

UNIVERSITY OF NAPLES FEDERICO II



PhD Course in Biology

XXXVI Cycle

**Molecular mechanisms underlying the functional role of
the human DKC1 gene in dyskeratosis congenita and cancer diseases**

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1 Index

ABSTRACT

2 Introduction

- 2.1 Dyskerin - general overview**
- 2.2 Dyskerin orthologs**
- 2.3 Structure of dyskerin**
- 2.4 Localization, functions and nuclear complexes**
 - 2.4.1 H/ACA ribonucleoproteins (RNPs)**
 - 2.4.2 The telomerase holoenzyme**
- 2.5 Dyskeratosis congenita**
- 2.6 Dyskerin and cancer**
- 2.7 The paradox of dyskeratosis congenita**

3 SECTION 1

- 3.1 Dyskerin Downregulation Can Induce ER Stress and Promote Autophagy via AKT-mTOR Signaling Deregulation**

4 SECTION 2

- 4.1 Morphological and functional impact of ER stress on mitochondria in dyskerin-downregulated cancer cell**

5 SECTION 3

- 5.1 New Insights into Dyskerin-CypA Interaction: Implications for X-Linked Dyskeratosis Congenita and Beyond**

6 Discussion

7 Conclusions and future work

8 References

ABSTRACT

Dyskerin, a highly conserved pseudouridine synthase, plays a pivotal role in diverse cellular functions by catalyzing the conversion of specific uridines to pseudouridines. The intricate associations of dyskerin with proteins like GAR1, NHP2, and NOP10, along with small non-coding RNAs, form functional RNPs, including active telomerase, H/ACA small nucleolar ribonucleoproteins (snoRNPs), and small Cajal body-specific ribonucleoproteins (scaRNPs). Dyskerin's involvement spans critical cellular processes such as telomere integrity, ribosome biogenesis, IRES-dependent translation, DNA damage response, vesicular trafficking, and energy metabolism. The focus of this study lies in the hereditary disorder X-linked dyskeratosis congenita (X-DC), stemming from hypomorphic mutations of the DKC1 gene encoding dyskerin. This research study has revealed a previously unknown interdependence between mTOR signaling and UPR during a pathological process initiated by reduced levels of dyskerin. The findings of this project highlight a unique and significant connection between dyskerin, autophagy, and UPR, which could potentially have far-reaching implications for our understanding of cellular mechanisms. This discovery sheds new light on the complex interplay of the mTOR signaling pathway and UPR, which could be important for developing new therapeutic interventions in the future. The present study endeavors to explore the functions of dyskerin, particularly its interaction with CypA, and the consequent potential opportunities to address the intricacies of dyskeratosis Congenita X-linked and allied cellular processes in various disease contexts.

2 Introduction

2.1 Dyskerin - general overview

Premature aging syndromes, bone marrow failure, and various types of cancer can occur due to defects in the expression and function of a protein called dyskerin. (1) (2) (3) (4).

Dyskerin is a nucleolar protein with a length of 514 amino acids; it is encoded by the DKC1 gene located at Xq28. (5). It was first identified in 1998 during a study of patients with X-linked recessive dyskeratosis congenita (X-DC), a genetic disorder that causes premature aging. The DKC1 gene mutations have been associated with several diseases, including Dyskeratosis Congenita, X-linked recessive aplastic anemia, and Hoyeraal-Hreidarsson syndrome.

The protein is involved in various cellular processes and plays a critical role in the biogenesis of ribosomes. Dyskerin is a component of a snoRNA ribonucleoprotein complex; it catalyses the formation of pseudouridine in ribosomal and certain small RNAs (6). It is also a part of the core telomerase complex (7), which is necessary for the biogenesis of hTERC. It is transcribed from the TERC gene and hTERC RNA contains a specific recognition domain for dyskerin.

The identification and characterization of the Dyskerin protein have significant implications for understanding the molecular mechanisms underlying these diseases and the development of potential therapeutic interventions.

2.2 Dyskerin orthologs

Dyskerin's remarkable evolutionary conservation is evidenced by the presence of orthologs in multiple organisms. The first dyskerin ortholog was found in yeast (Cbf5) where it was shown to have affinity for centromere DNA as well as a microtubule-binding site such that deletion of this site would cause cell cycle arrest (8). A few years later, Cbf5 was found to be involved in rRNA biogenesis which explains the cell cycle arrest caused by dyskerin mutations leading to reduced ribosomal synthesis. Additional orthologs were identified over the years such as in the rat (NAP57) (9), as well as in *Drosophila* (Nop60B) (10) and *Arabidopsis* (AtNAP57) (11) showing an important evolutionary conservation of the protein across species and highlighting its multiple key functions in regulating cellular processes. More strikingly, the deletion of these homologs is lethal for the organism (12) (13) (14). Furthermore, the presence of conserved counterparts, also known as Cbf5, in Archaea suggests its crucial role throughout the evolutionary process (15).

2.3 Structure of dyskerin

Dyskerin is an L-shaped protein of 514 residues with a molecular weight of ~58 kDa. A homology model of human dyskerin was created by using a template crystal structure from *Saccharomyces cerevisiae* which has a sequence identity of 73% with the human dyskerin. Three well-preserved

functional domains were identified: the Dyskerin-like domain (DKLD) with an unknown function; the TruB_N pseudouridine synthase catalytic domain which comprises the active site; this domain is directly associated with pseudouridylation and the pseudouridine synthase and archaeosine transglycosylase (PUA) RNA binding domain which has a role in recognizing RNAs of the H/ACA family. Additionally, two nuclear localization signals (NLSs) identified as lysine-rich sequences present in most metazoa, localized at the N- and C-termini, which is thought to ensure the typical nucleolar localization (2).

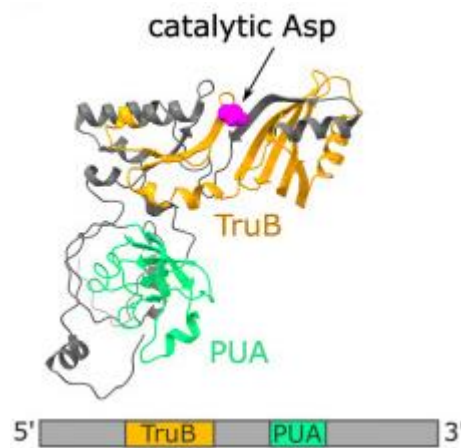


Figure 1: Description of dyskerin structure. The depiction visually represents human dyskerin (PDB: 7bgb), highlighting the TruB catalytic domain in orange. The catalytic aspartate is accurately portrayed by magenta spheres, while the PUA RNA binding domain is elegantly displayed in light green. The catalytic core of dyskerin is the upper, horizontal portion of the structure, consisting of multiple helices and strands.

Deletions in the last exon of the DKC1 gene are 100% lethal in mice. However, since the first generations of mice with no telomerase are viable, it is unlikely that the lethality is due to mutated dyskerin's effect on telomerase activity (16). These findings establish the biological relevance of this specific region in mammals and humans. In addition, truncated dyskerin isoforms without variable tracts of the C-terminal domain have been identified in *Drosophila* (17) and various human tissues and cell lines (18) (19). An alternative isoform, isoform 3, of dyskerin has been discovered by Angrisani et al. This isoform lacks the C-terminal NLS, but includes the catalytic and the PUA domains. It has shown a preference for cytoplasmic localization and may be involved in transporting pre-ribosomal structures from the nucleus to the cytoplasm. As a result, it may affect translation by contacting ribosomes or cytoplasmic mRNAs (18) (2). Overexpression studies of this isoform have

demonstrated significant effects, such as growth advantage, changes in cell morphology, and increased cell-cell and cell-substratum adhesion.

Recently, another truncated dyskerin (lacking the C-terminal) variant has been discovered. It is capable of boosting energy metabolism and enhancing respiration, which ultimately improves cellular ROS adaptive response and growth rate. These new findings indicate that DKC1 plays an unexpected role in energy metabolism and is involved in the regulation and homeostasis of cellular metabolism (20).

2.4 Localization, functions and nuclear complexes

2.4.1 H/ACA ribonucleoproteins (RNPs)

Dyskerin, also known as pseudouridine synthase, is an essential component of the H/ACA snoRNP complex. Dyskerin is responsible for catalytic activity, while the other components are required for the structural stabilization and positioning of the guide and substrate RNAs for catalysis (21) (22) (23). Therefore, understanding the function of dyskerin requires knowledge of H/ACA ribonucleoproteins. H/ACA snoRNPs and scaRNPs are small RNA–protein complexes that are crucial for the synthesis of larger RNPs such as ribosomes and telomerase (24) (25) (26) (27) (28). The mature H/ACA snoRNPs and scaRNPs are composed of a single or double hairpin guide RNA and four evolutionarily conserved proteins: Cbf5p, Nhp2p, Nop10p, and Gar1p in yeast; and dyskerin, NHP2, NOP10 and GAR1 in human (29) (30), and a function-specifying, noncoding H/ACA RNA. The main functions of H/ACA RNPs are telomerase assembly, stabilization, and posttranscriptional processing of nascent ribosomal RNA and spliceosomal RNA. Therefore, H/ACA RNPs are essential for ribosome biogenesis, pre-mRNA splicing, and maintenance of telomere. 100-200 different species of H/ACA RNPs are present in every cell, most of them residing in nucleoli and CBs, each consisting of the same four core proteins and one function-specifying H/ACA RNA (31). Most of H/ACA particles have site-specific pseudouridine synthases function in an RNA-guided mechanism; the guide RNA provides base-pairing interactions that specify the target uridine (32). Nevertheless, the H/ACA domain in telomerase is not required for pseudouridylation or TA as demonstrated in in vitro studies but is necessary for the in vivo accumulation and localization of the mature telomerase RNP (33) (34) (35).

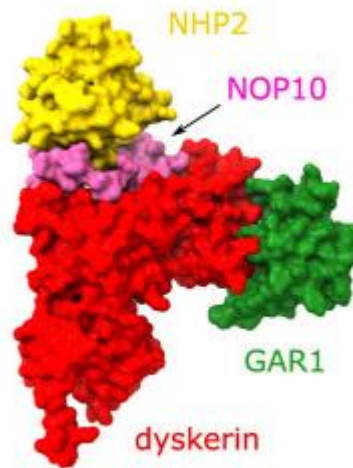


Figure 2: H/ACA snoRNP complex. Structure of the human H/ACA heterotetramer (PDB: 7bgb), Including NOP10 (pink), NHP2 (yellow), and GAR1 (dark green) to dyskerin (red).

2.4.2 The telomerase holoenzyme

Dyskerin has been proposed to be the third core component of the telomerase holoenzyme complementing the RNA component harboring the template region for telomere synthesis (hTR or hTERC) and a catalytic protein with reverse transcriptase activity, hTERT (36). Two sets of H/ACA core proteins (dyskerin, NOP10, NHP2, and GAR1) but only one copy of TERT and a single copy of the telomerase Cajal body protein 1 (TCAB1) associate with hTR to form a functional telomerase holoenzyme (37) (38). Cohen et al. (7) identified dyskerin as essential component of the complex. In fact, although there are at least 32 other proteins associated with the telomerase complex none of these, except dyskerin, was proved to be necessary for the catalytic activity. It is still unknown how dyskerin mutations lead to telomeres shortening, but both in mouse and in human cells has been observed that they lead to lower TERC levels (39) (40). Presumably, these reduced levels of TERC are not sufficient to maintain telomere length in the stem cells that give rise to blood, skin and other tissues renewable, causing the shortening of the same and, finally, the stem cells depletion.

2.5 Dyskeratosis congenita

Dyskeratosis Congenita (DC) is a rare genetic disorder that affects one in a million people. The disease causes premature aging and patients are usually diagnosed around the age of 10. Physical symptoms of DC include nail dystrophy, leukoplakia, and abnormal skin pigmentation. The consequences of DC affect all body systems; these may include the diagnostic triad of abnormal nails, reticular skin pigmentation, and oral leukoplakia; bone marrow failure, pulmonary fibrosis, liver disease, neurological and ophthalmic abnormalities, as well as increased risk of cancer also occur

(41) (42). Unfortunately, most patients die early due to deficiencies in highly replicative tissues which lead to stem cell depletion and bone marrow failure. This usually occurs around the age of 40-50 (43). The inheritance of DC can be X-linked recessive (XLR), autosomal dominant (AD), or autosomal recessive (AR). There is also a high frequency of sporadic cases, which are presumably due to new mutations in dominant genes. Six genes in the telomere biology pathway have been identified to date as mutated in patients with DC *DKC1*, *TERC*, *TERT*, *TINF2*, *NOLA2*, and *NOLA3* (44) (45) (46) (47) (48) (49). Approximately half the patients with DC have a mutation in one of these six genes, while the other half await discovery of mutations in additional telomere genes.

DC was originally discovered in the 1900s in the X-linked recessive form, arising from a mutation in the *DKC1* gene (50). It was initially hypothesized that the disease was caused by defects in ribosome maturation as dyskerin was mainly known as a pseudouridine synthase required for rRNA maturation and ribosome assembly until Mitchell et al. linked DC to telomerase defects. They found that DC patients have low levels of hTR and reduced telomerase activity as well as telomere length below average (51). Nowadays, other forms of the disease have been identified such as autosomal recessive and autosomal dominant forms caused by mutations in *NOP10* and *NHP2* or in the telomerase components hTERT and hTR respectively (52) (18) (53). The mutations in hTR are localized either in the pseudoknot, impairing telomerase activity, or at the 3' end impairing its association with the H/ACA scaffold proteins and therefore its stability and localization. Some of these mutations are localized in the H/ACA box (54). Mutating the CAB box impairs hTR localization to the CB, exemplified by the C408G mutant hTR identified in autosomal DC patients (55) (56). Other hTR mutations located in the CAB box are associated to bone marrow failure syndromes 114. The direct consequence of these mutations in DC patients is underlined in earlier research where the homozygous knock out of mouse TR displays DC-like phenotypes after a few generations (57). The clustering of DC-linked mutations in telomerase and telomere associated components underlines the importance of telomere misregulation in DC. Mutations in telomere associated components as well as dyskerin have also been linked to other premature aging diseases such as Hoyerlaal-Hreidarsson, a severe form of DC (58). Furthermore, several studies show DC patients are more prone to developing cancer than healthy individuals. The role and regulation of telomerase and its components, such as dyskerin are therefore essential for a better understanding of how it is implicated in aging and cancer and to translationally apply this knowledge to the development of therapeutic strategies.

2.6 Dyskerin and cancer

The first general link between dyskerin and cancer was observed in DC patients who have increased susceptibility to developing malignancies (59). The susceptibility is linked to the extreme shortening of telomeres in DC cells. As telomeres shorten, the cell eventually reaches the Hayflick limit and must

go through replicative senescence (60). Montanaro et al. conducted the first study looking at expression and function of dyskerin in human lung, breast and colon carcinomas (61). The analysis of dyskerin mRNA levels shows that dyskerin levels vary greatly between different cancer types with some having low levels of dyskerin mRNA. Additionally, they looked at rRNA pseudouridylation and found that it is positively correlated to dyskerin mRNA levels. Lower levels seem to increase neoplastic transformation suggesting dyskerin could act as a tumor suppressor (61) (60). Low dyskerin levels are however associated to reduced telomerase activity and they showed that breast tumors with lower dyskerin levels were associated with better prognosis (34). On the other hand, wild type dyskerin is often found to be upregulated in cancer cell lines. While some studies show that this upregulation mainly drives increased cell proliferation and protein biosynthesis in breast cancer and prostate cancer (62) (60), others associate the increased dyskerin protein level to increased telomerase activity resulting from increased levels of hTR. One study in neuroblastoma patient samples looked at enriched cluster of genes and identified snoRNA RNP-associated genes with DKC1 being at the top of the list. They saw a correlation between the expression of the telomerase complex and tumor survival and concluded that this increase in snoRNA RNPs in neuroblastoma causes elevated telomerase activity (63).

2.7 The paradox of dyskeratosis congenita

Proliferating tissues are affected by the defect in telomerase function and the alteration in ribosome biogenesis (64). Cells with a defective or slowed ribosome biogenesis are less able to proliferate, which is the case when dyskerin function is impaired (65) (40). This is because proliferating cells require an increased demand for protein synthesis, which is accomplished by increasing the rate of ribosome biogenesis (66). Moreover, telomerase is necessary for active DNA replication to prevent telomere attrition and the consequent activation of a DNA damage response (67). Hence, the effect of X-DC mutations on telomerase function is expected to limit the self-renewal of cells in labile tissues. However, it has been reported that in X-DC, defective proliferation is associated with increased tumour susceptibility (68) (59) (69). To explain this apparent paradox, it is essential to understand the specific function of dyskerin, which contributes to tumourigenesis when lost. The absence of telomerase function may lead to an increased susceptibility to cancer, through the induction of chromosomal instability as is observed in the late-generation *TERC*^{-/-} knockout mice. In this model, telomere attrition that is functionally evident after crossing the *TERC* null mice for five to six generations leads to a moderate incidence of spontaneous tumours. Although dyskerin is primarily associated with telomerase activity, studies have shown that a reduction in its expression can increase the incidence of certain types of tumors, such as breast, lung carcinoma, B-cell lymphomas in the DKC1 hypomorphic mutant mouse model. This mouse model exhibits a reduction of both telomerase

activity and rRNA pseudouridylation due to the decreased expression of dyskerin. However, the increased tumor susceptibility is observed in the early generations of DKC1 hypomorphic mice when the telomeres are still very long, indicating that there are telomerase-independent functions of dyskerin that play a crucial role in promoting tumorigenesis (65). It has been suggested that the alteration of ribosome function caused by defective rRNA uridine modification in the DKC1 hypomorphic mutant model could be involved in cancer development. The reduction in modified uridine residues in the ribosome may result in impaired translation of specific mRNAs, as the modification sites are critical for tRNA and mRNA binding (70). The mechanisms underlying cancer development from an alteration in ribosome function, however, are yet to be revealed.

There is a defect in the translation of specific mRNAs in cells from both X-DC patients and the DKC1 hypomorphic mice. This defect is due to the selective impairment of IRES-mediated translation (71). IRESs are nucleotide sequences that are present in some eukaryotic cellular mRNAs. They allow for independent translation of an mRNA without other elements generally required for translation initiation (72) (73). The defect in IRES-mediated translation in X-DC cells results in the relative impairment of the translation of specific mRNAs, especially under different types of stress.

The mRNAs encoding anti-apoptotic factors Bcl-xL and XIAP, and the tumour suppressor p27 were initially identified as translational targets of defective dyskerin (71). More recently, IRES-containing mRNAs encoding for the tumour suppressor p53 (74) have also been added to the list (75) (76). The defective synthesis of anti-apoptotic proteins may result in an increased death of haematopoietic progenitors, leading to bone marrow failure. Additionally, the defective synthesis of p27 and/or p53 may also play a role in bone marrow failure. The loss of function of p53 and p27 has been shown to affect the progression through the cell cycle of haematopoietic stem cells and more committed progenitors, respectively (77) (78). The defective synthesis of these tumour suppressors may alter the coordinated cell-cycle progression of haematopoietic progenitors, leading to their exhaustion.

There is an important question regarding whether the increased susceptibility to tumors in dyskeratosis congenita is due to the defective translation of mRNAs that encode for tumor suppressors. Studies have shown that the reduction of dyskerin levels impairs p53 mRNA translation under stress in breast cancer cells and normal breast epithelial progenitors. This diminishes the p53-mediated response to DNA damage. Additionally, mouse embryonic fibroblasts for DKC1 hypomorphic mice are more resistant to p53-mediated oncogene-induced senescence and are more easily transformed by oncogenic Ras expression (76). In mice, the hypomorphic mutation of DKC1 leads to a strong decrease of p27 mRNA translation in the pituitary gland and to an increase in susceptibility to pituitary adenomas in the p27 +/- knockout background.

3 SECTION 1

3.1 Dyskerin Downregulation Can Induce ER Stress and Promote Autophagy via AKT-mTOR Signaling Deregulation

The principal aim of this study is to evaluate the non-telomerase-related impacts of dyskerin depletion on cell physiology. Utilizing an inducible DKC1 downregulation system, we plan to swiftly reduce dyskerin expression and scrutinize the resulting effects on cells. The study will focus on the molecular processes that occur as a result of dyskerin depletion, with particular attention to the stress mechanisms of the endoplasmic reticulum (ER), the reticulum stress response (UPR), and autophagy. These mechanisms are essential for maintaining cellular homeostasis and play a critical role in the development and progression of various diseases, probably also in dyskeratosis congenita. The primary objective of this study is to understand the role of dyskerin in these processes and evaluate its potential as a therapeutic target for the treatment of dyskeratosis congenita. Specifically, this study could provide new insight into the molecular mechanisms underlying dyskeratosis congenita and identify potential therapeutic targets for the development of new treatments.



