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# A new human dyskerin isoform with cytoplasmic localization

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ABSTRACT

*Background:* The human DKC1 gene is causative of X-linked dyskeratosis congenita (X-DC), a syndrome characterized by mucocutaneous features, bone marrow failure, tumor susceptibility, perturbation of stem cell function, and premature aging. DKC1 is thought to produce a single protein, named dyskerin, which shows strict nucleolar localization and participates in at least two distinct nuclear functional complexes: the H/ACA small nucleolar ribonucleoproteic complex involved in RNA pseudouridylation and the active telomerase complex.
*Methods:* By bioinformatics and molecular analyses we identified a DKC1 splice variant able to encode a truncated form of dyskerin, confirmed its active expression in diverse human tissues by RT-PCR, and showed by immunoblotting and immunocytochemistry experiments that it actually encodes a novel protein. Stably transfected clones over-expressing the new isoform were analyzed for growth, morphology and adhesion properties.
*Results:* Our results show that DKC1 encodes a new alternatively spliced mRNA able to direct the synthesis of a variant dyskerin with unexpected cytoplasmic localization. Intriguingly, when over-expressed in HeLa cells, the new isoform promotes cell to cell and cell to substratum adhesion, increases the cell proliferation rate and leads to cytokeratin hyper-expression.
*Conclusions and general significance:* Our results highlight a novel degree of complexity and regulation of the human DKC1 gene and reveal that it can play a further, unpredicted role in cell adhesion. The identification of a dyskerin cytoplasmic variant reinforces the view that other mechanisms, in addition to telomere instability, can significantly contribute to the pathogenesis of the X-DC, and suggests that DKC1 nucleolar and cytoplasmic functions might cumulatively account for the plethora of manifestations displayed by this syndrome.



## 1. Introduction

Mutations in the human *DKC1* gene cause the X-linked form of DC, a bone marrow failure syndrome characterized by mucosal leukoplakia, nail dystrophy, abnormal skin pigmentation, premature aging, stem cell dysfunction and increased susceptibility to cancer [1,2]. *DKC1* loss of function also causes the Høyeraal–Hreidarsson syndrome, recognized as a severe X-DC allelic variant [3]. The *DKC1* gene, highly conserved from Archaea to mammals, encodes a 58 kDa multifunctional nucleolar protein, named dyskerin [4], which takes part in at least two types of distinct functional complexes: that of H/ACA snoRNPs, involved in rRNA processing and pseudouridylation, and that of the active telomerase [5]. Within the H/ACA snoRNPs, dyskerin associates with NOP10, NHP2 and GAR1 proteins and a molecule of snoRNA carrying the box H/ACA sequence motifs [5] and acts as catalytic pseudouridine synthase. Isomerization of uridines to pseudouridines is one of the most abundant RNA modifications present in eukaryotic cells. Although rRNA represents the major target of pseudouridylation, additional RNAs, such as spliceosomal RNAs and tRNAs, may be subjected to this modification [5]. Even if the specific function remains elusive, pseudouridylation is believed to influence the stability, folding and functionality of RNA molecules [6–8]. Beyond its catalytic role, dyskerin binding stabilizes H/ACA snoRNAs [9–11], so that its loss might possibly elicit a variety of effects directly related to drop in snoRNA levels. This issue acquired particular relevance after the recent finding that snoRNAs could also act as potential microRNA precursors [12–16], or be processed into smaller

*Abbreviations:* DC, Dyskeratosis Congenita; X-DC, X-linked Dyskeratosis Congenita; snoRNA, small nucleolar RNA; snoRNPs, small nucleolar RNA ribonucleoproteins; hTERT, human telomerase reverse transcriptase; hTR, human telomerase RNA component; IRES, Internal Ribosome Entry Site; EST, expressed sequence tag; UTR, untranslated region; CDS, protein coding region; NLS, Nuclear Localization signal; DKLD, Dyskerin Like Domain; TruB_N, N-terminal catalytic domain of *E. coli* tRNA pseudouridine 55 synthase B; PUA, RNA binding domain from archaeal and eukaryotic pseudouridine synthases and archaeosine transglycosylase; LR, Lysine Rich domain; HeLa, human cervical carcinoma cell line; POLR2A, largest subunit of RNA polymerase II; HIER, Heat-Induced Epitope Retrieval

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RNA able to regulate alternative splicing [17], suggesting that these molecules can play a wider spectrum of functional roles than previously suspected.

Dyskerin is also an essential component of the active human telomerase complex [18,19] where, in association to hTERT and hTR, which presents an H/ACA motif at its 3′ region, contributes to telomere stabilization and maintenance. However, the protein has also been involved in a variety of disparate cellular functions, although its exact role in these processes still remains to be defined. For example, the rat homolog protein, named NAP57, has been implicated in nucleo-cytoplasmic "shuttling" [20], while in both mouse and human cells dyskerin has been involved in the promotion of cap-independent mRNA translation [21–24] starting at IRESs.

Despite its involvement in such a wide range of biological processes, the *DKC1* gene has not been extensively characterized at the molecular level, and a single constitutive transcript of 2.6 kb encoding nucleolar dyskerin has been so far described [4]. Considering that alternative splicing is widely used by eukaryotic genes, we wondered whether the variety of cellular functions attributed to this gene may, at least in part, be based on the production of alternative transcripts. Here we report that after a detailed bioinformatics and molecular analysis of the *DKC1* transcriptional activity we were able to identify and characterize a novel *DKC1* mRNA able to encode a variant protein with an unexpected cytoplasmic localization. Active expression of this splice mRNA isoform was effectively confirmed in diverse human tissues and cell lines; moreover, its overexpression in HeLa cells indicated that it can play biological functions in cell growth and adhesion.

