide

- **3XF-Iso3**

**B. FAD/NAD(P)H ratio**

|          | 3XF-Mock | 3XF-Iso3 |
|----------|----------|----------|
| Mean     | 25.934   | 28.389   |
| SD       | 5.917    | 5.873    |
| T test   | 0.075    |          |

**C. Malonate treatment 100 µM 12h**

- **Control**
- **Treated**

**D. H2DCFDA**

| Sample  | Count |
|---------|-------|
| 3XF-Mock | 16518 |
| 3XF-Iso3 | 16950 |

**E. MitoSOX**

| Sample  | Count |
|---------|-------|
| 3XF-Mock | 15145 |
| 3XF-Iso3 | 14946 |

(caption on next page)
2.9. Statistical analysis

Experiments were performed in biological triplicates; the Mann-Whitney test for variance analysis was applied. A difference of \( p < 0.05 \) was considered statistically significant.

3. Results

3.1. Dyskerin isoform 3 boosts energy metabolism

In our previous study, we showed that a truncated dyskerin variant, named Isoform 3 (hereafter called Iso3) retained all dyskerin functional domains, with the exception of the C-terminal bipartite nuclear localization signal (NLS) (see Fig. 1A), and displayed a peculiar subcellular localization, being predominantly cytoplasmic [19]. To gain more information about its impact on cell metabolism, we took advantage of previously obtained stably-transfected HeLa clones (hereafter called 3XF-Iso3) that overexpress this Flag-tagged isoform under the control of the CMV promoter [19]. First, we performed a (MTT) reduction assay, commonly used for assessing cell proliferation and metabolic activities. As shown in Fig. 1B, the reduction of tetrazole to formazan salt markedly increased in 3XF-Iso3 with respect to control cells (carrying the p3XFLAG-CMV-10 empty vector; hereafter called 3XF-Mock). Since MTT reduction is mainly dependent on mitochondrial reductases [22], we next evaluated the total oxygen consumption rate and found that it was raised significantly in 3XF-Iso3 cells, indicating that cellular respiration was upregulated (Fig. 1C). Given that mitochondrial activity generally correlates directly with mitochondrial number/mass, we next calculated the mitochondrial/nuclear DNA ratio by qPCR. As shown in Fig. 1D, the fluorescence was found substantially unchanged compared to control cells. To reaffirm this point, we stained cells with MitoTracker green, which is insensitive to mitochondrial membrane potential (Δψ) and allows the direct visualization of the mitochondrial network. In these experiments, confocal images of 3XF-Iso3 and 3XF-Mock cells were acquired with the same parameters and subjected to quantitative analysis by ImageJ tools, to precisely quantify the mitochondrial mass. To take into consideration the distinct morphologies of 3XF-Iso3/3XF-Mock cells [19], the intensity of the fluorescent signal was normalized per cell area. Consistent with previous observation, this analysis indicated that the mitochondrial mass does not increase in 3XF-Iso3 cells (Fig. 1E). We then used reverse qRT-PCR to check the expression of members of the PGC-1 family of positive regulators of cellular respiration. Transcription of peroxisome proliferator-activated receptor gamma co-activator-1 alpha (PGC-1α) was found modestly induced (≈1.8 fold) in 3XF-Iso3 quiescent cells, while that of peroxisome proliferator-activated receptor gamma co-activator-related 1 (PPARC1) slightly decreased (Fig. 1F). PGC-1α expression was further checked by western blotting analysis (Fig. 1G; Supplemental Fig. 1A). PGC-1α is a master regulator of mitochondrial activity whose induction can be due to different physiological context and stimuli; it can exert a positive impact on cellular respiration by either increasing the number of mitochondria in cells or by improving the respiratory capacity of individual mitochondria, remodeling their composition (reviewed in [27]). Given that direct measurements showed that the mitochondrial mass was unaffected by Iso3 over-expression (Fig. 1D, E), we presumed that PGC-1α induction in 3XF-Iso3 quiescent cells might contribute to mitochondrial remodeling. To note, PGC-1α also promotes peroxisomal biogenesis and remodeling and stimulates the increase in content of ROS-detoxifying enzymes, thereby improving cell survival during conditions of oxidative stress [27]. Instead, lowered PPRC1 expression has been associated with elevated basal metabolic rate and increased energy expenditure [28]. Altogether, these results lead to focus our attention on mitochondrial activity, prompting us to measure the mitochondrial membrane potential. When TMRE-stained cells were analyzed by Flow cytometry, a significant increase of mitochondrial membrane polarization was observed in 3XF-Iso3 cells (Fig. 1H). Considering that mitochondrial Δψ is directly linked to the proton gradient generated through the electron transport chain (ETC), and is a direct indicator of the efficiency of coupling between respiration and ATP production, these results converged to indicate that Iso3 overexpression stimulates respiration efficiency and energy metabolism.