Dyskerin Downregulation Can Induce ER Stress and Promote Autophagy via AKT-mTOR Signaling Deregulation

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Citation: Maiello, D.; Varone, M.; Vicidomini, R.; Belli, V.; De Rosa, M.; Dama, P.; Furia, M.; Turano, M. Dyskerin Downregulation Can Induce ER Stress and Promote Autophagy via AKT-mTOR Signaling Deregulation. *Biomedicines* **2022**, *10*, 1092. <https://doi.org/10.3390/biomedicines10051092>

Academic Editor: Nina Entelis

Received: 31 March 2022

Accepted: 4 May 2022

Published: 8 May 2022

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Abstract: Dyskerin is an evolutionarily conserved nucleolar protein implicated in a wide range of fundamental biological roles, including telomere maintenance and ribosome biogenesis. Germline mutations of *DKC1*, the human gene encoding dyskerin, cause the hereditary disorders known as X-linked dyskeratosis congenita (X-DC). Moreover, dyskerin is upregulated in several cancers. Due to the pleiotropic functions of dyskerin, the X-DC clinical features overlap with those of both telomeropathies and ribosomopathies. In this paper, we evaluate the telomerase-independent effects of dyskerin depletion on cellular physiology by using inducible DCK1 knockdown. This system allows the downregulation of *DKC1* expression within a short timeframe. We report that, in these cellular systems, dyskerin depletion induces the accumulation of unfolded/misfolded proteins in the endoplasmic reticulum, which in turn induces the activation of the PERK branch of the unfolded protein response. We also demonstrate that the PERK-eIF2a-ATF4-CHOP signaling pathway, activated by dyskerin downregulation, triggers a functional autophagic flux through the inhibition of the PI3K/AKT/mTOR pathway. By revealing a novel unpredicted connection between the loss of dyskerin, autophagy and UPR, our results establish a firm link between the lowering of dyskerin levels and the activation of the ER stress response, that plays a key role in the pathogenesis of several diseases.

Keywords: dyskeratosis congenita; DKC1; autophagic flux; UPR; LC3 puncta

1. Introduction

Dyskerin is the major evolutionarily conserved pseudouridine synthase involved in the conversion of specific uridines to pseudouridines on ribosomal RNAs (rRNAs), small nuclear RNAs (snRNAs) and messenger RNAs (mRNAs) [1]. The protein associates with the three highly conserved proteins GAR1, NHP2 and NOP10, and with small ncRNAs bearing the H/ACA motifs (mostly snoRNAs), to form diverse functional nuclear ribonucleoprotein (RNPs) complexes, including active telomerase, H/ACA small nucleolar ribonucleoproteins (snoRNPs) and the small Cajal body-specific ribonucleoproteins (scaRNPs). These complexes are involved in several biological functions that include both the safeguarding of telomere integrity and ribosome biogenesis [2]. Dyskerin is also involved in a wide range of biological processes, including IRES-dependent translation, [3–5], DNA damage response [6–8], vesicular trafficking [9] and energy metabolism [10]. In addition to these

functions, dyskerin has been found upregulated in several human sporadic cancers [11–13] and its overexpression has been linked to poor prognosis [14]. Considering the multiple biological functions in which the protein is involved, it is not surprising that hypomorphic mutations of DKC1, the human gene encoding dyskerin, causes the hereditary disorder X-linked dyskeratosis congenita (X-DC) [15]. The main symptoms of this pathology are the progressive failure of proliferating tissues, premature aging and increased susceptibility to various types of cancers [16,17]. Due to the pleiotropic functions of dyskerin [18], the X-DC clinical features overlap with those of both telomeropathies and ribosomopathies. For example, cells from X-DC patients display a telomere shortening, but also altered snoRNA regulation and rRNA modification [18]; accordingly, certain dyskerin pathogenic X-DC variants substantially reduce the accumulation of hTR, but, in some cases, also that of other H/ACA RNAs [19]. In transgenic mice carrying hypomorphic DKC1 mutations, impaired ribosomal RNA pseudouridylation was observed before the reduction in telomere length, suggesting that deregulated ribosome function is important in the initiation of X-DC [17]. In addition, in organisms without telomerase, in which telomeres are maintained by retrotransposons, mutations in the human DKC1 orthologue cause context-dependent defects, suggesting that the telomerase-independent functions of pseudouridine synthases are important both for development and tissue homeostasis [20,21].

The use of cellular systems that allow the detection of which physiological/metabolic alterations are triggered by dyskerin loss of function, before a significant shortening of the telomeres occurs, represents a useful tool to evaluate the telomerase-independent functions of dyskerin. The timely identification of early alterations could lead to the identification of new molecular targets and, possibly, new therapeutic approaches to mitigate the most severe symptoms of this disease. In this paper, to detect the early (telomerase-independent) effects caused by dyskerin reduction, we use an inducible cellular silencing system capable of depleting dyskerin in human colon carcinoma (RKO) [9] and human embryonic kidney 293 cells (HEK 293T). Since a previous study has shown that ribosomes purified from dyskerin-depleted cells display altered translational fidelity when added to a cell-free system [5], our study focused on testing, in cellular systems, whether the depletion of dyskerin causes an accumulation of misfolded/unfolded proteins with the consequent activation of the pathways responsible for the mitigation of such accumulation.

Importantly, consistent with the accumulation of misfolded/unfolded proteins, we observe that dyskerin depletion triggers endoplasmic reticulum (ER) stress with the consequent activation of the unfolding protein response (UPR), eIF2 α phosphorylation and ATF4 production. In addition, by using a GFP-LC3-RFP reporter transgene coupled with our own custom R-pipeline designed to precisely analyze the ratio RFP/GFP signal of LC3 puncta, we also discovered that dyskerin depletion causes a strong upregulation of the autophagic flux, which correlates with a strong downregulation of the mTOR signaling.

Altogether, our data show that the early effects triggered by dyskerin silencing concern the alteration of three important cellular pathways, namely UPR, autophagy and mTOR signaling. This leads us to suggest that players involved in these pathways, such as PERK (the kinase that phosphorylates eIF2 α), or mTORC1/mTORC2, could represent molecular targets to be evaluated for the early diagnosis of X-DC, before the manifestation of late severe symptoms, such as bone marrow failure, might occur.

2. Materials and Methods

Generation of Stable, Dox-inducible DKC1 Silenced Cell Lines

To generate stable cell lines, HEK 293T cells were seeded in a 60 mm dish one day before transfection in a complete growth medium to achieve a confluence of 60–80% at the time of transfection. On the following day, 3 μ g of pLKO-Tet-On-shDKC1 plasmid [9] and 12 μ L of Polyethylenimine (PEI) (Polysciences, Valley Rd, Warrington, PA, USA) were diluted in 0.5 mL of serum-free Dulbecco Modified Eagle Medium (DMEM), respectively. After 5 min of incubation at room temperature, the diluted DNA and the diluted PEI were mixed and incubated for 30 min at room temperature, in order to allow the formation

of the DNA/PEI complexes. Cells were then incubated with 2 mL of growth medium without antibiotics and 1 mL of DNA/PEI complexes at 37 °C in a humidified atmosphere of 5% CO₂ for 5 h. After incubation, the culture medium was replaced with a fresh culture medium containing 20% fetal bovine serum (FBS), 1% penicillin-streptomycin (P/S) and 1% L-glutamine. Following 48 h of transfection, cells were passaged 1:10 into complete medium containing 750 ng/mL puromycin (Sigma-Aldrich, St. Louis, MO, USA). After 20 days of puromycin selection, during which the culture medium was changed every 2 days, all transformed cell clones were pooled together and the inducible stable cell line (HEK-shDKC1) was expanded. Silencing was triggered by adding doxycycline (Dox, Sigma-Aldrich, St. Louis, MO, USA) (400 ng/mL) to the medium.

Cell Culture and Treatments

RKO and HEK 293T (ATCC, Manassas, VA, USA), RKO-shDKC1 and HEK-shDKC1 cell lines were maintained at 37 °C in a humidified atmosphere of 5% CO₂ in complete DMEM containing 10% Tet-free fetal bovine serum (FBS), 1% penicillin-streptomycin and 1% L-Glutamine (Euroclone, Milano, Italy). In addition, the culture media of the RKO-shDKC1 and HEK-shDKC1 cell lines were supplemented with 500 ng/mL of puromycin (Sigma-Aldrich, St. Louis, MO, USA).

For 3BDO (3-Benzyl-5-((2-nitrophenoxy)methyl)-dihydrofuran-2(3H)-one) treatment, cells were washed in 1X DPBS and resuspended in complete Tet-free culture medium containing 400 ng/mL Dox. Following 72 h of incubation at 37 °C in a humidified atmosphere with 5% CO₂, the culture medium was replaced with complete Tet-free culture medium containing 400 ng/mL Dox and 100 µM of 3BDO (Sigma-Aldrich, St. Louis, MO, USA) and cells were grown for a further 24 h.

Transfection with GFP-LC3-RFP Reporter Transgene

To detect the autophagic flux, both untreated and 48 h Dox-treated HEK-shDKC1 and RKO-shDKC1 cells, seeded on glass coverslips placed in 35 mm dishes, were transfected with 3 µg of GFP-mRFP-LC3 plasmid (GFP-LC3-RFP-Plasmid, Addgene, Arsenal Way, Watertown, USA, 21074, depositing lab Tamotsu Yoshimori) and 12 µL of PEI (Polysciences, Valley Rd, Warrington, PA, USA) as described above. Following 48 h of transfection, coverslips were rinsed in 1X DPBS, fixed with 4% PFA (paraformaldehyde) (Biochemica, Billingham, UK, A3813), mounted on glass slides with DAPI solution and then examined under a Zeiss confocal fluorescence microscope Zeiss LSM 700 (Zeiss, Oberkochen, Germany). Images of control and depleted cells were always acquired at the same settings.

Reverse Transcription Quantitative PCR (RT-qPCR)

Total RNA was extracted from RKO and HEK cells using the QIAzol Lysis Reagent (Qiagen, Hilden, Germany, 79306), according to the manufacturer's guidelines. Reverse Transcription Quantitative PCR (RT-qPCR) was carried out as previously described [22] using the following primer pair sequences: DKC1 forward, 5'-GGCGGATGCGGAAGTAAT-3', DKC1 reverse, 5'-CCACTGAGACGTGTCCAAC-3'; ATG12 forward, 5'-CTTTGCTCCTTCC CCAGACC-3', ATG12 reverse, 5'-CACGCCCTGAGACTTGCAGTA-3'; ATG5 forward, 5'-TGTCCTTCTGCTATTGATCCTGA-3', ATG5 reverse, 5'-CCGGGTAGCTCAGATGTTCA-3'; HPRT1 forward, 5'-TGGTCAAGGTCGCAAGCTT-3', HPRT1 reverse, 5'-AGTCAAGGGCA TATCCTAC-3'; HSP70 forward, 5'-TTTTTGTGGCTTCCTTCGTT-3', HSP70 reverse, 5'-GAGTAGGTGGTGCCAAGATCA-3'; and HSP90 forward, 5'-GTTTGAGAACCTCTGCAA AAT-3', HSP90 reverse 5'-CATGGAGATGTCACCAATCG-3'. Relative quantifications were normalized to the expression of HPRT1 endogenous control.

Flow Cytofluorimetric Analyses

HEK 293T-shDKC1 and RKO-shDKC1 cells untreated and treated with Dox for 96 h or with 60 mM LiCl for 24 h were trypsinized, counted, washed three times in DPBS 1X and fixed in ice-cold ethanol at -20 °C overnight. The cells were then washed twice with cold

DPBS, resuspended in a hypotonic solution (0.1% Na-Citrate, 50 µg/mL RNase, 50 µg/mL propidium iodide) and incubated for 30 min in the dark at RT. The DNA content of the labeled cells was measured using a BD Accuri C6 flow cytometer and data were analyzed using BD Accuri™ C6 Software.

Protein Analysis

For protein extraction, cells were pelleted by centrifugation at 2500 rpm for 5 min at 4 °C and resuspended in a RIPA lysis buffer (50.0 mM Tris pH 7.5, 150.0 mM NaCl, 5.0 mM EDTA, 1% NP40) supplemented with anti-phosphatase and anti-protease (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, Sigma-Aldrich, St. Louis, MO, USA). After 30 min of incubation in ice, cell lysates were centrifuged at 14,000 rpm for 15 min at 4 °C to eliminate insoluble material. The supernatants were transferred in clean tubes and stored at −80 °C. The protein concentration of extracts was determined by Bio-Rad Protein Assay (Dye Reagent Concentrate, Biorad, Hercules, CA, USA, following the manufacturer's instructions. For Western blot analysis, samples were run on SDS-PAGE under reducing conditions, transferred onto 0.45 µm nitrocellulose filters (GE Healthcare, Little Chalfont, UK) and blocked with 5% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBST). After blocking, blots were incubated for 2 h with the primary antibody diluted in TBST and then for 2 h with horseradish peroxidase-conjugated anti-rabbit/mouse/IgG. The following primary antibodies were used in this study: dyskerin (Elabscience Biotechnology Inc., Houston, TX, USA, E-AB-31251; 1:100), GAPDH (Abcam, CA, USA, ab56788; 1:100), GRP78/BiP (Thermo Fisher Scientific, Waltham, MA, USA, PA5-11418; 1:1000), phospho PERK (Elabscience Biotechnology Inc., Houston, TX, USA, E-AB-21296; 1:1000), phospho eIF2α (Cell Signaling Technology (CST), Heidelberg, Germany, 9721; 1:1000), ATF4 (Santa Cruz Biotechnology; 1:500), CHOP (DSHB—Developmental Studies Hybridoma Bank, University of Iowa, Iowa city, USA; CPTC-DDIT3-1-S; 1:500), HSP70 (Santa Cruz biotechnology, sc-25837; 1:1000), HSP90 (Stressgen Biotechnologies Corporation, Ann Arbor, MI, USA, SPA-846; 1:1000), LC3 (Abcam, CA, USA, ab51520; 1:2000), Beclin 1 (Elabscience Biotechnology Inc., Houston, TX, USA, E-AB-33743; 1:1000), PARP (Santa Cruz Biotechnology, Dallas, TX, USA, sc-7150; 1:1000), BAX (Cell Signaling Technology (CST), Heidelberg, Germany 2772; 1:500), phospho 4E-BP1 (Cell Signaling Technology (CST), Heidelberg, Germany, 2855; 1:1000), phospho p70 S6K (Cell Signaling Technology (CST), Heidelberg, Germany 9205; 1:1000), phospho AKT (Cell Signaling Technology (CST), Heidelberg, Germany, 9271; 1:1000), and phospho GSK3β (GeneTex, Inc. (North America), Alton Pkwy Irvine, CA, GTX-59576; 1:1000). The following secondary antibodies used in this study were: Anti-Mouse-HRP (Bethil Laboratories, Montgomery, TX, USA, A90-144P; 1:10,000) and Anti-Rabbit-HRP (Bethil Laboratories, Montgomery, TX, USA, A120-100P; 1:10,000). Bands were visualized by ECL (Biorad, Hercules, CA, USA, 1705061) using the ChemiDoc XRS+ System (Bio-Rad) and quantified with Image Lab Software (Bio-Rad).

Immunostaining and Image Capturing

Cells were seeded on glass coverslips placed in 6-well plates and treated for 96 h with Dox. They were then fixed with 3.7% paraformaldehyde for 10 min, permeabilized in 100% ice-cold methanol for 10 min at −20 °C and finally blocked with DPBS supplemented with 3% bovine serum albumin (BSA) for 30 min. After each step, cells were rinsed in DPBS and incubated with primary antibodies overnight at 4 °C, following which the cells were washed in DPBS and incubated with secondary antibodies at RT for 1 h on the next day. Glass coverslips were counterstained with DAPI, mounted on glass slides and examined under a Zeiss confocal fluorescence microscope (Zeiss LSM 700). The primary antibodies used were the following: Calreticulin (D3E6) XP (Cell Signaling Technology (CST), Heidelberg, Germany, 12238; 1:250), Dyskerin (Santa Cruz Biotechnology, Dallas, TX, USA, sc-373956; 1:1000), GRP78/BiP (Thermo Fisher Scientific, Waltham, MA, USA, PA5-11418; 1:500) and LC3 (Abcam, CA, USA, ab51520; 1:3000). The secondary antibodies used were: Cy3-AffiniPure Goat Anti-Rabbit IgG (H + L) (Invitrogen, Van Allen Way

Carlsbad, CA, USA, A10520; 1:500) and Alexa Fluor 488 donkey anti mouse IgG (H + L) (Invitrogen, Van Allen Way Carlsbad, CA, USA, A21202; 1:1000).

Immunoreactivity Quantification

Dyskerin—To quantify dyskerin accumulation levels, confocal z-stack images of cells stained with DAPI and anti-dyskerin were converted to maximum projection images using ImageJ (Fiji distribution; <https://imagej.net/software/fiji> accessed on 30 March 2022). The DAPI and dyskerin channels were separated and the background was removed by applying a threshold and a mask specific for each channel. The nucleus volume of the each analyzed cell was selected by manually drawing a ROI surrounding the DAPI fluorescence. For each ROI/Nucleus, the RawIntDen in the dyskerin channel was measured. This value represents the total dyskerin immunoreactivity and is an estimation of the total amount of dyskerin contained in a nucleus (DAPI volume). The RawIntDen values were split by condition (Dox- and Dox+ 96 h), normalized and reported relative to the Dox-. The statistical analysis was performed using PRISM software applying a one-tailed *t*-test.

CRT and GRP78—ImageJ was used to convert the confocal z-stack images of cells stained with anti-CRT or anti-GRP78 in maximum projections. ROIs surrounding each of the captured cells were manually drawn. The background was removed by applying a threshold and a mask, both specific for the examined channel.

For each ROI/cell the RawIntDen in the CRT or GRP78 channel was measured. In order to have a measure of the amount of CRT or GRP78 not dependent on the cell size, the RawIntDen values were divided by the areas of the obtained mask (CRT or GRP78) inside each cell. The value RawIntDen/CRTArea (or RawIntDen/GRP78 Area), defined also as CRTdensity (or GRP78 density), is an estimation of the CRT accumulation levels in each cell. The statistical analysis of the normalized RawIntDen/CRTArea (or RawIntDen/GRP78Area) values, split by condition (Dox- and Dox+ 96 h), was performed using PRISM software applying a one-tailed *t*-test.

LC3—To measure LC3 levels, the RawIntDen/LC3 Area value for each cell was calculated as described above for CRT and GRP78. In addition, the values were split by condition (Dox- and Dox+), normalized and reported relative to the Dox- cells. Additionally, the find maxima function from ImageJ (Fiji version) was used to automatically detect the number of LC3 puncta per cell. Dividing the LC3 area in each cell by the number of LC3 puncta, a measurement of the size of LC3 puncta was obtained and, by dividing the RawIntDen/LC3 area by the number of LC3 puncta, a measurement of the amount of LC3 per puncta was obtained. The statistical analyses for all the calculated parameters were performed by using a one-tailed *t*-test using Prism software comparing the Dox- and the Dox+ 96 h conditions.

Evaluation of the Autophagic Flux Using the GFP-mRFP-Reporter

For quantifying the autophagic flux, for both conditions (Dox- and Dox+ 96 h), five of the imaged GFP-mRFP-LC3 transfected cells were manually isolated with ImageJ software (Fiji distribution; <https://imagej.net/software/fiji> accessed on 30 March 2022). For each of the isolated cells, the channels were separated and, for both of them (GFP and mRFP), the background was removed by subtracting the filtered median image (radius = 8 pixels) from the original image. After merging the two resulting channels, linear ROIs (of about two microns) were manually centered on each of the detected puncta in the RFP channel. The multiplot profile function (Fiji) was used to convert each cell's punctum information into tables of pixel intensity changes along linear regions of interest (ROI) for each detected punctum. The procedure was applied once for RFP intensity, and once FOR GFP intensity for each punctum in each cell. Subsequently, all tables were imported into RStudio, and the area under RFP- and GFP-plot profile curves for each punctum was calculated using the function trapz from the pracma package. Indeed, in acidic lysosomes, the ratio GFP/RFPArea is strongly reduced due to the quenching of GFP. The GFP/RF area ratio for each punctum was then labelled according to the cell condition (Dox- or Dox+ 96 h), and all the labelled values were aggregated into a single table for each signal (Tables S1 and

S2 for RFP and GFP, respectively). Significant ratio differences between Dox- and Dox+ conditions were estimated using a one-tailed *t*-test in Prism software. Finally, the ggplot2 package plots all RFP simultaneously as well as GFP-intensity profiles and ROIs. The ggplot2 package was used to simultaneously plot all RFP- and GFP-intensity profiles along the ROIs. The reproducible procedure has been reproduced as R script (Supplementary Materials S3: R code).