## 2. Material and methods

### 2.1. Bioinformatic analyses

The human EST databases at the NCBI was searched by using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi), entering the *DKC1* human genomic sequence (GenBank ID: NC_000023) and that of the 2.6 kb *DKC1* ubiquitary mRNA (isoform 1 GenBank ID: NM_001363). Positive matches were verified using ClustalW (http://align.genome.jp/); when more than one EST was positive, only the longest was taken into account for successive analyses. The coding potential of the new isoform was evaluated using the translate tool at the ExPASy web site (http://www.expasy.ch/tools/dna.html).

### 2.2. RNA extraction and analysis

Total RNA was isolated from cells and tissues using 'TRIZOL reagent' (Invitrogen). 2 μg of total RNA was reverse transcribed using 100 ng of random hexamer primers and Super Script III Reverse Transcriptase (Invitrogen). PCR reactions were then carried out using 5 ng cDNA as template and the DreamTaq enzyme (Fermentas), in a total volume of 25 μl, under the following conditions: one cycle at 94 °C for 5 min, followed by 40 cycles of 20 s at 94 °C, 30 s at 60 °C and from 30 to 100 s, depending on the fragment's length, at 72 °C, with a final cycle at 72 °C for 7 min. Human tissue RNA samples were obtained from Clontech and examined by RT-PCR semiquantitative analysis in the conditions described above, except that the number of cycles was reduced to 28 for *DKC1* isoform 1 and POLR2A, and to 32 for *DKC1* isoform 3. In order to avoid amplification of genomic DNA contamination, primers used in RT-PCR reactions were designed to span exon/exon borders; their sequences are listed in the Supplementary Table 1. All reagents, kits and enzymes were employed according to manufacturer's instructions. For quantitative analyses of dyskerin mRNA isoforms, total RNA (1 μg) was reverse transcribed using 100 ng random hexamers and Super Script III Reverse Transcriptase (Invitrogen). Real Time RT-PCR (qRT-PCR) experiments were performed using the iQ 5 Multicolor Real-Time PCR Detection System (Bio-Rad); PCR reactions were in a final volume of 15 μl, using 5 ng of cDNA and iQ SYBR Green Supermix 2X (Bio-Rad). Reagents and enzymes were employed according to manufacturer's instructions. PCR cycling profile consisted of a cycle at 95 °C for 10 min and 40 two-step cycles at 95 °C for 10 s and at 60 °C for 60 s. Different amounts of the two Flag-tagged expression vectors (Flag-Isoform 1 and Flag-Isoform 3; see Section 2.3) were used to create calibration lines for absolute quantitation of *DKC1* isoforms 1 and 3 in different cell lines. Primers were chosen using Primer3 software (http://frodo.wi.mit.edu/primer3/) and designed to span exon/exon borders; their sequences are listed in the Supplementary Table 1. Three different RNA preparations were tested for each sample, and each reaction was run in triplicate. The data is representative of three independent experiments.

To define the full length structure of the new isoform 3, RT-PCR fragments were purified by the Nucleospin Extract II (Macherey-Nagel), cloned into the pGEM T-Easy Vector system (Promega), and sequenced by the external company Primm.

### 2.3. Expression vector construction

Flag-tagged expression vectors (Flag-Isoform 1 and Flag-Isoform 3) were constructed as follows: the entire coding regions of isoforms 1 and 3 were amplified using the same forward primer containing at its 5′ end a HindIII restriction site coupled with isoform-specific reverse primers containing at their 5′ end an EcoRI restriction site; primer sequences are listed in the Supplementary Table 1. PCR reactions were carried out using cDNA obtained from HeLa cells as a template; PCR products were purified, digested with HindIII and EcoRI enzymes and then cloned into HindIII and EcoRI sites of the p3XFLAG-CMV-10 expression vector (Sigma). Obtained clones were confirmed by sequencing.

### 2.4. Cell culture and transfection

HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen) and grown at 37 °C in a humidified atmosphere containing 5% $CO_2$. For transfection, 8 μg of either p3xFLAG-Isoform 1, p3xFLAG-Isoform 3 or the p3XFLAG-CMV-10 empty vector plasmid (Sigma) were added to lipofectamine 2000 Reagent (Invitrogen) following the manufacturer's instructions. For stable transfection, after 2 weeks of selection in the presence of 1 mg/ml G418 (Invitrogen), drug-resistant clones were isolated and grown in the presence of 0.5 mg/ml G418.

### 2.5. Western blot analysis

HeLa cells were lysed in a buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA (pH 8.0), 1% v/v NP40, supplemented with Complete inhibitor cocktail (Roche). Total lysates were incubated for 30 min on ice and insoluble material was removed by centrifugation at 10000×*g* at 4 °C for 15 min. Protein concentration was measured using the Bio-Rad protein assay. Western blottings were probed with anti-Flag (1:5000; Sigma), anti-dyskerin (1:1000; Sigma SAB2104539); and anti-actin antibodies (1:1000; Sigma A2066); the Page ruler (Fermentas) was used as protein ladder.

### 2.6. Immunocytochemistry

HeLa cells cultured on coverslips were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature, permeabilized and blocked with 0.5% Triton X-100 3% BSA in PBS for 15 min at room temperature. After washing, cells were incubated with either mouse anti-Flag antibody (1:5000; Sigma) or rabbit N-terminal anti-dyskerin antibody (1:1000, Sigma SAB2104539) for 1 h at room temperature, washed and incubated with Texas Red conjugated anti-mouse IgG (1:250) or Cy3 conjugated anti-rabbit IgG (1:1000) for 1 h at room temperature. After final rinsing, nuclei were counterstained with DAPI