3.2. Dyskerin Iso3 modulates global redox ratio and ROS level

To further investigate the relationship between respiratory efficiency and increased growth rate, we treated cells with rotenone, which blocks the ETC by specifically inhibiting complex I [29], and analyzed them by FACS after propidium iodide staining. As shown in Fig. 2A, 3XF-Iso3 cells were more sensitive to rotenone-induced cytotoxicity compared to controls, suggesting that their enhanced growth rate is primarily dependent on a higher respiratory efficiency. Next, we determined the cellular global redox level, which is regulated by the equilibrium between the oxidized and reduced forms of NADH/NAD + , NADPH/NADP + + {altogether NAD(P)H/NAD(P)+ } and FADH2/FAD, which are the main cellular redox cofactors. NAD(P)H and FAD are the only two ETC cofactors to be intrinsically fluorescent, with their fluorescence depending on binding to metabolic enzymes. In fact, NAD (P)H quantum yield increases, whereas FAD quantum yield decreases, when they are bound to proteins [30–32]. To precisely determine the redox ratio, we utilized multiphoton confocal laser microscopy following near-infra red excitation, that allows for the evaluation of the NAD(P)H and FAD autofluorescence in live cells with high resolution, while avoiding any staining or cell manipulation [26]. While NAD(P)H fluorescence was found substantially unchanged in 3XF-Iso3 cells, FAD fluorescence increased significantly with respect to the control (Fig. 2B). This resulted in a significant rise in the redox ratio [expressed as FAD/(FAD + NAD(P)H)], and suggested a more active reduction of ubiquinone to ubiquinol by complex II, the reaction coupled with FADH2 oxidation. This view is further supported by the observation that treatment with dimethyl malonate, a permeable precursor of the SDH inhibitor malonate, reduced more severely the vitality of 3XF-Iso3 compared to controls, as observed by Trypan Blue viable cell count (Fig. 2C).

Next, we checked total ROS and mitochondrial superoxide levels by Flow Cytometry analysis of cells stained with H2DCFDA or MitoSOX Red, respectively. As shown in Fig. 2D–E, both levels increased upon Iso3 over-expression. Interestingly, Flow Cytometry of MitoSOX stained cells revealed that 3XF-Iso3 and control cells were both composed of two subpopulations, specifically characterized by low or high mitochondrial superoxide levels (note the double peaks in Fig. 2E). However, the fraction of cells that incorporate higher levels of MitoSOX increases markedly in 3XF-Iso3 cells (by SE Dymax calculation, more
than 36% of 3XF-Iso3 cells resulted positive with respect to the control), with the superoxide high-producer population consisting of about three times the number of cells with respect to that of the control (see inset in Fig. 2E and compare ‘% cells gate 2’). Since increased mitochondrial function and ROS generation correlate with metabolic rate [33], this finding suggests that Iso3 can promote an endogenous metabolic rewiring resulting at a higher respiratory rate.

3.3. Dyserkerin Iso3 induces a ROS adaptive response

To check whether the higher respiratory rate displayed by 3XF-Iso3 cells correlated with overexpression of OXPHOS components, we followed the expression of key ETC enzymes: the NADH-coenzyme Q reductase (NDUFS3; Complex I), the sucrinate dehydrogenase subunit b (SDHB; Complex II), and the monomeric cytochrome c (CTSC; Complex III). Indeed, all these enzymes showed increased levels in 3XF-Iso3 cells (Fig. 3A; Supplemental Fig. 1B), supporting the notion that ETC activity was improved. We then checked the expression of key regulators of the mitochondrial membrane, that detach from mitochondria and transport selected cargo to peroxisomes [40] and lysosomes [41].

Next, we focused on TOM20, a key marker of the outer mitochondrial membrane that acts as a receptor for the import of mitochondrial pre-proteins (reviewed by [38]). Intriguingly, expression of this protein was found to be significantly up-regulated in 3XF-Iso3 cells, as shown by both western blotting and LUT quantitative analyses of confocal images (Fig. 3A, B). Since TOM20 upregulation can have beneficial effects on cell growth [39], it is plausible that this trait may contribute to improving mitochondrial functionality. Note that, although TOM20 expression is usually taken as a marker of increased mitochondrial biogenesis, it is known that it has additional localization on vesicles that detach from mitochondria and transport selected cargo to peroxisomes [40] and lysosomes [41].