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, Inc. La Jolla, CA, USA). Differences among multiple groups were compared using a one-way ANOVA. Differences were considered statistically significant when $p < 0.05$. The level of statistical significance is indicated by asterisks * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. All experiments were performed in triplicate with the exception of the lithium chloride treatment, whose results were confirmed by two different methods (Western blot and flow cytometry).

Results

Dyskerin Downregulation Leads to the Accumulation of HSP90, HSP70 and Calreticulin Chaperones

A previous study has shown that under-pseudouridylated ribosomes purified from dyskerin-depleted human cells showed altered translational fidelity when added to a cell-free system [5]. However, the effects of this telomeric-independent impairment of translational fidelity on cellular physiology remain to be determined. Recently, to study the telomerase-independent functions of human dyskerin, we developed an inducible cellular silencing system (pLKO-Tet-On-shDKC1 silencing vector) capable of depleting dyskerin in human colon carcinoma (RKO) cells upon the addition of tetracycline or doxycycline in culture media [9]. In this paper, we applied this system to test the possibility that the reduced translational fidelity of under-pseudouridylated ribosomes may induce the accumulation of unfolded/misfolded proteins and consequently lead to ER stress. In order to uncover phenomena not restricted to a specific cell type, we also generated a HEK 293T-shDKC1 cell line in this study. To obtain this line, we transfected human embryonic kidney 293T cells (HEK 293T) with a pLKO-Tet-On-shDKC1 silencing vector [9]. Once stably transformed lines were obtained, to minimize clonal differences, all subsequent experiments were carried out on pools of transformed cell clones (see Materials and Methods).

To verify whether dyskerin depletion could trigger the production of incorrectly folded proteins in general, we evaluated both mRNA and protein levels of Hsp70 and Hsp90 after the induction of DKC1 silencing in RKO and HEK 293T cells. Hsp70 and Hsp90 are chaperones that are known to play central roles in the folding of newly synthesized proteins and in the refolding of misfolded or misaggregated proteins, thus orchestrating the so-called heat shock response [23–25]. Importantly, our RT-qPCR and Western blot analyses show that Hsp70 and Hsp90 are upregulated at both the transcriptional and translational levels in dyskerin-silenced cells (Figure 1A–D). Specifically, we observed that, in both RKO-shDKC1 and HEK 293T-shDKC1 cells, a reduction of ~70% of DKC1 mRNA, obtained after 96 h of induction, causes a significant increase in Hsp70 and Hsp90 mRNA accumulation levels (Figure 1A,C). Consistent with an increased folding capacity, these transcriptional changes are translated to increased Hsp70 and Hsp90 protein levels during a time course of 96 h (Figure 1B,D).

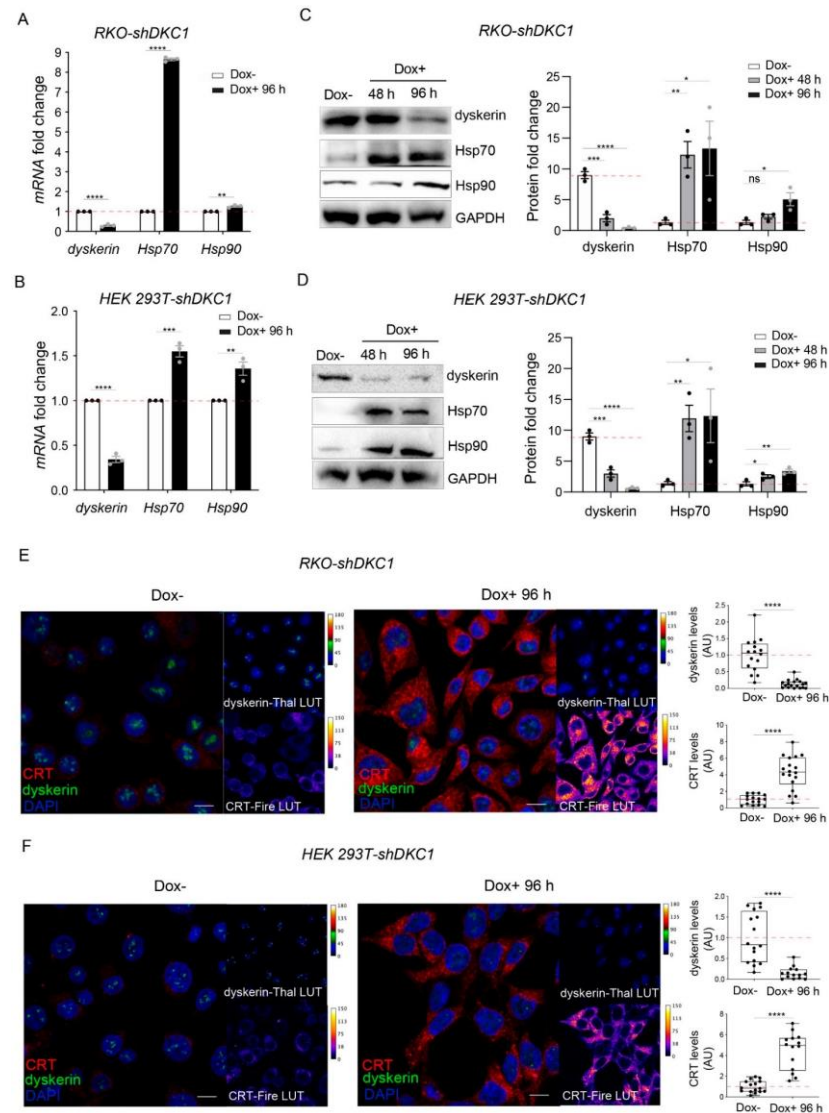


Figure 1. Dyskerin silencing stimulates the expression of chaperones involved in protein refolding. (A,B) qRT-PCR analysis showing the accumulation of Hsp70 and Hsp90 mRNAs in RKO-shDKC1 (A) and HEK 293T-shDKC1 cells (B) after DKC1 silencing induced by 96 h of Dox treatment. Data shown are relative to the untreated (Dox-) condition and as mean $\pm$ SEM ($n = 3$). (C,D) Western blot analysis showing an increased accumulation of Hsp70 and Hsp90 in RKO-shDKC1 (C) and HEK 293T-shDKC1 cells (D) after dyskerin depletion induced by 48 or 96 h of Dox treatment. The intensity of the bands was normalized to GAPDH and the differential accumulations evaluated with respect to the Dox- condition. Data are presented as mean $\pm$ SEM ($n = 3$). (E,F) Confocal images showing the increase in calreticulin (CRT) in RKO-shDKC1 (E) and HEK 293T-shDKC1 cells (F) treated with Dox for 96 h (Dox+) compared to their untreated control cells (Dox-). Scale bars 10 μ m. Nuclei were counterstained with DAPI. CRT immunoreactivity is in red or Fire-Lut (false color Fire-Lookup Table) in separate channels. Dyskerin immunoreactivity is in green or in Thal-Lut (false color Thal-Lookup Table). CRT and dyskerin accumulation levels were quantified in multiple cells ($n = 16$ RKO-shDKC1 Dox-; $n = 18$ RKO-shDKC1 Dox+ 96 h; $n = 16$ HEK 293T-shDKC1 Dox-; $n = 14$ 293T HEK 293T-shDKC1 Dox+ 96 h), normalized and reported relative to the Dox- condition as mean $\pm$ SEM. An unpaired one-tailed t -test was used in pairwise comparisons with respect to the Dox- condition in all statistical analyses in this figure (**** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns, $p > 0.05$).

These data demonstrate that the activation of the heat shock response after dyskerin depletion is not restricted to one specific cell type. In addition, since our analyses refer to a time window of 96 h, a time too short for a significant shortening of telomeres to occur, the induction of heat shock response upon dyskerin silencing is not due to the telomerase-dependent dyskerin function and appears at the early onset of the X-DC pathology.

To test whether the observed increased folding capacity induced by DKC1 silencing is directly due to the accumulation of unfolded/misfolded proteins synthesized by under-pseudouridylated ribosomes, we verified if dyskerin depletion correlates with an increased immunoreactivity of calreticulin (CRT). CRT is an ER-localized Ca^{2+} -binding chaperone that binds to misfolded glycoproteins to promote their refolding and is thus considered an endoplasmic reticulum stress (ERS) marker [26,27]. Our confocal analyses show a considerable upregulation of CRT in both RKO and HEK 293T dyskerin-silenced cells (Figure 1E,F). Specifically, in both systems, we found that, in comparison with uninduced controls, 96 h of dyskerin silencing caused an 84% reduction in dyskerin immunoreactivity and more than a 4-fold increase in CRT immunoreactivity.

Hence, it is reasonable to conclude that the lack of translational fidelity caused by dyskerin downregulation results in a telomerase-independent accumulation of unfolded proteins and in the consequent induction of chaperones involved in their refolding upon ERS.

Dyskerin Depletion Triggers Unfolding Protein Response

Since the accumulation of unfolded/misfolded proteins can activate the UPR pathway, with enormous consequences on the general metabolism of the cell, we evaluated, upon dyskerin depletion, the expression of the glucose-regulated protein 78 (GRP78 also referred as Bip or HSPA5), a master regulator of UPR pathways [28–30].

Consistent with the idea that dyskerin depletion causes the activation of UPR, our confocal analyses showed that a 60% reduction in dyskerin immunoreactivity (induced by 96 h of doxycycline administration to RKO cells) triggers a strong increase (more than 7-fold) in GRP78 immunoreactivity (Figure 2A). The GRP78 accumulation following dyskerin depletion in RKO was further confirmed by Western blot analysis (Figure 2B).

To confirm that dyskerin downregulation triggers UPR, we analyzed, by Western blot, the accumulation levels of the key markers of the PERK pathway pPERK, p-eIF2 α , ATF4 and CHOP, during a time course of 96 h of DKC1 silencing in RKO cells [31]. As expected, we found that dyskerin depletion results in increased levels of pPERK and p-eIF2 α , concomitantly with the accumulation of ATF4 and CHOP. Importantly, we also found that a time course of 96 h of dyskerin depletion induces a similar UPR response in HEK cells (Figure 2C).

Altogether, these findings indicate that dyskerin depletion promotes UPR activation through the PERK pathway. Since this activation is not related to the shortening of telomeres, the physiological and metabolic changes caused by UPR activation could represent important steps occurring before the onset of X-DC typical features.

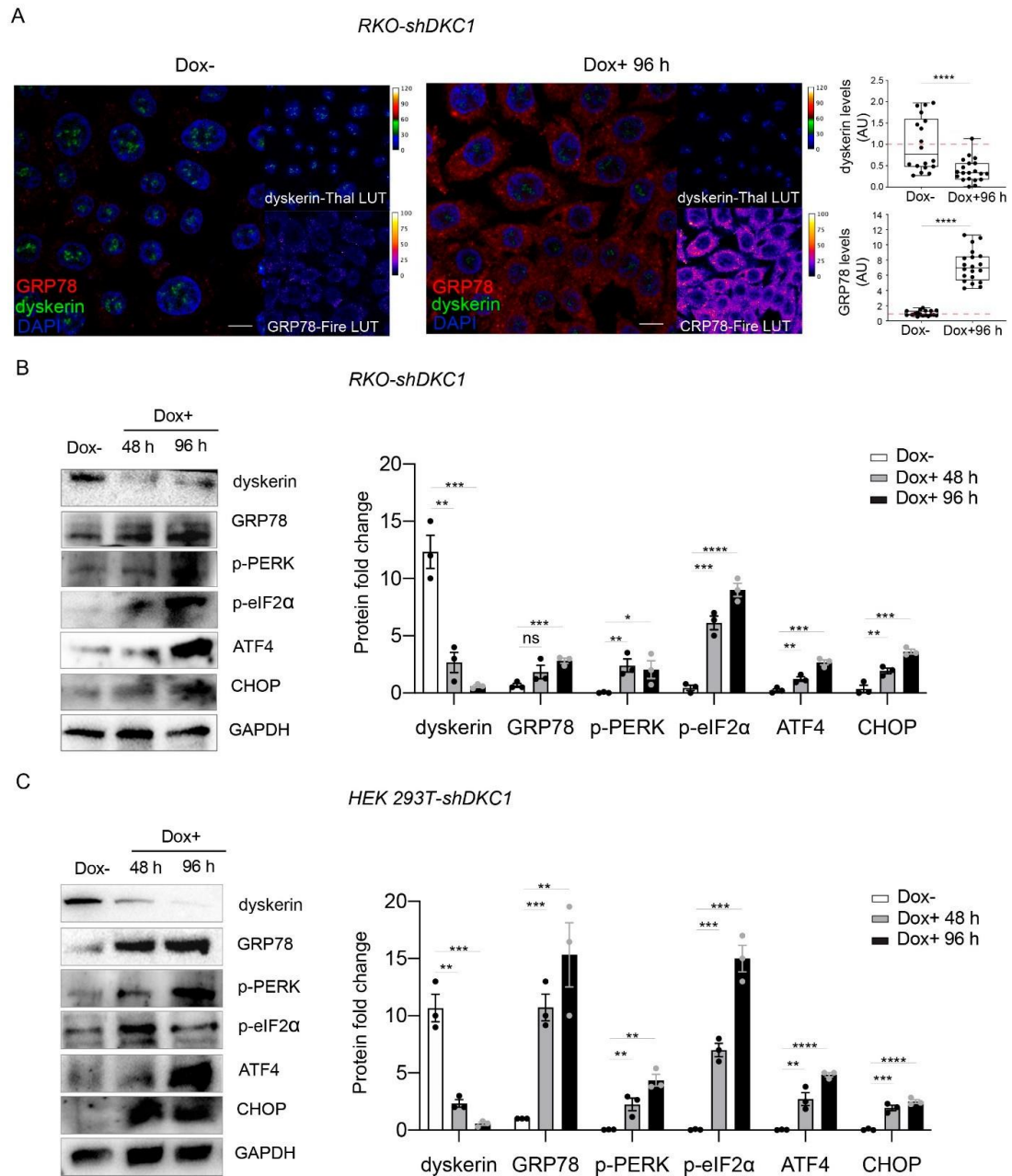


Figure 2. Dyskerin depletion induces ERS and increases UPR markers. (A) Confocal images showing the dramatic increase in GRP78/BiP (red or Fire-Lut) expression in RKO-shDKC1 cells treated with Dox for 96 h (Dox+, right panel) compared to untreated control cells (Dox-, left panel). Dyskerin (green or Thal-Lut) downregulation is evident from the strong reduction in the relative signal in Dox+ cells. Nuclei were counterstained with DAPI. GRP78/BiP and dyskerin levels were quantified in multiple cells ($n = 18$ RKO-shDKC1 Dox-; $n = 20$ RKO-shDKC1 Dox+ 96 h), normalized and reported relative to the Dox- condition as mean $\pm$ SEM. Scale bars 10 μ m. (B,C) Western blot analysis confirming the upregulation of GRP78/BiP and showing the induction of key components of the PERK branch of UPR in RKO-shDKC1 (B) and HEK 293T-shDKC1 (C) Dox-treated (Dox+) cells for 48 or 96 h. Protein levels were normalized to GAPDH. Data are shown as mean $\pm$ SEM ($n = 3$). All statistical analyses were performed using an unpaired one-tailed t -test in pairwise comparisons with respect to the Dox- condition (**** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns, $p > 0.05$).

Dyskerin Depletion Does Not Induce Apoptosis

Severe and prolonged ERS conditions that prevent cells from restoring ER homeostasis can lead the UPR to activate the apoptotic program, leading to cell death [32,33]. ERS-induced apoptosis can occur via different pathways [34]. In order to evaluate if dyskerin depletion could trigger apoptosis, we examined the silenced cells directly by flow cytometry analysis. To set up the optimal conditions and discriminate apoptotic cells from live cells, we used RKO and HEK 293T untreated cells as internal controls in these experiments. When these cells were induced to undergo apoptosis by a 24 h treatment with 60 mM LiCl, a GSK3 β inhibitor known to induce apoptosis in several human cell lines [35–37], they exhibited a high percentage of subdiploid cells (Figure 3A). In contrast, when Dox-treated RKO and HEK 293T cells were analyzed for their DNA content, they presented only a negligible percentage of the subdiploid peak (Figure 3A), indicating that they are not subjected to apoptosis. Next, we checked by Western blotting the cleavage of poly (ADP-ribose) polymerase-1 (PARP-1), a known apoptotic marker, and found it was not activated by dyskerin depletion in HEK 293T cells. In contrast, its induction was efficiently triggered by LiCl treatment (Figure 3B). Accordingly, no induction of the apoptotic marker Bax was observed upon dyskerin silencing, while it was sharply induced upon LiCl treatment (Figure 3B). Similar results were previously obtained by our group in dyskerin-depleted RKO cells [9].

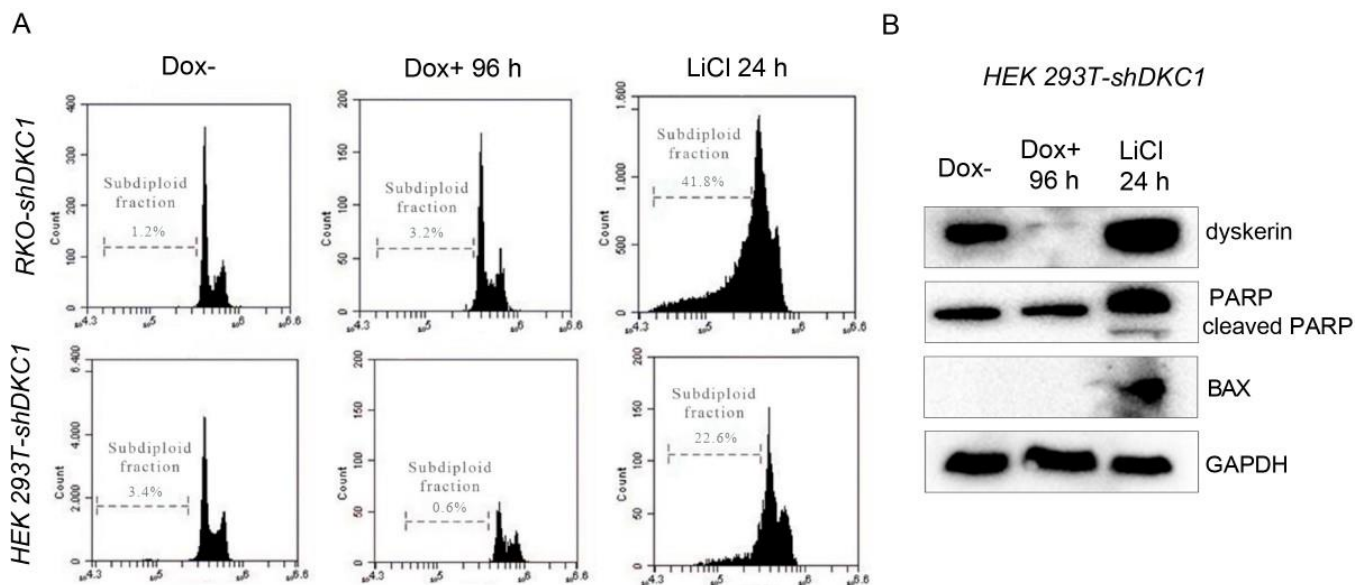


Figure 3. Dyskerin depletion does not activate apoptosis. (A,B) Induction of apoptosis was measured by calculating the percentage of control (Dox-) and silenced (Dox+) cells displaying subdiploid DNA content following propidium iodide staining. As a positive control, RKO-shDKC1 and HEK 293T-shDKC1 cells were treated for 24 h with LiCl (60 mM), an inducer of apoptosis. (C) Western blot analyses of the apoptotic markers PARP and BAX in control (Dox-), dyskerin-depleted cells (Dox+ 96 h), and control cells treated with LiCl (60 mM) for 24 h; the latter served as a positive control.

Collectively, the above findings indicate that ERS caused by dyskerin depletion does not activate apoptosis, at least not after 96 h of DKC1 silencing.

Dyskerin Downregulation Promotes Autophagy

It is clear from a previous study that the accumulation of misfolded proteins can activate autophagy as a defensive mechanism for maintaining ER homeostasis [29]. We thus wondered whether the activation of the PERK pathway observed in the silenced cells could also promote autophagy. Therefore, we evaluated by Western blot the levels of Beclin-1 marker and, as shown in Figure 4A,C, found that its accumulation level dramatically

increased more than 6-fold in both types of dyskerin-depleted cells after 96 h of silencing (92% of depletion efficiency).

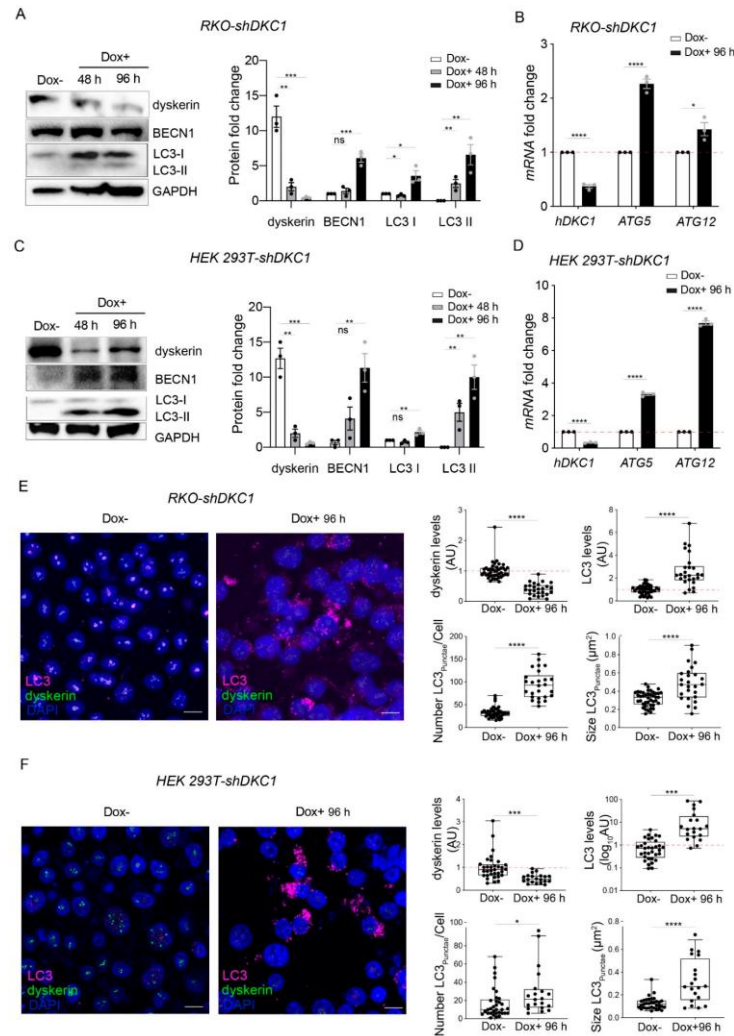


Figure 4. Dyskerin downregulation induces a set of autophagic markers. (A,C) Western blot analyses of BECN1 and LC3 proteins following 48 or 96 h of dyskerin depletion. Dyskerin depletion induces LC3-II in both RKO-shDKC1 (A) and HEK 293T-shDKC1 (C) silenced cells (Dox+). Upon silencing, LC3-I, whose level is not indicative of autophagic induction, also appeared upregulated in RKO cells. BECN1 levels increased in both cellular systems at the times considered. Protein levels were normalized to GAPDH. Results are expressed as mean $\pm$ SEM ($n = 3$). (B,D) qRT-PCR analyses in both RKO-shDKC1 and HEK 293T-shDKC1 ((B,D), respectively) cells show an increase in the transcription of *ATG12* and *ATG5* genes, which participate in the elongation of autophagosomes, following 96 h of Dox-induced dyskerin silencing. Results are expressed as mean $\pm$ SEM ($n = 3$). (E,F) Confocal micrographs of control (Dox-) and silenced (Dox+) RKO-shDKC1 (E) and HEK 293T-shDKC1 (F) cells, immunolabeled with anti-LC3 (magenta) and anti-dyskerin (green) antibodies. Scale bars 10 μ m. Nuclei were counterstained with DAPI. LC3 and dyskerin levels were quantified in multiple cells ($n = 42$ RKO-shDKC1 Dox-; $n = 26$ RKO-shDKC1 Dox+ 96 h; $n = 33$ HEK 293T-shDKC1 Dox-; $n = 20$ 293T HEK 293T-shDKC1 Dox+ 96 h), normalized and reported relative to the Dox- condition as mean $\pm$ SEM. The cytoplasmic LC3 puncta number and size were also calculated for the same cells. Both LC3 puncta and size were significantly upregulated after 96 h of Dox treatment. An unpaired one-tailed *t*-test was used in pairwise comparisons with respect to the Dox- condition in all statistical analyses in this figure (**** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns, $p > 0.05$).

We then evaluated the conversion of LC3-I to LC3-II that is commonly used as a marker for the activation of autophagy [38]. Intriguingly, we observed that, while in unsilenced cells LC3-II is undetectable, dyskerin depletion strongly increases LC3-II accumulation levels even after 48 h of silencing (83% depletion efficiency) (Figure 4A,C). However, during this time course, LC3-I levels are only slightly increased in both cell types, implying that dyskerin depletion causes autophagosome formation.

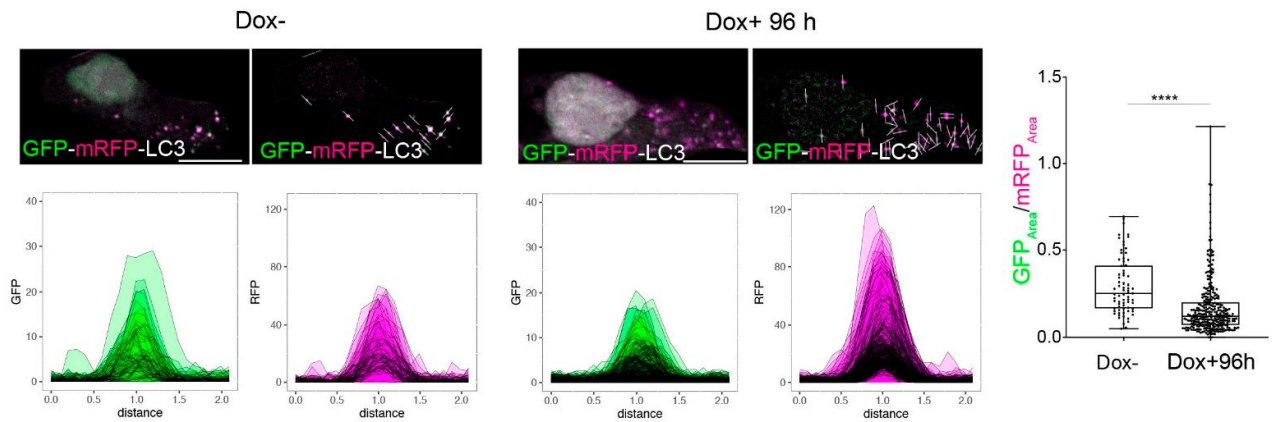
The conjugation between ATG12 and ATG5 is essential for LC3 lipidation to form LC3-II, and therefore for autophagosome formation [39]. We therefore evaluated the expression of ATG5 and ATG12 by qRT-PCR and found an increase in their expression in the silenced cells (Figure 4B,D).

To confirm that dyskerin reduction induces autophagosome formation, we evaluated, by confocal imaging LC3 immunoreactivity, the cellular area occupied by LC3 puncta, the number of LC3 puncta per cell (which represents the most commonly used assay for measuring the autophagosome number [40]), the puncta size and the intensity of LC3 in each detected punctum in both the control and silenced cells. Importantly, we observed that a ~60% reduction in nucleolar dyskerin in RKO cells leads to an about 3-fold increase in both the total levels of LC3 per cell and the number of LC3 puncta per cell (from ~30 to ~90, Figure 4E). This LC3 increment following dyskerin depletion was further confirmed by a similar increase in the LC3Area/CellArea (Figure S1). In addition, we observed a 30% increase in the amount of LC3 contained in each punctum (Figure S1) and a 1.5 fold increase in the puncta size (from 0.32 to 0.49 μm^2 ; Figure 4E).

Although we observed a similar response to dyskerin depletion in HEK cells, there were some differences with the results obtained with RKO cells. Specifically, we estimated that, in HEK cells, a 50% reduction in dyskerin caused a considerable increase (~17 fold) in the total levels of LC3 per cell, but only a ~2-fold increase in LC3 puncta per cell (Figure 4F). This indicates that each punctum contains a considerable amount of LC3 or that, in this cell type, our confocal resolution is not sufficient to separate LC3 puncta that are very close to each other. This hypothesis was confirmed by our estimation of LC3 puncta size (increased by ~3-fold, from ~0.12 to 0.32 μm^2 ; Figure 4F) and by the LC3 amount contained in each detected punctum or overlapped puncta (increased by more than 4-fold upon dyskerin reduction; Figure S1).

Altogether, our data clearly demonstrate that, as a general phenomenon, dyskerin depletion induces autophagosomes accumulation. This accumulation could be either due to an induction of autophagy or its blockage (i.e., inefficient fusion or decreased autophagosome degradation) [41]. To rule out the possibility that the observed increase in LC3-II could derive from an accumulation of autophagosomes due to the block of autophagosome-lysosome fusion, we transiently transfected control and silenced cells with the mRFP-GFP-LC3 tandem construct. This construct permits monitoring the different stages of autophagic flux, thereby allowing one to discriminate autophagosomes from autolysosomes [40,42]. When the tandem-tagged fluorescent proteins accumulated in these cells, both green and red fluorescent signals were detectable at the early stages of the autophagic process; however, at later stages, the GFP signal was quenched inside the acidic autolysosomes, while the RFP signal was still retained. Following silencing, a downward shift in the distribution of GFPArea/RFPArea values (Figure 5A,B) in both RKO-shDKC1 and HEK 293T-shDKC1 cells was clearly observed.

A

RKO-shDKC1

B

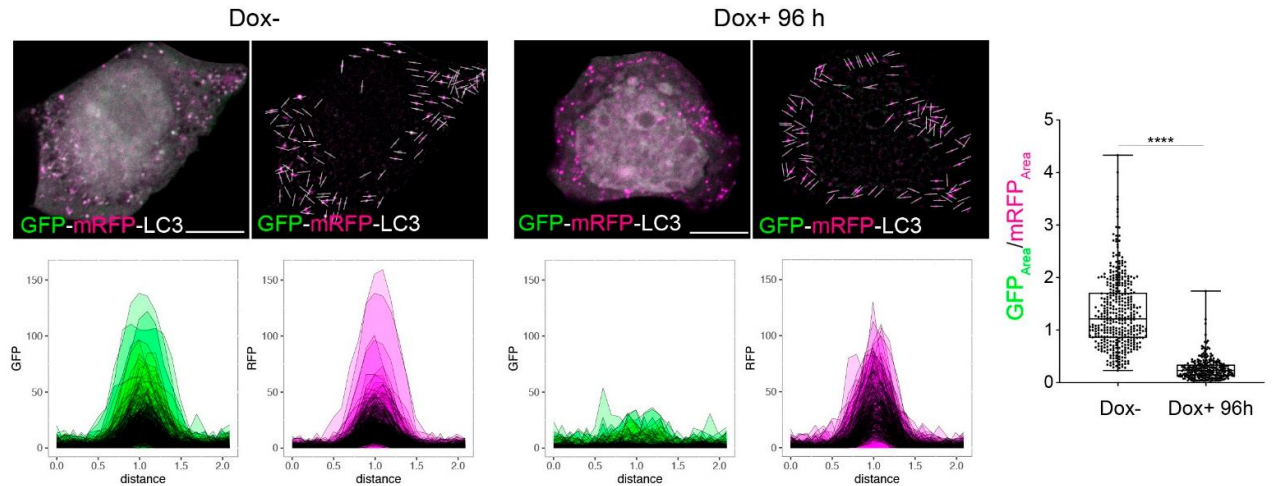
HEK 293T-shDKC1

Figure 5. Dyskerin depletion promotes a functional autophagic flux. RKO-shDKC1 and HEK 293 T-shDKC1 Dox⁻ cells were transiently transfected with the GFP-mRFP-LC3 tandem construct (containing LC3-I fused to mRFP and GFP). After 5 h of transient transfection, untreated (Dox⁻/GFP-mRFP-LC3) or treated (Dox⁺/GFP-mRFP-LC3) cells were grown for 96 h and then fixed and analyzed by confocal microscopy to detect individual GFP-mRFP-LC3 puncta (see materials and methods). For each single punctum, the area under the plot-profile in both GFP and mRFP channels and the GFPArea/RFPArea ratios were calculated. **(A)** GFP-mRFP-LC3 puncta analyses in RKO-shDKC1 cells. One representative cell is shown per condition (left panels) and linear ROIs centred on each of detected punctum in this cell are shown as well (right panel). Scale bars 10 μ m. GFP and mRFP plot-profile curves for all the detected puncta are shown for both conditions ($n = 73$ puncta from 5 RKO-shDKC1 Dox⁻ cells; $n = 316$ puncta from 5 RKO-shDKC1 Dox⁺ 96 h cells). The GFPArea/RFPArea ratio is significantly decreased after 96 h of Dox treatment, demonstrating the majority of the LC3 puncta detected in DKC1 silenced cells represent phagosomes fused with lysosomes. **(B)** GFP-mRFP-LC3 puncta analyses in HEK 293T-shDKC1 cells. Scale bars 10 μ m. The GFPArea/RFPArea ratio is significantly reduced after 96 h of Dox treatment, demonstrating an increased autophagic flux upon dyskerin depletion ($n = 400$ puncta from 5 HEK 293T-shDKC1 Dox⁻ cells; $n = 264$ puncta from 5 HEK 293T-shDKC1 Dox⁺ 96 h cells). All statistical analyses were performed using an unpaired one-tailed t -test in pairwise comparisons with respect to the Dox⁻ condition (**** $p < 0.0001$).

Taken together, these results unequivocally indicate that dyskerin depletion induces a functional autophagic flux, which is characterized by an efficient mature autophagolysosome formation.

In this paper, we propose a hybrid image-processing/statistical analysis pipeline for autophagic flux evaluation based on the quantification of the GFPArea/RFPArea. Coupling the pipeline with the experimental design presented in this paper allowed the successful evaluation of autophagic flux in silenced cells and highlighted the suitability of the ratio as an autophagy proxy.

Dyskerin Downregulation Promotes Autophagy through the Inhibition of AKT/mTOR Signaling

The mechanistic target of rapamycin (mTOR) is the master regulator of autophagy [43], and recent studies have shown that ERS induces autophagy through the suppression of this pathway [44–47]. Thus, in order to explore the possibility that dyskerin depletion could induce autophagy by mTOR inhibition, we investigated the protein expression of its downstream substrates, i.e., 4E-BP1 and P70S6K, by Western blot analysis. As shown in Figure 6A,B, the expression of both these mTOR downstream targets was markedly reduced in both RKO and HEK silenced cells, indicating that dyskerin depletion effectively inhibited TORC1.

Considering that ERS and UPR are interlaced with the mTOR pathway, and that a large number of studies demonstrated that ERS downregulates AKT activity [45], we hypothesized that, upon dyskerin downregulation, the activation of the ERS/UPR pathways inhibited AKT/mTOR, which in turn could be responsible for the activation of autophagy. To confirm this hypothesis, we analyzed by Western blot the expression of pAKT in the dyskerin-silenced cells and found a significant decrease in the expression of this protein compared to their untreated controls (Figure 6A,B). To further assess the role played by pAKT in this context, we treated the silenced cells with the mTOR activator 3-benzyl-5-((2-nitrophenoxy) methyl)-dihydrofuran-2(3H)-one (3BDO). Accordingly, treating the silenced cells with 3BDO rescued the expression of p-AKT, p-4EBP1 and p-P70S6K and also prevented the accumulation of LC3 II (Figure 6C).

These findings fully support the conclusion that the inhibition of the AKT/mTOR signaling pathway plays a key role in triggering the autophagic process observed in the dyskerin-silenced cells.

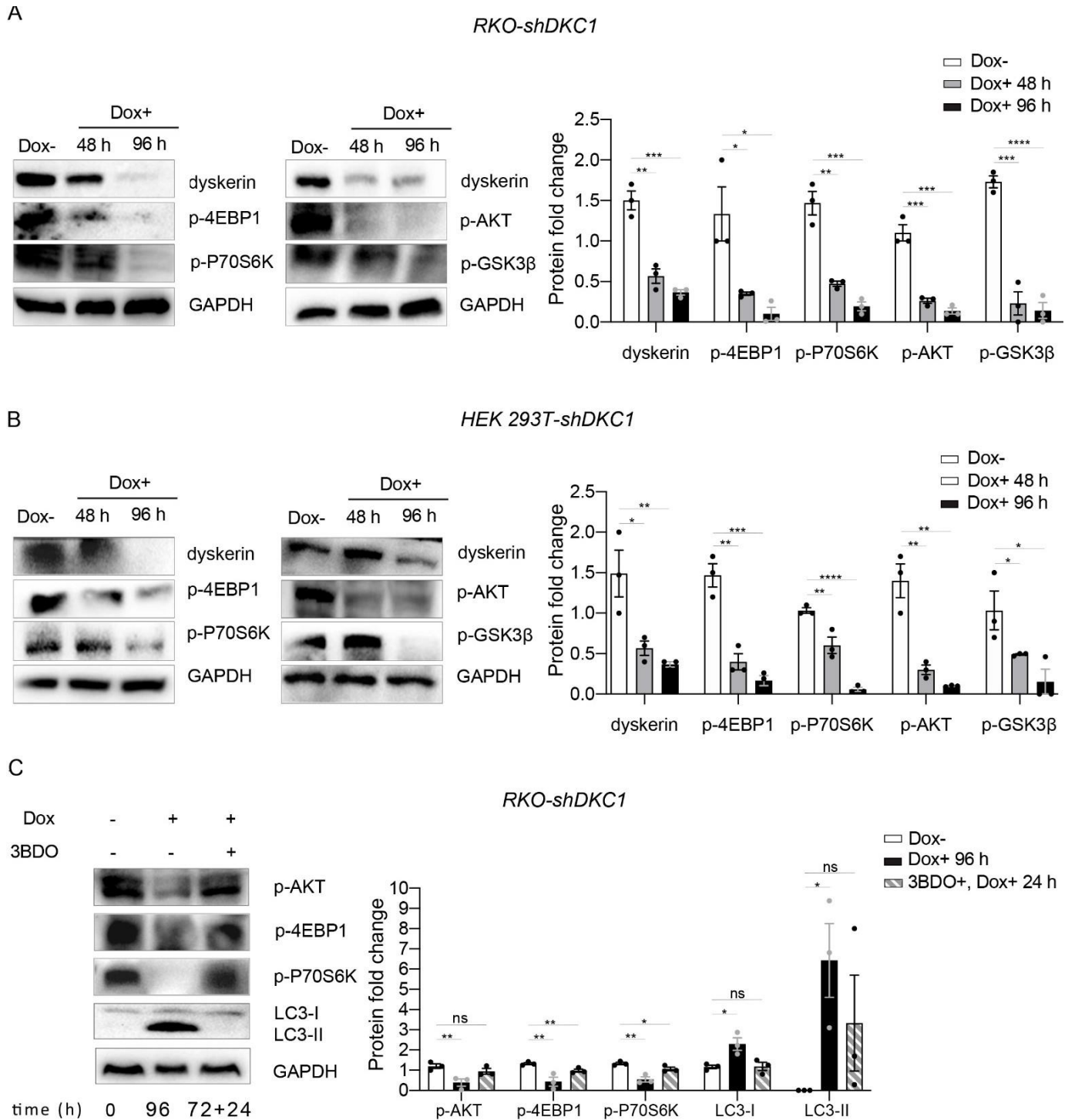


Figure 6. Dyskerin downregulation inhibits AKT/mTOR signaling. (A,B) Western blots showing the downregulation of p-4EBP1, p-P70S6K, p-AKT and p-GSK3 β upon dyskerin depletion. Band intensities for each protein were normalized to the band intensity of GAPDH and the results are expressed as mean $\pm$ SEM ($n = 3$). (C) The 3BDO treatment rescued the expression levels of p-AKT, p-4EBP1 and p-P70S6K and prevented the accumulation of LC3 II. In the timeline, 72 + 24 h indicates that 72 h of Dox treatment were followed by 24 h of Dox + 3BDO treatment. For quantification, the band intensity for each protein was normalized to the band intensity of GAPDH. Results are expressed as mean $\pm$ SEM ($n = 3$). An unpaired one-tailed t -test was used in pairwise comparisons with respect to the Dox- condition (**** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns, $p > 0.05$).

Discussion

In this study, by using stably transformed RKO and HEK 293T cell lines able to trigger DKC1 silencing in inducible manner, we detected early cellular alterations due to dyskerin depletion.

Since it is reasonable to consider that the telomere length is not significantly reduced during 96 h of induction, it is likely that the alterations we identified do not depend on the role that dyskerin plays as a component of the telomerase complex.