We thus wondered whether 3XF-Iso3 cells could counteract ROS-induced deleterious effects by improving their anti-oxidant defenses. The expression of key detoxifying enzymes, such as those of the NADPH oxidase (NOX) family, can contribute to increased ROS formation; however, if this was true, in 3XF-Iso3 cells the NAD(P)H/NAD(P)+ ratio would have shifted towards its oxidized form. Conversely, this ratio was unchanged, while the FAD/(FAD + NAD(P)) ratio increased. In addition, the findings that rotenone and dimethyl malonate treatments have shifted towards its oxidized form, providing evidence for the potential benefit of increased mitochondrial biogenesis. Since mitochondrial respiratory activity is associated with increased ROS production, we hypothesized that Iso3 might fuel OXPHOS through this mechanism. Indeed, we observed that Iso3 is upregulated by incubation with rotenone, a specific inhibitor of mitochondrial Complex I, suggesting a potential role for Iso3 in promoting mitochondrial biogenesis and ROS generation.

4. Discussion

We showed that Iso3, a truncated dyserkerin splice-variant, exhibits new uncanonical functions, having the ability to promote a metabolic shift that enhances the respiratory rate without significantly altering the mitochondrial volume. Since mitochondrial respiratory activity is associated with increased ROS production, we hypothesized that Iso3 might fuel OXPHOS through this mechanism. Indeed, we observed that Iso3 is upregulated by incubation with rotenone, a specific inhibitor of mitochondrial Complex I, suggesting a potential role for Iso3 in promoting mitochondrial biogenesis and ROS generation. Since mitochondrial respiratory activity is associated with increased ROS production, we hypothesized that Iso3 might fuel OXPHOS through this mechanism. Indeed, we observed that Iso3 is upregulated by incubation with rotenone, a specific inhibitor of mitochondrial Complex I, suggesting a potential role for Iso3 in promoting mitochondrial biogenesis and ROS generation.
route. Although further experiments need to be performed to define the specific molecular pathways stimulated by Iso3 overexpression, it is reasonable to suppose that the protein could have a general impact on snoRNA-mediated processes, including pseudouridylation, thereby concomitantly regulating multiple processes connecting energy metabolism with cell homeostasis. Several considerations support this view: first, snoRNAs are assuming expanding roles in the regulation of cell homeostasis [51]; second, the mammalian transcriptome is known to be pseudouridylated in a specific manner according to diverse metabolic conditions [52]; third, Drosophila dyskerin is deeply implicated in the regulation of cell homeostasis in vivo [53–55].

We also demonstrated that the boost in ROS production is efficiently counterbalanced by antioxidant defenses, allowing 3XF-Iso3 cells to acquire a ROS adaptive tolerance. Note that this finding suggests novel mechanisms by which DKC1 overexpression might contribute to cancer aggressiveness, particularly in cancer cell types known to synthesize ATP mainly through mitochondrial respiration (reviewed by [56]). Mitochondrial ROS have been implicated in mitochondria-nucleus signaling, regulating the expression of detoxifying enzymes, with hydrogen peroxide appearing to be the main ROS with such signaling properties. Peroxidases have a high affinity for hydrogen peroxide and act as localized sensors of this molecule in specialized compartments [47]. Indeed, the NADPH-dependent cytosolic PRDX-2 enzyme is likely to play a key role in activating the ROS protective response of 3XF-Iso3 cells, and possibly in stimulating their proliferative rate. In fact, in many types of cancer cells characterized by high level of ROS production, PRDX-2 up-regulation serves as a mechanism of defense against oxidative damage, assuring survival and proliferation [57,58]; in addition, PRDX-2 has been identified as the molecular link that propagates the ROS beneficial signal in the mitohormetic pathway [59].

Taken together, the data reported here reveal a new moonlight function for the DKC1 gene, whose more abundant protein isoform is restricted to the nuclei, where it is enriched in the nucleoli and the Cajal bodies (reviewed by [5]). Several proteins thought to have specific nuclear functions have subsequently been found to have an additional localization in the cytoplasm, or more specifically in the mitochondria, and play distinct roles in their diverse subcellular locations (reviewed by [60]). The most striking example is that of telomerase reverse transcriptase (TERT), whose mitochondrial localization improves repression and protects cells from oxidative stress and apoptosis (reviewed by [61]). Considering that dyskerin participates with TERT in the formation of the nuclear telomerase complex, the ability of its truncated variant to enhance mitochondrial functionality further reinforces the functional relationship between telomerase and mitochondrial status. It will be interesting to see whether, owing to its peculiar cytoplasmic localization, Iso3 can transduce a nucleus-mitochondria signal, or interact with TERT in the cytosol, or even in mitochondria.

Acknowledgements

Nothing to claim.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2017.11.003.