Importantly, we found that both cell lines used in this study respond early to dyskerin depletion by increasing their protein folding capacity, as evidenced by the observed concomitant strong upregulation of HSP70, HSP90 and calreticulin chaperones. The induction of these chaperones, which are present in abundance within the ER [48], suggested that the loss of function of dyskerin leads to an accumulation of misfolded/unfolded proteins. Consistently with this idea, we found that the GRP78 chaperone, a main marker of UPR activation [49], rapidly accumulated upon DKC1 silencing, demonstrating that the lowering of dyskerin levels can stimulate immediately both the ERS and UPR homeostatic responses. These results fit well with previous data indicating that ribosomes purified from dyskerin-depleted cells were intrinsically altered in translation [5], thus being able to promote the synthesis of proteins bearing altered reading frames, whose presence is known to affect proper folding [50]. Accordingly, it can be presumed that the activation of the ERS and UPR pathways is likely to be due to the reduced translation fidelity caused by dyskerin depletion, with the consequent accumulation of misfolded/unfolded proteins in the ER lumen.

In parallel with the increased accumulation of GRP78, we found that several key effectors of the PERK-dependent UPR pathway are upregulated upon dyskerin depletion. Importantly, in both RKO-shDKC1 and HEK 293T-shDKC1 cells, reduced levels of dyskerin were able to induce eIF2 α phosphorylation and to increase ATF4 levels. PERK belongs to the eIF2 α kinase subfamily and represents one of the major ER transmembrane stress-responsive proteins [51]. Upon disturbances in protein folding, PERK is activated by a trans-autophosphorylation [52–54]. Activated PERK phosphorylates eIF2 α [55–57] with the consequent attenuation of global protein synthesis [58,59] and, on the contrary, the enhanced translation of only selective sets of mRNAs, including ATF4 [5]. In turn, ATF4 induces the multifunctional C/EBP-homologous protein (CHOP) transcription factor [60].

It is worth noting that the reduced ribosome pseudouridylation (caused by either DKC1 pathologic mutations or reduced dyskerin levels) has to date been reported to differently modulate IRES-dependent translation [3,4,61]; in addition, ribosomes derived from dyskerin-depleted cells, when tested in an *in vitro* system, proved to significantly affect only IRES-mediated translation. Nonetheless, the increased level of eIF2 α phosphorylation we observed in the dyskerin-silenced cells suggests that the lowering of dyskerin levels could impact the rate of cap-dependent translation in cellular systems. Indeed, it is possible that the results obtained in an *in vitro* system do not fully reproduce the real context of the growing cells, where potential response mechanisms may be activated dynamically.

To the best of our knowledge, this is the first time that dyskerin depletion is reported to correlate with an increase in PERK and eIF2 α phosphorylation. The overactivation of PERK-eIF2 α phosphorylation, which occurs in several models of human diseases [51], led to the prediction that the pharmacological modulation of the PERK-dependent UPR signaling pathways may contribute to ameliorate the symptoms of X-DC. Importantly, several pieces of evidence have demonstrated that eIF2 α phosphorylation is a central event for the stimulation of autophagy [62]. For example, it has been established that the induction of the autophagy flux by different compounds is accompanied by eIF2 α phosphorylation, and that the blockage of this phosphorylation, by using a non-phosphorylatable mutant of eIF2 α , strongly abolishes the autophagy. Conversely, the inhibition of eIF2 α phosphatase is sufficient to stimulate autophagy [62]. Altogether, these data demonstrate that eIF2 α phosphorylation is sufficient to induce autophagy, even if the molecular details of this induction remain unknown. Based on the relationship between eIF2 α phosphoryla-

tion and autophagy, it is reasonable to believe that the autophagic process we observed upon dyskerin depletion relies on p-eIF2 α and it is downstream of the PERK-dependent UPR pathway.

Several studies reported that the main function of UPR signaling pathways is to protect cells from unfavorable conditions caused by pathological changes. Moreover, UPR protects the organism by eliminating cells that were exposed to extreme ER stress via apoptosis. Indeed, once ERS cannot be resolved, the persistence of UPR is known to activate the apoptotic program [63]. Since during a time course of 96 h we did not detect any apoptosis in the DKC1 silenced cells, it is possible to conclude that the activation of the PERK-dependent UPR pathway upon DKC1 silencing has an adaptive and not a pro-apoptotic role. Accordingly, the induction of apoptosis was never observed by our and other groups in several different human cancer cell lines subjected to different dyskerin depletion systems [9,12,64,65].

Consistent with the adaptive role of the PERK-dependent UPR pathway activated following dyskerin depletion, we also found that dyskerin downregulation induces a functional autophagic flux, which is characterized by an efficient mature autophagolysosome formation. To follow the efficiency of the process, we carefully evaluated autophagosome–lysosome fusion by coupling imaging and R data analysis. Autophagy acts as a pro-survival mechanism to remove misfolded proteins and restore ER homeostasis.

Importantly, the UPR-induced deactivation of mTOR contributes to the downregulation of AKT/TSC/mTOR pathway and promotes ERS-induced autophagy [45,66]. Similarly, the inhibition of AKT and mTORC1 by several agents, including the monoclonal antibody cetuximab, can induce autophagy [67–75]. Furthermore, the PERK–CHOP mediated induction of the AKT inhibitor TRB3 may also contribute to the inhibition of AKT upon severe ERS [76,77].

Consistent with the idea that a chronic activation of UPR induces autophagy by inhibiting mTORC1, we found that the accumulation level of 4E-BP1 and p70S6K is markedly reduced in both RKO-shDKC1 and HEK 293T-shDKC1 silenced cells. Intriguingly, in our experiments the treatment of the silenced cells with the mTOR activator 3BDO was able to rescue not only the expression of the autophagy marker LC3 II, but also that of p-AKT and of the two mTOR targets p-eIF4E-BP1 and p-p70S6K. This finding indicates that the activation of autophagy in the dyskerin-depleted cells is mediated by the inhibition of the AKT/mTOR pathway.

This study, for the first time, revealed an interdependence between mTOR signaling and UPR during a pathological program triggered by reduced levels of dyskerin. It is worth noting that the interplay between mTOR and chronic UPR has been associated with various pathologies [71,78–81], justifying the preclinical evaluations of combined therapies [66]. For example, preliminary studies suggest that treatment with ER-stress-alleviating “chemical chaperones”, such as 4-phenylbutyric acid (PBA), combined with a low dosage of the mTORC1 inhibitor (everolimus or rapamycin) could be used for the treatment of nephropathies and for TSC patients who are not suitable for surgical intervention [80–82].

The results we present in this paper link, for the first time, the loss of dyskerin with autophagy, UPR and mTOR signaling. In addition to further strengthening the role played by this multifunctional protein in the cellular homeostasis and in the stress response [10], these data suggest that a combined use of drugs aimed at relieving/suppressing the UPR (such as PBA and the PERK antagonist GSK2606414) and inhibiting the mTOR pathway (rapamycin or everolimus) might prevent or delay some severe manifestations of X-DC. These approaches might be useful especially in cases in which the onset of severe X-DC symptoms occur late, largely beyond childhood [83,84].

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/biomedicines10051092/s1>, Figure S1: Dyskerin depletion increases LC3 cytoplasmic levels; Table S1: Dataframes (distance; pixel-intensity) obtained from linear ROIs centered on detected puncta in silenced and control RKO-shDKC1 cells; Table S2: Dataframes

(distance; pixel-intensity) obtained from linear ROIs centered on detected puncta in silenced and control HEK 293T-shDKC1 cells; S3: R code to obtain GFPArea/RFPArea values for each detected punctum.

Author Contributions: Conceptualization, M.T. and M.F.; formal analysis, P.D. and R.V.; investigation, D.M., M.V. and V.B.; data curation, R.V.; writing—original draft preparation, M.T. and M.F.; writing—review and editing, M.T., M.F., R.V. and M.D.R.; supervision, M.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

Acknowledgments: We are grateful to Thomas Brody for the English revision of the manuscript. We also thank Bruno Hay Mele for the fruitful discussion on statistical and coding aspects.

Conflicts of Interest: The authors declare no conflict of interest.

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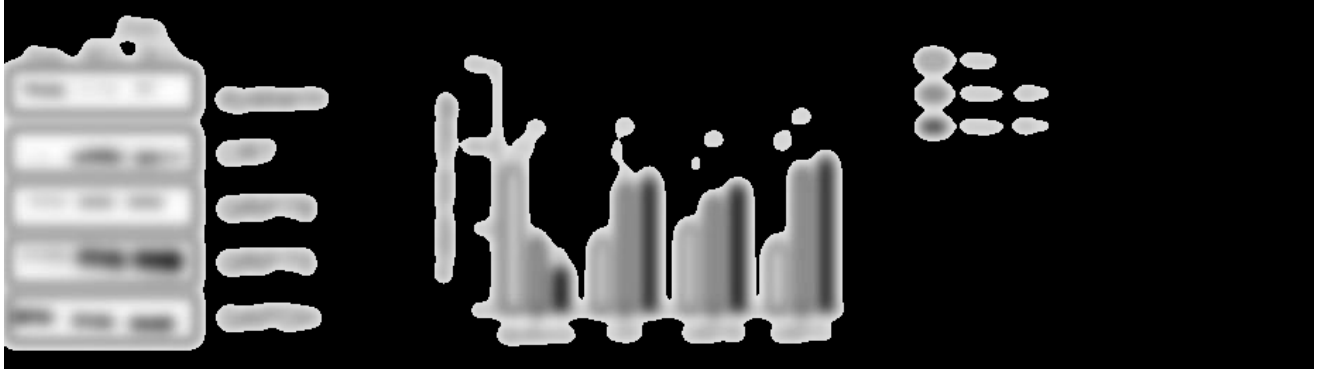
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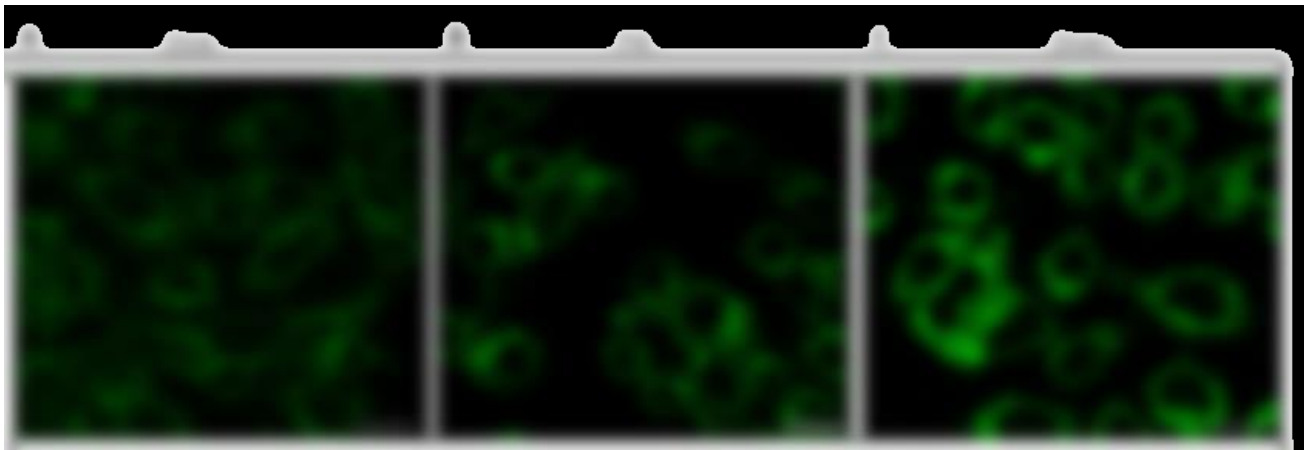
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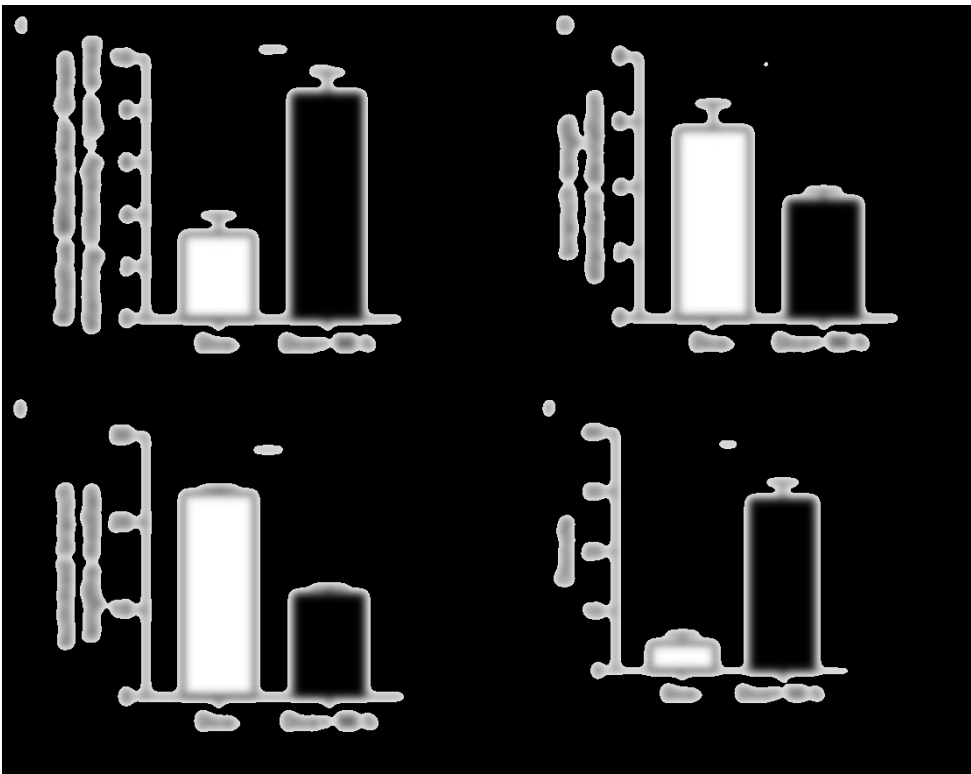
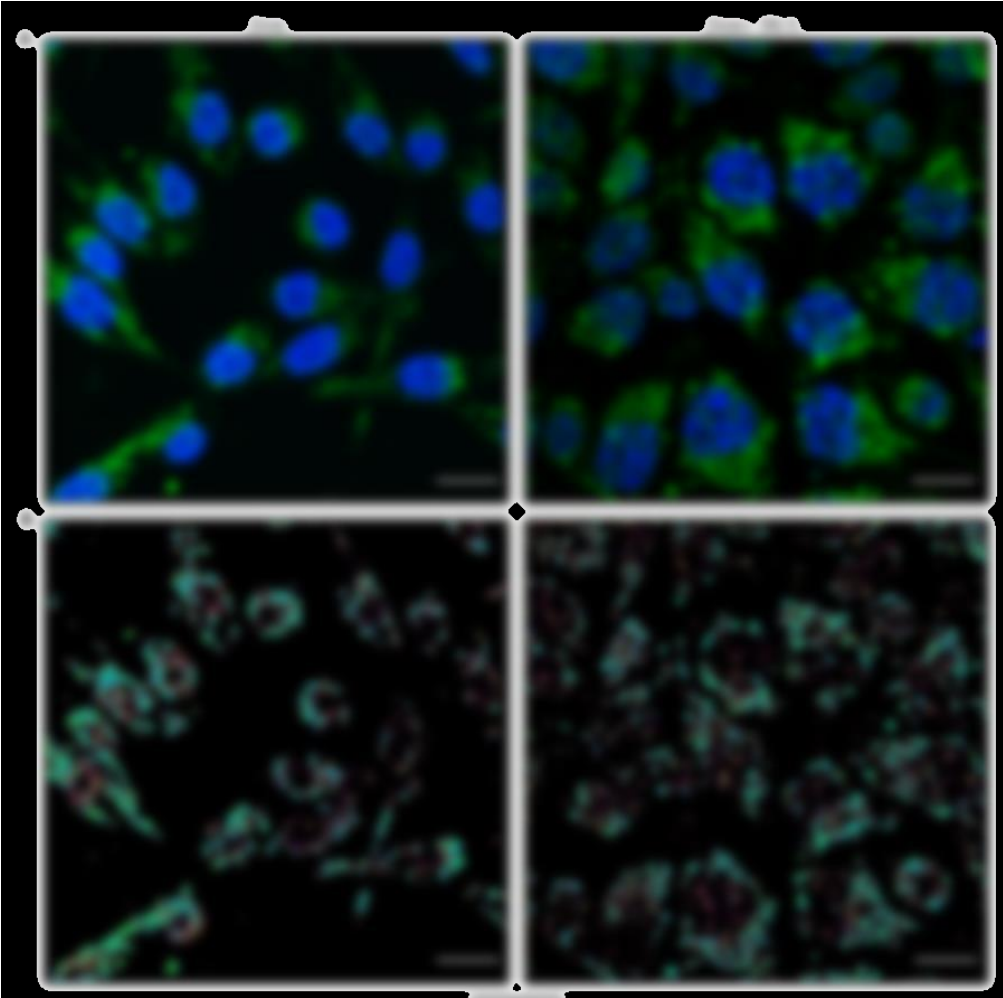
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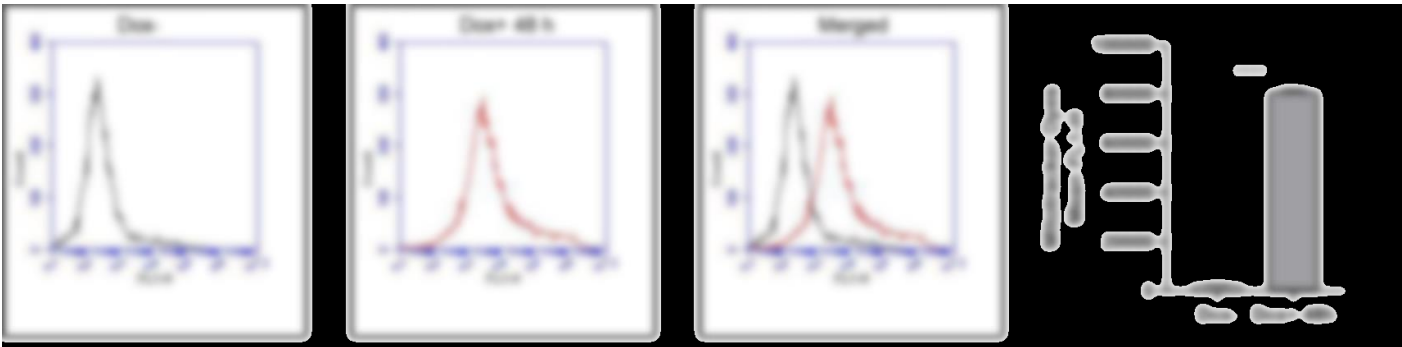
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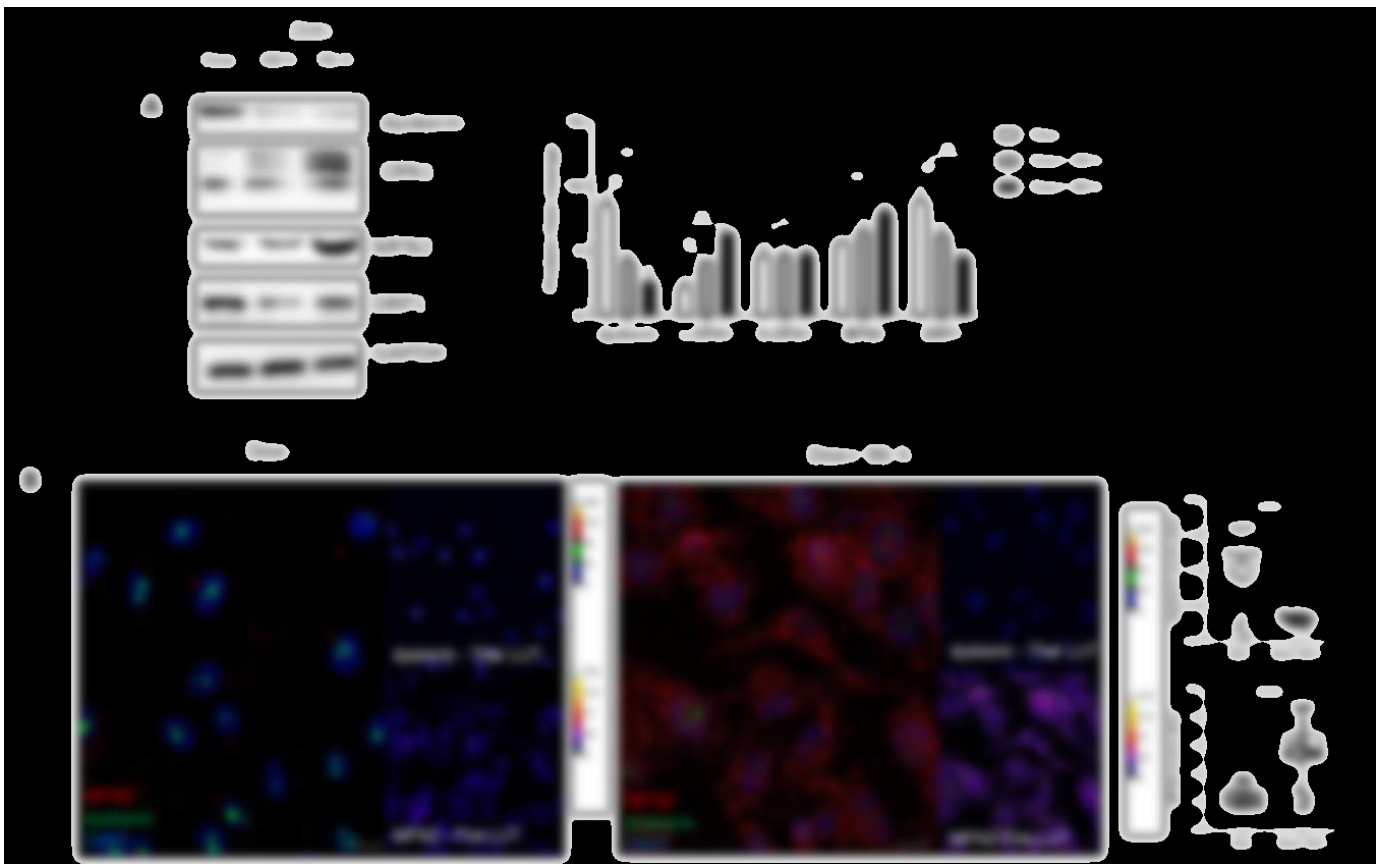


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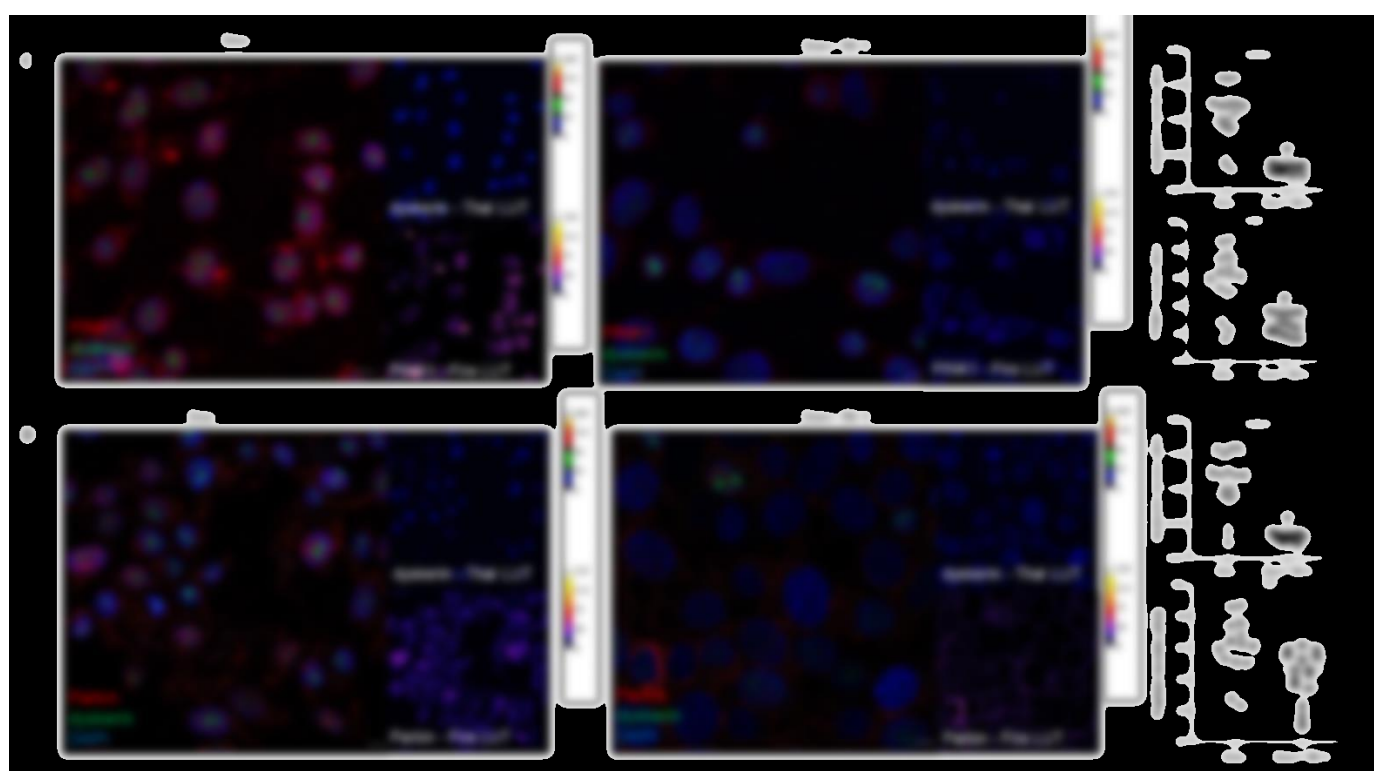
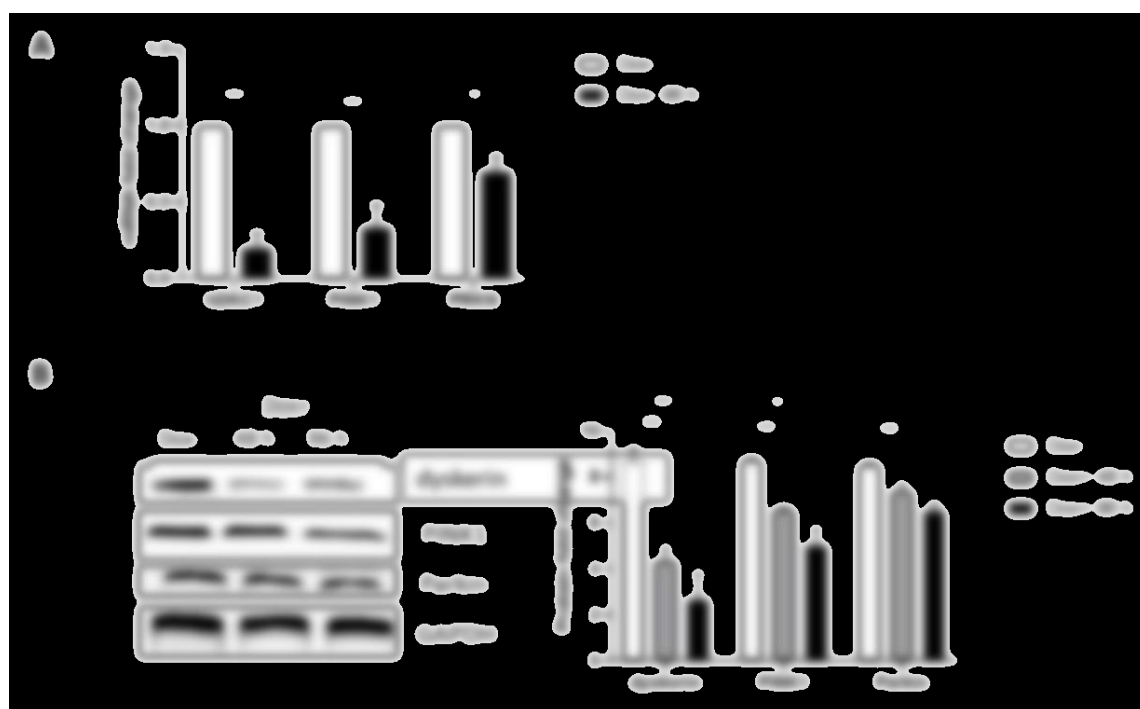
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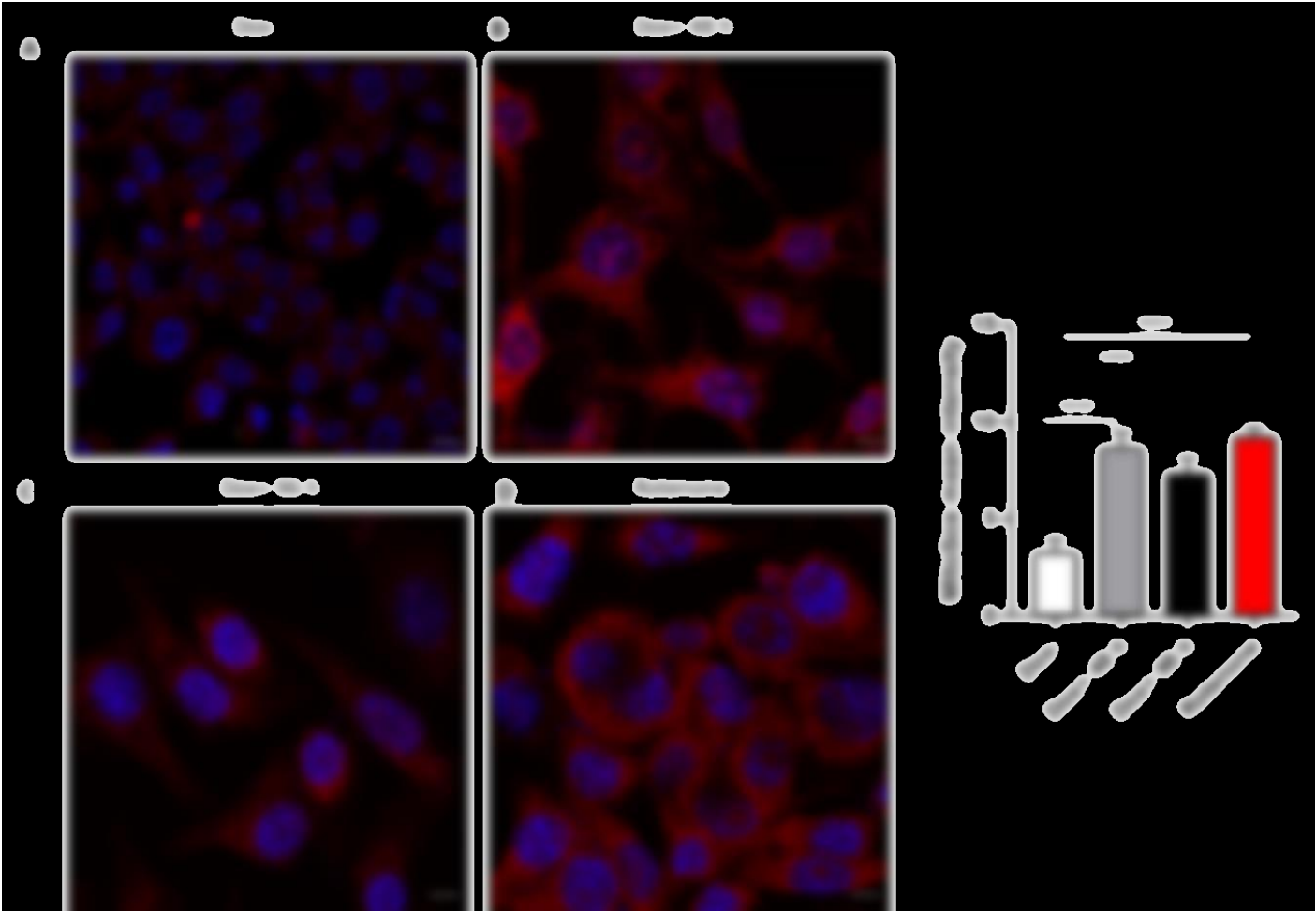
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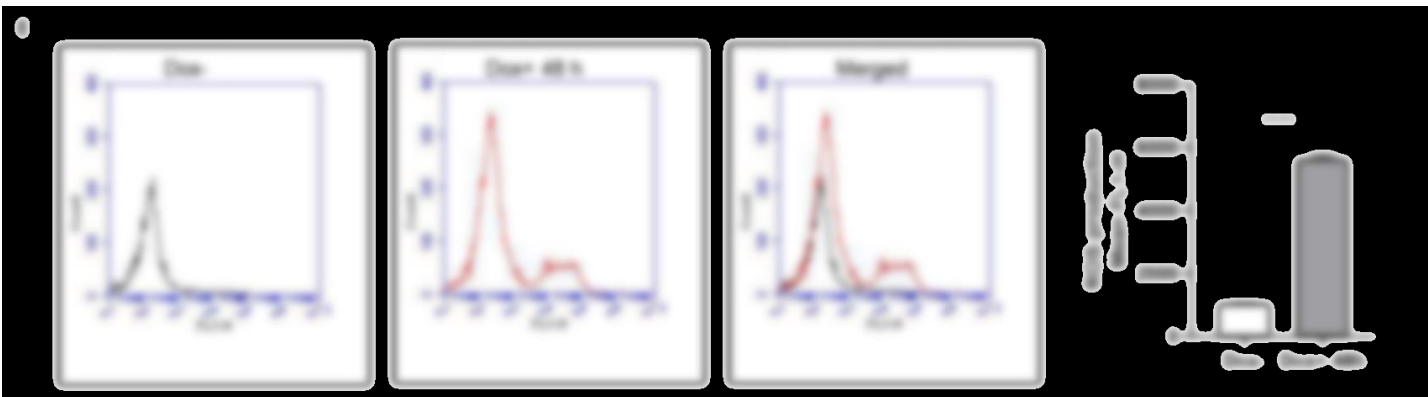
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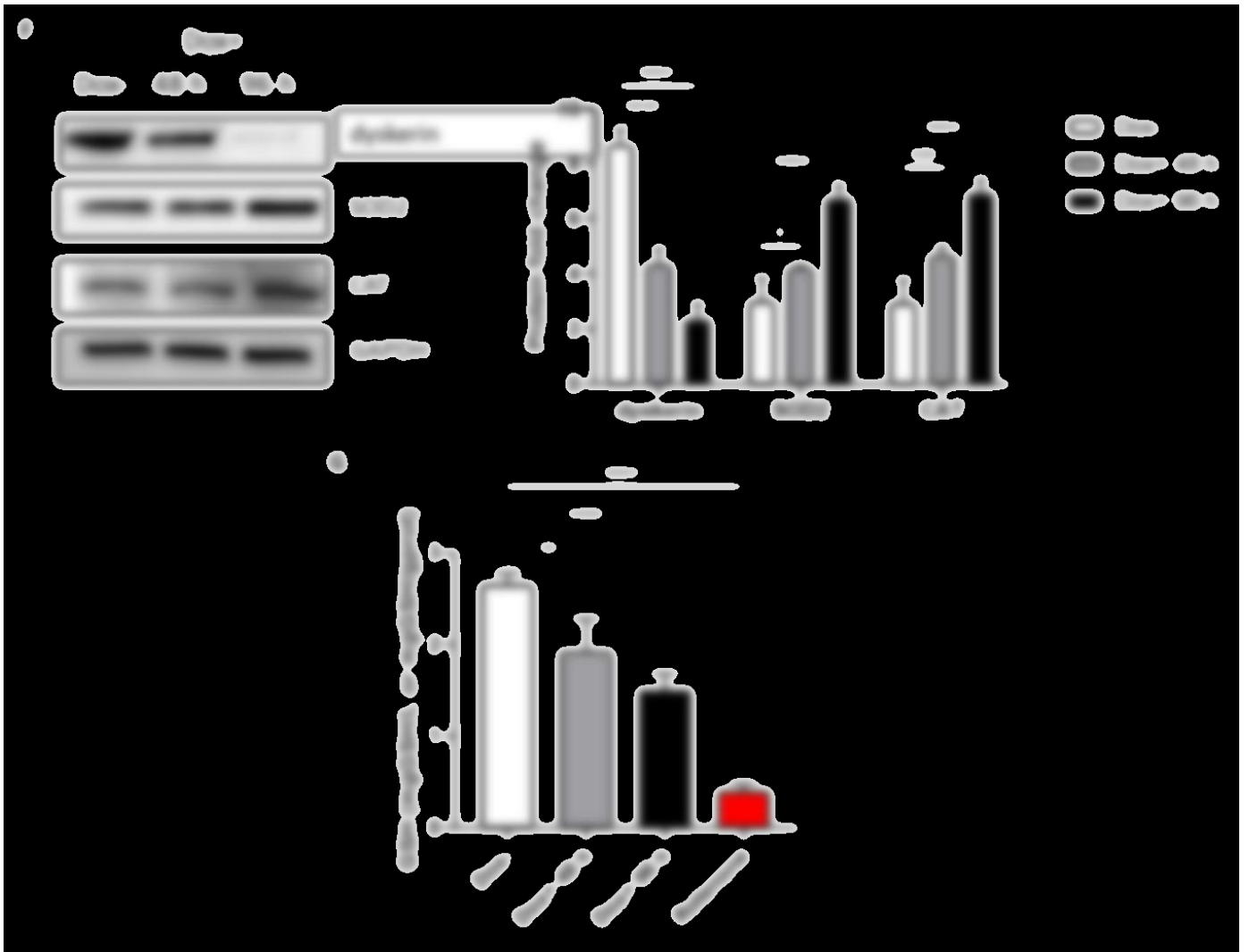
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cutaneous squamous cell carcinoma (cSCC) displaying low levels of dyskerin tend to halt glucose

consumption and instead rely on LDL cholesterol molecules for survival (28). This suggests that the

accumulation of these lipid particles might function as an energy reserve for the cell.

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5 SECTION 3

5.1 New Insights into Dyskerin-CypA Interaction: Implications for X-Linked Dyskeratosis Congenita and Beyond

The objective of the project is to provide new insights into the functional repertoire of the multifunctional chaperone, CypA, in relation to dyskerin. The study aims to demonstrate CypA's localization in the nucleoli and its interaction with both the canonical and Iso3 truncated variants of human pseudouridine synthase dyskerin, suggesting a potential role for CypA in nucleolar dynamics. The discovery of the CypA-dyskerin interaction holds significant relevance for patients affected by Dyskeratosis Congenita X-linked (X-DC), a rare genetic disorder caused by mutations in the dyskerin-encoding gene hDKC1. The identification of CypA-dyskerin interactions unveils an unknown dimension of dyskerin's regulation, shedding light on potential disease-contributing pathways, and presenting an avenue to explore modulating dyskerin's activity to potentially alleviate symptoms or slow disease progression.

Article

New Insights into Dyskerin-CypA Interaction: Implications for X-Linked Dyskeratosis Congenita and Beyond

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Abstract: The highly conserved family of cyclophilins comprises multifunctional chaperones that interact with proteins and RNAs, facilitating the dynamic assembly of multimolecular complexes involved in various cellular processes. Cyclophilin A (CypA), the predominant member of this family, exhibits peptidyl–prolyl cis–trans isomerase activity. This enzymatic function aids with the folding and activation of protein structures and often serves as a molecular regulatory switch for large multimolecular complexes, ensuring appropriate inter- and intra-molecular interactions. Here, we investigated the involvement of CypA in the nucleus, where it plays a crucial role in supporting the assembly and trafficking of heterogeneous ribonucleoproteins (RNPs). We reveal that CypA is enriched in the nucleolus, where it colocalizes with the pseudouridine synthase dyskerin, the catalytic component of the multifunctional H/ACA RNPs involved in the modification of cellular RNAs and telomere stability. We show that dyskerin, whose mutations cause the X-linked dyskeratosis (X-DC) and the Hoyeraal–Hreidarsson congenital ribosomopathies, can directly interact with CypA. These findings, together with the remark that substitution of four dyskerin prolines are known to cause X-DC pathogenic mutations, lead us to indicate this protein as a CypA client. The data presented here suggest that this chaperone can modulate dyskerin activity influencing all its participated RNPs.

Keywords: PPIase A; proline isomerization; H/ACA RNPs; dyskerin; DKC1; X-DC; ribosomopathies; redox response

Introduction

Cyclophilins are highly conserved proteins known for their molecular chaperone activity, which facilitate protein folding and trafficking [1]. Peptidyl–prolyl cis–trans isomerase A (PPIase A), also named cyclophilin A (CypA), the most abundant member of this family, is widely recognized for its intracellular role and is a major target of the immunosuppressive drug cyclosporin [2] that is widely used for the treatment of several tumors. Beyond its involvement in immune modulation, CypA is implicated in various cellular processes and has been associated with numerous pathological conditions, including aging, stress, cancer, cardiovascular diseases, and neurodegeneration [3]. Founded on its ability to also bind to RNA, the protein plays additional roles in the progress of viral infections [4] and is needed for the replication of several coronaviruses, including SARS-CoV19 [5,6]. While CypA is distributed between the cytoplasm and the nucleus [7], its nuclear functions remain to be fully explored. However, studies have shown that CypA plays a crucial role in regulatin



Citation: Belli, V.; Maiello, D.; Di Lorenzo, C.; Furia, M.; Vicidomini, R.; Turano, M. New Insights into Dyskerin-CypA Interaction: Implications for X-Linked Dyskeratosis Congenita and Beyond. *Genes* **2023**, *14*, 1766. <https://doi.org/10.3390/genes14091766>

Academic Editors: Maria Monticelli, Bruno Hay Mele and Giuseppina Andreotti

Received: 1 August 2023

Revised: 27 August 2023

Accepted: 4 September 2023

Published: 6 September 2023



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the composition and architecture of different heterogeneous nuclear ribonucleoprotein (RNP) complexes, which are involved in chromatin remodeling, RNA dynamics, and nuclear receptor signaling [7]. This regulatory role is further enhanced by the fact that, as with other cyclophilins, CypA is endowed with a peptidyl-prolyl cis-trans isomerase (PPIase) activity that is known to give an additional contribution to protein conformational stability. Peptidyl-prolyl isomerases play important roles not only in protein trafficking and secretion, but also in the assembly of large complexes, by favouring proper inter/intra-molecular interactions [8,9].

Unlike most chaperones, PPIase are specific in their respective targets: they preferentially bind proteins that contain conformationally heterogeneous proline residues, whose conformation change often acts as a switch in the regulation of specific functions [10]. Such types of configuration changes are known to be essential to the dynamic influx-outflux of the various components of membrane-less (ML) foci, biological condensates that coalesce through multiple transient interactions and act as compartmentalized functional hubs within the cell [11]. Thanks to the lack of an external membrane, ML bodies assume a liquid droplet-like behaviour, so that their components can continuously be interchanged and their structure and organization rapidly rearranged [12].

Proteins harbouring Intrinsically Disordered Regions (IDRs) are known to have a relevant role in the assembly of these foci [13,14], since IDRs can mediate transient assembly with different interacting partners and promote Liquid-Liquid Phase Separation (LLPS), that is widely recognized as the main mechanism driving the coalescence of ML bodies [15,16]. Inside the nucleus, many ML bodies act as specialized subcompartments composed of locally concentrated RNA-protein assemblies where specific functions take place. The nuclear membrane-less (NML) bodies ensemble includes primarily nucleoli, nuclear speckles, paraspeckles, PML, Sam68, and Cajal bodies [17]. While an increasing number of these NML foci are continuously revealed by modern imaging/molecular techniques, the nucleoli still appear to be those playing the most prominent cellular roles. In addition to representing the milieu where ribosomes assemble their molecular components, these multifunctional organelles act as hubs in the coordination of many cellular responses, being able to dynamically change structure and composition in response to various stimuli, such as growth conditions, stress, DNA damages, signaling events, and viral infections [18]. Many key nucleolar proteins are known to contain IDRs, and their dysregulation proved to impact the LLPS maintenance of the nucleolar structure [19]. Given that IDRs are particularly rich in prolines [14], PPIase activity emerges as a post-translational modification that can modulate LLPS [11]. Notably, CypA has been identified as a client protein of the Upstream Binding Transcription Factor (UBTF) and other key nucleolar proteins, suggesting its major role in LLPS-based nucleolar organization [20]. This notion is supported by the close connection between rDNA transcription, ribosome biogenesis, and the initial steps of nucleolar phase separation [21]. This view is well consistent with the observation that rDNA transcription, together with the initial steps of ribosome biogenesis, is connected to the primary events of nucleolar phase-separation [21] and is further supported by recent NMR spectroscopic studies showing that CypA plays a regulatory role in the phase organization of droplets formed by proteins harboring proline-rich IDRs [22]. As is well known, the nucleolus has a liquid droplet tripartite structure [23,24] composed of the internal fibrillar center (FC), containing rDNA chromatin, the surrounding dense fibrillar component (DFC), where most processes important for transcription, processing, and modification of ribosomal RNAs take place, and the external granular component (GC) containing the immature ribosomes [25].

Continuous trafficking occurs not only between the nucleolus and the surrounding nuclear regions, but also within its tri-layered body. This dynamic interchange is assisted by multiple resident chaperones and co-chaperones that act by favoring the structural arrangement of large macromolecular aggregates, allowing protein-protein and protein-nucleic acid interactions to be continuously renewed and reestablished.

Here, we indicate CypA as one of the chaperones involved in these nucleolar dynamics. We report that this protein can localize within the nucleoli of human cell lines and that it interacts with dyskerin (UniProt # 060832), a highly conserved nucleolar protein belonging to the family of RNA-guided pseudouridine synthases. Dyskerin, whose sequence includes several IDRs [26], is a multifunctional RNA-binding protein that, in association with the highly conserved GAR1, NHP2, and NOP10 proteins, enters in the composition of the H/ACA RNPs active tetramer [26,27]. Within the nuclei, dyskerin concentrates at the nucleoli and the Cajal bodies (CBs), two intimately connected NMLs bodies [28]. At these two sites reside or transit the main dyskerin-participated functional complexes: the telomerase holoenzyme and the small Cajal body RNPs (scaRNPs), specifically involved in the modification of spliceosomal snRNAs and the small nucleolar RNPs (snoRNPs) that control ribosome biogenesis and RNA pseudouridylation [27]. By elucidating the interaction between CypA and this multi-faced pseudouridine synthase, our data suggest that CypA assists in the assembly, organization, and trafficking of dyskerin-participated RNPs. This discovery not only expands our understanding of the repertoire of CypA functions, but also holds clinical significance. In fact, mutations of dyskerin, encoded by the *DKC1* gene (OMIM: #305000), cause two extremely pleiotropic congenital ribosomopathies: the X-linked dyskeratosis (X-DC) [29] and the more severe Hoyeraal–Hreidarsson syndrome (HHS) [30], while the protein over-expression characterizes several types of sporadic cancers and is valued as a marker of poor prognosis [31].

Materials and Methods

Cell Culture, Treatments and Bioinformatic Data

RKO, HEK, and U2OS cell lines were maintained at 37 °C in a humidified atmosphere of 5% CO₂ in Dulbecco Modified Eagle Medium (DMEM) containing 10% Tet-free fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% L-Glutamine (Euroclone, Milano, Italy). To generate HEK 293T and U2OS (ATCC, Manassas, VA, USA) transfected cells, 3 µg of pMGIB-3xFLAG-dyskerin (Addgene) and 12 µL of Polyethylenimine (PEI) (Polysciences) were added to the medium; following 30 min of incubation, cells were put on plate for 5 h; the medium was then replaced by DMEM supplemented with 20% FBS. Heat shock was performed by exposing cells at 45 °C for 30 min; cells were allowed to recover for 30 min at 37 °C and then analyzed by immunofluorescence or immune precipitation as described below.

HeLa cells over-expressing dyskerin isoform 3 (3XF-Iso3) were previously obtained [32] and cultured and subjected to sodium arsenite treatment as described [33]. Unless otherwise specified, all published protein interactions were retrieved from the BioGrid database version 4.4.223 (<http://thebiogrid.org> (accessed on 7 July 2023)).

Immunofluorescence Analysis

For immunofluorescence experiments, cells were directly seeded onto glass coverslips then permeabilized and exposed to antibodies as previously described [33]. Immunofluorescence micrographs were taken by Zeiss LSM 700 microscope (Zeiss, Oberkochen, Germany) using EC Plan-Neofluar 40× or Plan-Apochromat 63x/1.40 Oil immersion objectives. Images were analyzed by ImageJ (<http://imagej.nih.gov/ij/> (accessed on 5 June 2023; 21 August 2023)) or Zeiss Zen software tools. Nuclei were counterstained by DAPI. The primary antibodies used were anti-DKC1 H-3 (SC-373956), Santa Cruz Biotechnology, Santa Cruz, CA, USA; anti-CYPA/PPIA (Genetex 104698); anti-FLAG (F1804–200UG, Sigma-Aldrich, St. Louis, MO, USA); and anti-PRDXII (Ab133481, ABCAM). The secondary antibodies used were anti-Rabbit-Cy3 (A10523) and anti-Mouse-AlexaFluor 488(A21202) from Invitrogen and anti-Rabbit-Cy3 (A120-201C3), anti-Rabbit-FITC (A120-101F), anti-Goat-Cy3 (A50-201C), anti-Mouse-FITC (A090-138F), and anti-Mouse-Cy3 (A90-516C3) from Bethyl Laboratories, Montgomery, AL, USA. Nuclei were counterstained by DAPI. Each experiment was repeated at least three times independently.

Pull-Down ASSAY

In these assays, HEK and U2OS cells were transfected for 48 h with 3xFLAG-dyskerin plasmid (Addgene), as described above and then transferred in RIPA lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 5 mM EDTA, 1% NP40) supplemented with anti-phosphatase and anti-protease (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, Sigma-Aldrich, Merck, Rahway, NJ, USA). Lysates of transfected cells (about 1 mg) were added to 20 µL of TBS 1x-rinsed beads (ANTI-FLAG M2 Affinity Gel A2220-SIGMA ALDRICH) and incubated overnight at 4 °C. Following a 1 min spin at 8000 × g at 4 °C, unbound proteins were removed by rinsing beads with 200 µL of TBS 1x wash buffer three times. Pulled-down proteins were then analyzed by immunoblotting. The experiments were repeated three times independently. The primary antibodies used were anti-CYP A/PPIA (104698, Genetex); anti-PRDXII (Ab133481, ABCAM); anti-Zo-1 (bs1329, Bioss); anti-GAR1 (PA5-63656, Thermofisher); and anti-FLAG (F1804-200UG, Sigma-Aldrich, St. Louis, MO, USA). Secondary antibodies were anti-Rabbit (A120-100P) and anti-Mouse (A90-137P), Bethyl Laboratories, Montgomery, AL, USA.

Results

CypA Can Colocalize with the Dyskerin Pseudouridine Synthase in the Nucleoli

Several proteins with various intracellular localizations play different roles at the distinct subcellular locations. To provide new insight into the roles played by CypA within the nucleus, we conducted a detailed analysis of its intracellular localization using confocal immunofluorescence microscopy (IF). When cells from the RKO, U2OS, and HeLa lines were examined, we found that, although in each line CypA appeared to be widely diffused throughout the whole cell bodies, its nuclear pattern showed some cell-type specificity. In fact, in the RKO and U2OS cells, the protein's immunosignal marked intensely the nucleoplasm, where many positive puncta were detected, whereas in the HeLa cells the nucleoplasm remained nearly unlabelled (Figure 1A–C). Despite these cell-type differences, in each line a fraction of the CypA immunosignal concentrated within the nuclei at main bodies that, as shown by their colocalization with the nucleolar marker dyskerin, corresponded to the nucleoli (Figure 1).

Focusing on CypA-dyskerin co-stained cells, we noted that the CypA's immunosignal, although generally concentrated in the fibrillar center, also labelled peripheral foci, suggest a lively transit throughout the nucleolar tri-layered organization (Figure 1). The more pronounced peri-nucleolar localization of CypA in HeLa cells as compared to RKO and U2OS cells, as depicted in Figure 1, may indeed reflect the complex interplay between cellular context, metabolic conditions, and protein dynamics.

One plausible explanation could be attributed to the inherent heterogeneity in cellular environments, such as differences in organelle composition, signaling pathways, and gene expression profiles. It is well-known that different cell types may exhibit unique subcellular distributions of various proteins, which can arise from variations in their functional roles and regulatory mechanisms.

Furthermore, post-translational modifications and interactions with partner molecules can also significantly influence protein localization. Given that dyskerin is also highly concentrated at CBs, where the assembly of the dyskerin-participated telomerase holoenzyme initiates and the mature H/ACA SnoRNPs are thought to be gathered [34], we also looked at these sites. CBs shape as a consequence of an elevated concentration of both snRNPs and snoRNPs and, not being essential, may even be undetectable in the same cells [35]. In our experiments, putative CBs, labelled by the dyskerin antibody, were hardly visible within the small nuclei of RKO cells, instead being well evident in the U2OS and HeLa cells. Yet, in both HeLa and U2OS cells the CypA immunosignals still partially overlap at these sites (Figure 1A',B', white arrows). Based on the different intensity at which the CypA immunosignal was simultaneously detected in the same cells (Figure 1), the nucleoli should be expected to represent the major sites where CypA concentrates. Indeed, it is plausible that the lowering of CypA concentration might favour the locally assembled

mature H/ACA RNPs in stabilizing their active conformational structures. To this regard, we noted that another cyclophilin member, CypB, was reported to localize at the nucleoli of the HeLa and human primary fibroblast cells, whereas it remained undetected at CBs in normal growth conditions [36]. Thus, it is possible that a specific major nucleolar concentration might represent a common pattern for nuclear cyclophilins.

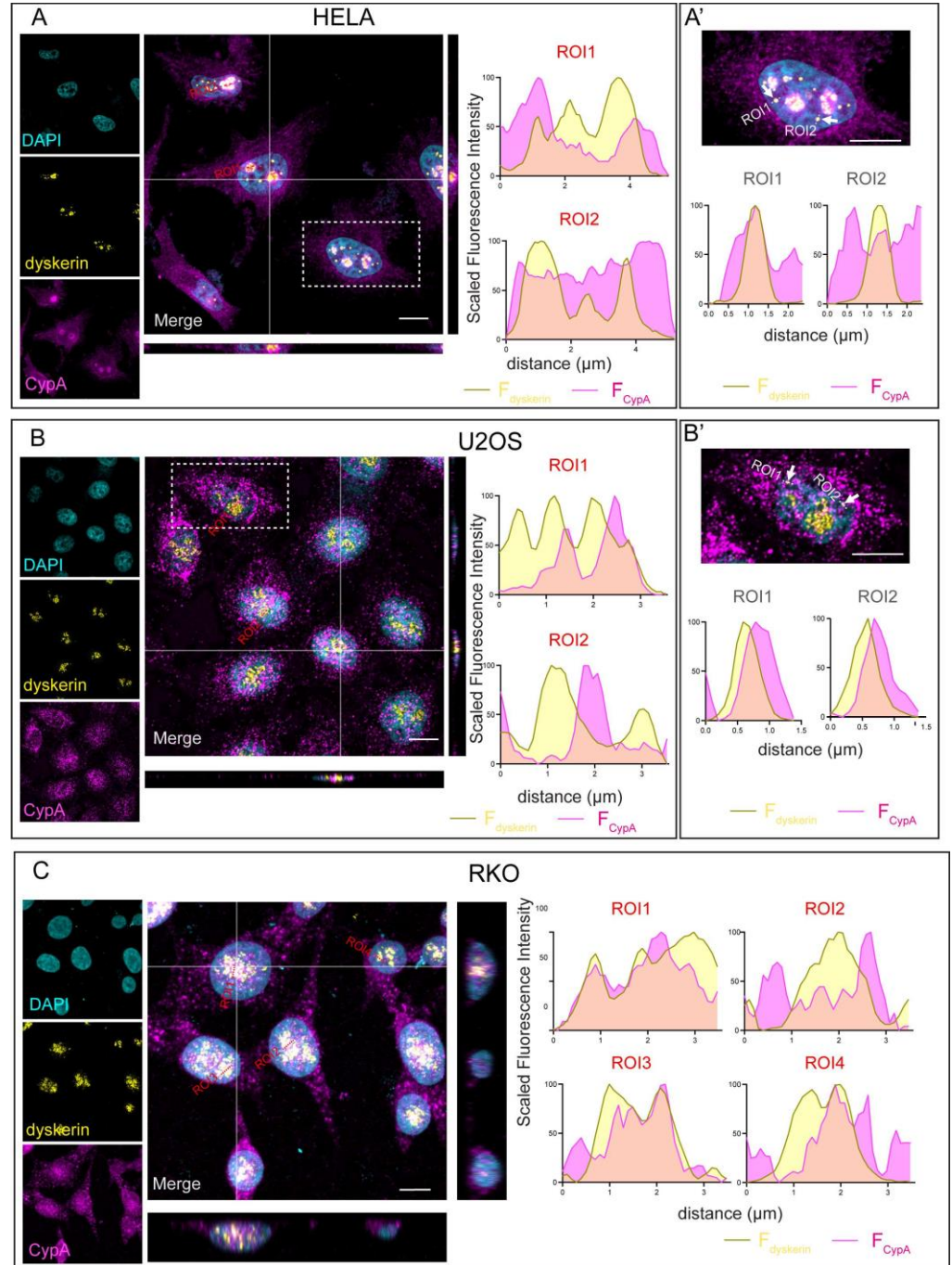


Figure 1. Intracellular Localization of CypA. Confocal microscopy images depicting distinct cell types, namely HeLa (A), U2OS (B), and RKO (C), immunostained with anti-dyskerin (yellow) and anti-CypA (magenta) antibodies, and counterstained with DAPI (cyan). In HeLa cells (A), as validated through Z-stack analysis (refer to XZ and YZ projections), the immunoreactive signal of CypA demonstrates a distribution spanning both the cytoplasm and the nucleus. Notably, fluorescence intensity profiles along two linear ROIs centered on nucleoli reveal CypA fluorescence exhibiting partial overlap with the immune signal of dyskerin (a nucleolar marker), indicating that CypA can assume a partial nucleolar

localization. As expected, nuclear bodies (marked by white arrows), putatively corresponding to CBs, are also marked by dyskerin's immune signal. (A') Detailed analysis of fluorescence intensity profiles of the cell shown in the top enlargement (highlighted in (A)) along two linear ROIs centered on two putative CBs (ROI1 and ROI2—indicated in gray) illustrates CypA again surrounding and partially overlapping with dyskerin. (B) In U2OS cells, the distribution of the CypA signal similarly spans both the nucleus and the cytoplasm and, along two linear ROIs centered on nucleoli also exhibits a varying degree of overlap with nucleolar dyskerin signal. (B') Here, again, analysis of fluorescence intensity profiles (cell on top enlargement, highlighted in (B)) shows that CypA and dyskerin signals are closely aligned and partially overlap along two linear ROIs (ROI1 and ROI2, (indicated in gray) centered on putative CBs (marked by white arrows in the magnified view). (C) Additionally, in RKO cells the immunoreactivity of CypA appears to be distributed between the cytoplasm and the nucleus. The analysis of fluorescence profiles along four linear ROIs (highlighted in red), each centered on a nucleolus, demonstrates a varying degree of overlap between the signals of CypA and dyskerin. Unlike the previous cell types, putative Cajal's bodies are not clearly evident in this cellular context. ROI: Region of Interests; CBs: Cajal Bodies.

In addition to its potential involvement in dyskerin-RNP dynamics, we speculate that the nucleolar concentration of CypA might have, per se, a functional relevance for the redox response of this organelle [37]. In fact, although CypA participates in the maintenance of cell homeostasis in several ways, a significant contribution is given by its ability to act as an endogenous electron donor. With its reducing action, the protein is known to actively support the antioxidant activity of mammalian peroxiredoxins (PRDXs), being able to bind them and help to restore their redox activity [38,39]. Interestingly, PRDX-2, a relevant member of this ROS scavenger family, has been found to localize at the nucleoli [40,41]; (Figure 2). This suggests that, under certain conditions, CypA and PRDX-2 may act together as localized ROS sensors, thereby contributing to the orchestration of the nucleolar antioxidant response. It is worth noting that dyskerin has also been implicated in the modulation of oxidative metabolism, further connecting it to these non-canonical functions [35,40,42]. Consistent with this, by analyzing the fluorescence intensity of dyskerin and PRDX-2 immunoreactivities along a rectangular region of interest (ROI) containing the nucleoli, it became evident that the peaks of PRDX-2 fluorescence significantly overlap with the peaks of the dyskerin signal, suggesting a potential cooperation between the two proteins in redox-related cellular processes [Figure 2].

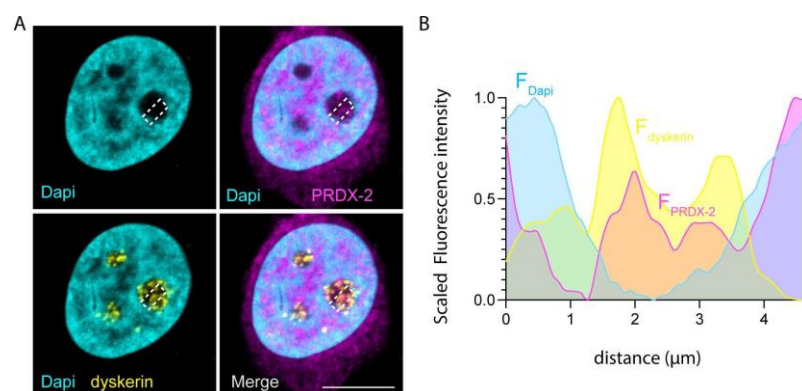


Figure 2. PRDX-2 intranuclear localization. (A) Confocal microscopy analyses of HeLa cells labelled with dyskerin (yellow) and PRDX-2 (magenta) antibodies and counterstained with DAPI (cyan). Note that PRDX-2 is widely diffused in the nucleoplasm and localizes within the nucleoli and the putative CBs. White bars correspond to 10 μm . (B) Graph showing the fluorescence intensity values for DAPI (cyan), dyskerin (yellow), and PRDX-2 (magenta), plotted as a function of distance along a linear ROI (indicated as a dotted rectangular outline in (A)). Dyskerin signal is observed within the nucleolus region, where the DAPI signal is decreased. A clear and evident overlap is observed between the fluorescence profiles of PRDX-2 and dyskerin.

Dyskerin as a Putative Novel CypA Client

Since CypA has been correlated to specific nucleolar functions, such as rDNA transcription and ribosome biogenesis [20,21], we investigated the possibility that it could directly interface with dyskerin. To explore this, we transfected both HEK and U2OS cells with Flag-tagged full-length dyskerin and tested the obtained lysates with the anti-FLAG® M2 Magnetic Beads in a dyskerin pull-down assay (see Methods). As shown in Figure 3A, CypA was effectively co-pulled down with the Flag-tagged dyskerin; it was also recovered in the immunoprecipitate after a mild heat shock exposure, indicating that this condition did not disrupt this interaction. In these experiments the protein GAR1, a core component of the H/ACA snoRNP mature complex, was also co-pulled, providing a positive control; a suitable negative control was instead furnished by the absence in the pulled immunocomplex of the membrane protein Zo-1 (Figure 3A,B).

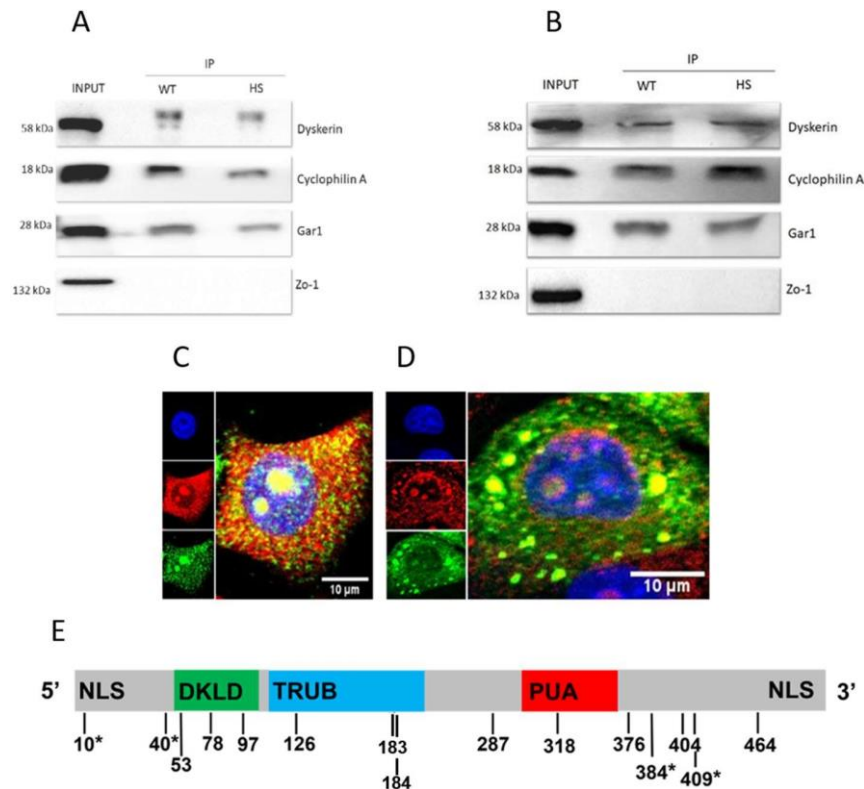


Figure 3. CypA-dyskerin interfacing. (A,B) Pull-down experiment supporting CypA-dyskerin interaction. U2OS (A) and HEK (B) cells were transfected with the 3xFLAG-dyskerin plasmid and the lysates incubated with the ANTI-FLAG M2 Affinity Gel beads, as described in Methods. Pulled-down complex was analyzed by immunoblotting; transfected dyskerin was detected by the anti-FLAG antibody, CypA, GAR1, and Zo-1 proteins by their specific antibodies. Abbreviations: immunoprecipitation (IP); total extract (INPUT); wild-type (WT); and heat shock (HS). (C,D) Confocal immunofluorescence pictures of 3xF-Iso3 cells (that overexpress the Iso3 dyskerin splice variant) show Iso3 and CypA co-localization in absence (C) or presence (D) of oxidative stress. Iso3 (in green) is labelled by FLAG antibody, CypA (in red) by its specific antibody. Nuclei were counterstained with DAPI (blue). Scale bars correspond to 10 μm. (C) Note the overlay of Iso3 and Cypa immunosignals (in yellow) at both the nuclear and cytoplasmic compartments. (D) Sodium arsenite exposure triggers Iso3 recruitment at SGs and leads CypA to similarly assume a cytoplasmic granular pattern, with its immunosignal labelling a subset of Iso3-positive cytoplasmic foci. (E) Schematic representation of dyskerin sequence and its functional domains; the position of proline residues that might potentially be targeted by CypA PPLase is indicated below. The prolines whose missense substitutions proved to be X-DC pathogenic (Pro10Leu, Pro40Arg, Pro384Ser, Pro384Leu, Pro409Leu, Pro409Arg) are marked by asterisks.

To further investigate the interfacing of CypA with dyskerin, we extended IF analyses to the low abundance dyskerin splice isoform 3 (thereafter called Iso3), whose expression is characterized by a different intracellular pattern. This truncated protein, 420 aa long, maintains all dyskerin functional domains with exception of the C-terminal NLS, whose absence allows a variegated dual cytoplasmic–nuclear localization; nevertheless, within the nuclei, Iso3 concentrates into nucleoli and CBs, similarly to canonical dyskerin [32,33,43]. Like dyskerin [44–46], this isoform is actively involved in the stress/DNA damage response [33,47] and, upon oxidative stress, is efficiently recruited into stress granules (SGs), as components of mature H/ACA snoRNPs [33]. SGs are ML cytoplasmic bodies that promote adaptation to stress conditions and cell survival [48], whose nucleating factor is the RNA-binding protein Ras-GTPase-activating protein (G3BP1) [49,50]. To follow the expression of CypA and Iso3 in the absence or presence of oxidative stress, we subjected the previously obtained stably transfected Hela cell lines that overexpress the FLAG-tagged Iso3 (3XF-Iso3 cells) to IF analysis [32]. Strikingly, in the unstressed condition, Iso3 (marked by the FLAG antibody) and CypA immunosignals were found to colocalize not only into the nucleoli, but also in the cytoplasm (Figure 3C). This indicates that the truncated dyskerin variant includes all sequences eventually required for the interaction with CypA, and that the interfacing is not restricted to the nuclei. Given that the canonical dyskerin, although in trace amounts, also resides in the cytoplasm [33,51] this issue suggested that CypA might have a chaperoning role for both newly synthesized isoforms, possibly contributing to their immediate stabilization and/or preventing them from unspecific and incorrect interaction isoforms also in this cell compartment. Considering the presence of two nuclear localization signals (NLS) in dyskerin, it is also plausible that CypA might translocate to the nucleus through an interaction with this protein. Next, we exposed 3XF-Iso3 cells to sodium arsenite, which is known to induce oxidative stress and is widely used to trigger SGs formation. In this condition not only Iso3, but also CypA coalesced into SGs, as previously noticed by other authors [9,52]. As shown in Figure 3D, the CypA's immunosignal overlapped with that of Iso3 at a subset of SGs, indicating that co-localization of these two proteins persisted upon oxidative stress. It is plausible that the number of Iso3-positive SGs not marked by CypA might be due to the high level of Iso3 overexpression in the 3XF-Iso3 cells and/or from the general structural plasticity of SGs, whose individual components are known to continuously shuttle from them to the cytoplasm or to other cytoplasmic bodies [53]. It is worth noting that the coalescence of CypA into SGs fits well not only with its role of molecular chaperone in the refolding of denatured proteins following oxidative stress [7], but also with the knowledge that many proteins that have a nucleolar localization are recruited into these bodies in response to stress stimuli [54]. Finally, it adds functional significance to the remark that G3BP1 is a dyskerin-CypA common interactor (as listed at the www.thebiogrid.org (accessed on 7 July 2023), version 4.4.223). Considering the evidence supporting the close association between CypA and dyskerin, we hypothesized that the peptidyl–prolyl isomerase (PPLase) activity of CypA might specifically target dyskerin at certain proline residues. Structural information on eukaryotic H/ACA RNPs is still incomplete, so we focused on the analysis of sequence conservation and the mutational hot spots of dyskerin protein sequences. As shown in Figure 3E, dyskerin harbors several highly conserved proline residues, most of which are embedded within its main functional domains: the dyskerin-like domain (DKLD; 48–106 aa), whose function remains so far elusive and the TruB pseudouridine synthase domain (110–226 aa), which acts catalytically in the RNA pseudouridylation process and the PUA RNA binding domain (297–370 aa), which allows RNA recognition [55]. Dyskerin-prevalent nuclear localization is also ensured by two Nuclear Localization Signals (NLSs) localized, respectively, at the N- and C-ends. Specifically, five conserved proline residues lie within the N-terminal moiety that includes the DCLD domain, three in the TRUB catalytic domain, one in the RNA-binding PUA domain, and five in the subsequent C-terminal moiety (Figure 3E). While these prolines are distributed across various domains of dyskerin, it is plausible that the interaction between CypA and dyskerin may involve specific structural motifs and conformational changes

rather than a uniform recognition of all proline residues. Considering that structural information on eukaryotic H/ACA RNPs is still incomplete, we next focused on the analysis of sequence conservation and the mutational hot spots of dyskerin protein sequence. We next compared the position of these prolines with the distribution of the known X-DC pathogenic missense mutations and remarked that they overlap each other at four codons (Pro10Leu, Pro40Arg, Pro384Ser, Pro384Leu, Pro409Leu, Pro409Arg, all labelled by an asterisk in Figure 3E). Substitutions of these four prolines are known to affect dyskerin functions [30,56], in good accordance with the possibility that they might represent putative CypA targets. The observation that these codons all map within the sequence of the truncated Iso3 isoform that proved to retain the ability to interact with CypA further supports this hypothesis. Noticeably, two different single nucleotide substitutions fall at two of these codons (Pro384 and Pro409), giving rise to two diverse pathogenic missense mutations at each site (Pro384Ser, Pro384Leu, Pro409Leu, Pro409Arg). This raises the possibility that the substitution of Pro384 and Pro409 might potentially play relevant roles in dyskerin activity. Indeed, these two residues map within the PUA downstream flanking region, which is highly conserved among eukaryotes and is required for the interaction with SHQ1, the assembly factor involved in the early steps of H/ACA RNP biogenesis [57]. Although direct evidence is still lacking, the data presented here strongly support the hypothesis that the PPLase activity of CypA might specifically target dyskerin, potentially modulating its activity and H/ACA RNPs dynamics, in line with the role attributed to CypA on the establishment of the LLPS-based nucleolar architecture.

Overlapping of CypA, Dyskerin and Fibrillarin Interactomes

H/ACA and C/D box SnoRNPs are the major resident nucleolar complexes involved in rRNA maturation and processing, with dyskerin and fibrillarin representing their respective catalytic core members and those that primarily contact RNAs [29]. Both RNP families are assembled throughout dynamic multistep pathways characterized by a lively intranuclear trafficking between nucleoli and CBs [29,58]. These interchanges rely on transient interactions and are regulated by a diverse set of chaperones [29,58,59]. Given the involvement of chaperones in these processes, we explored the potential role of CypA in assisting the dynamics of both RNP families, possibly during transient assembly events. To this aim, we analyzed the human interactomes of CypA, as well as the other H/ACA RNPs core components at the BioGRID Database of Protein Interactions. These RNPs include, in addition to dyskerin, the highly conserved NOP10 and NHP2 proteins; a fourth protein, NAF1, is transiently recruited at the site of transcription to be subsequently replaced by GAR1 [58]. After this exchange that is thought to occur at CBs [34], the mature complexes move back to the nucleolus, where they are actively involved in the processing and the modification of rRNA.

First, we compared the human interactomes of CypA, dyskerin, and fibrillarin. This comparison showed that a significant fraction of fibrillarin (72 interactors) and dyskerin (49 interactors) registered partners were shared with CypA (Figure 4A,B), suggesting that these proteins could participate in common assemblies. Remarkably, a consistent number of partners (i.e., 34 members) was shared by all three proteins, delineating a possible context of common interactions.

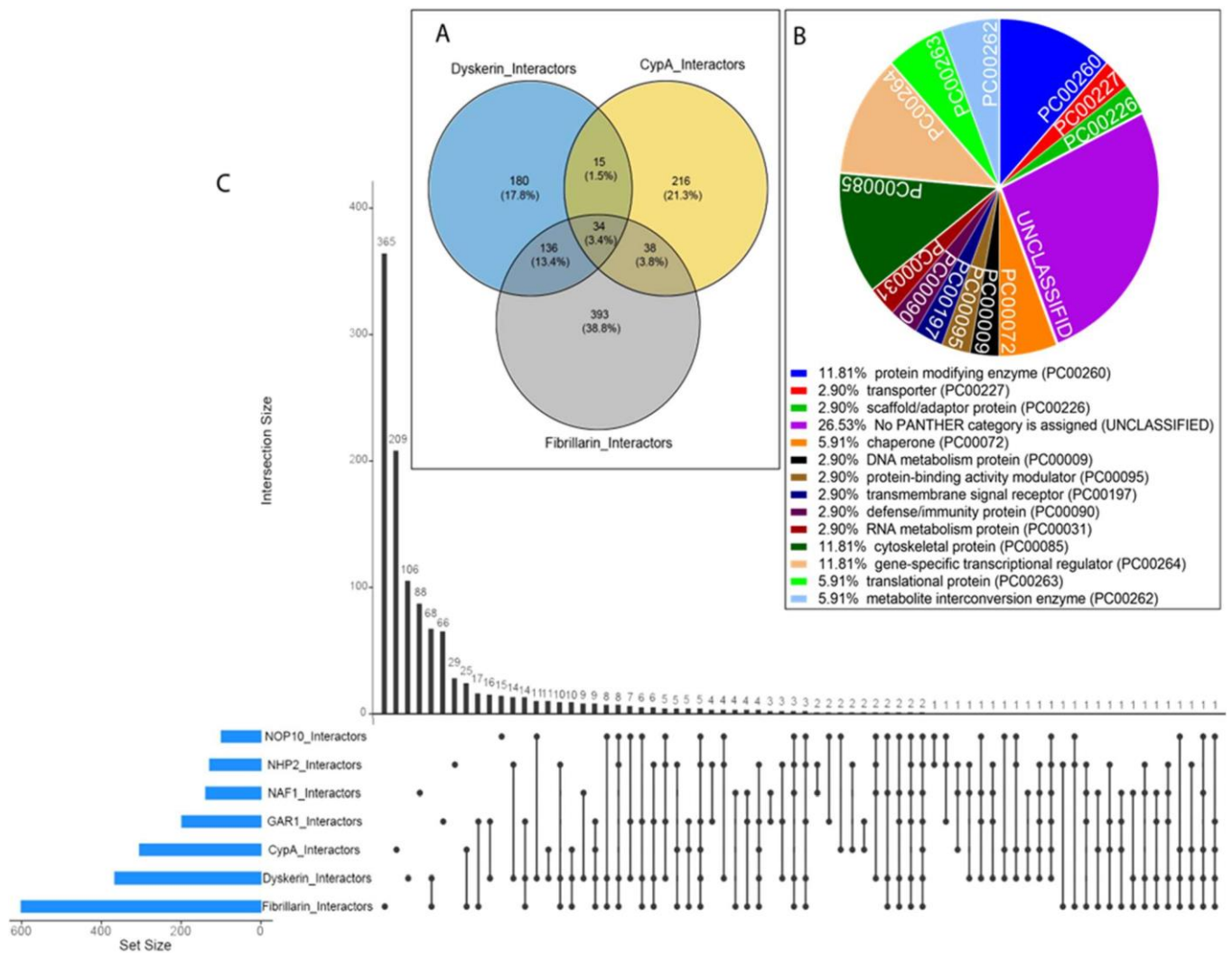


Figure 4. Overlay between CypA's interactome and those of core components of nucleolar snoRNPs. (A) Percentage of components of the dyskerin, fibrillarin, and CypA interactomes. (B) Interactors common to CypA, fibrillarin, and dyskerin clustered in protein classes according to PANTHER classification system (<http://www.pantherdb.org> (accessed on 7 July 2023)). (C) Relationship between interactors of dyskerin, CypA, NAF1, Fibrillarin, Nop10, NHP2, and Gar1: an UpSet plot analysis (supplementary materials S1 and S2). The plot provides an analysis of the relationships among the interactors associated with dyskerin, CypA, NAF1, Fibrillarin, Nop10, NHP2, and Gar1, offering a comprehensive view of the data's intersection patterns and revealing how these interactors are interconnected. The specific lists of interactors were obtained from BioGrid (<https://thebiogrid.org> (accessed on 7 July 2023)). The plot includes horizontal blue bars representing the sets and the size of the compared groups. Intersecting lines or "intersections" connect the sets, indicating the presence of interactors shared between the corresponding groups. A single dot without any intersection line represents interactors unique to a specific group. The height of the vertical column corresponds to the number of interactors in a specific group, which is indicated by a dot above the column. The height also represents the number of interactors shared among two or more groups, which is indicated by an intersecting line above the column.

Interestingly, we noted that the dysfunction of almost all these common partners (30/34; 88.23%), despite their variegated functional roles, was invariantly related to diseases (Table 1).

Table 1. Function of a subset of CypA, dyskerin, and fibrillarin common interactors and their related diseases. Protein class (PC) accession number derived from the site <http://www.panther.org> (accessed on 7 July 2023). For proteins marked by the asterisk, PC was unavailable at that site and functional classification is in accordance with the uniprot.org site or the <https://www.ncbi.nlm.nih.gov/gene> (accessed on 7 July 2023) site. Associated diseases were derived from the www.genecards.org (accessed on 7 July 2023) site.

Protein Name	Function	Associated Disease
ARRB2	Transmembrane receptor regulatory/adaptor protein	Synovium Neoplasm; Cecum Lymphoma
BIRC3	Enzyme Modulator (PC00095)	Lymphoma, Mucosa-Associated; Lymphoid Type Lymphoma
BTF3	Transcription Factor (PC00090)	Ciliary Dyskinesia
CIT	Non-Receptor Serine/Threonine Protein Kinase (PC00167)	Microcephaly 17, Primary, Autosomal; Primary Autosomal; Recessive Microcephaly
COX15	Chaperone (PC00072)	Mitochondrial Complex IV Deficiency, Nuclear Type 6; Leigh Syndrome with Leukodystrophy
CUL3	Scaffold/Adaptor Protein (PC0026)	Pseudohypoadosteronism, Type IIe; Neurodevelopmental Disorder, With or Without Autism or Seizures
CUL7	Scaffold/Adaptor Protein (PC00226)	Three M Syndrome 1; Shox-Related Short Stature
DDRKG1	Ubiquitin-Protein Ligase (PC00234)	Spondyloepimetaphyseal Dysplasia Shohat Type; Spondyloepimetaphyseal Dysplasia
DLST	Transferase (PC00220)	Paragangliomas 7 and Hereditary Paraganglioma–Pheochromocytoma Syndromes
EFTUD2	Translation Elongation Factor (PC00222)	Mandibulofacial Dysostosis, Guion–Almeida Type; Esophageal Atresia
FANCD2 *	DNA Repair, Chromosomal Stability	Fanconi Anemia, Complementation Group D2 and Fanconi Anemia, Complementation Group A
G3BP1	RNA Metabolism Protein (PC00156)	Chikungunya and Mouth Disease
HSD17B10	Oxidoreductase (PC00176)	Hsd10 Mitochondrial Disease and Syndromic X-Linked Intellectual Disability Type 10
KIF14	Microtubule Binding Motor Protein (PC00156)	Meckel Syndrome 12 and Microcephaly 20, Primary, Autosomal Recessive.
KIF20A	Microtubule Binding Motor Protein (PC00156)	Cardiomyopathy, Familial Restrictive, 6; Familial Isolated Restrictive Cardiomyopathy
KIF23	Microtubule Binding Motor Protein (PC00156)	Anemia, Congenital Dyserythropoietic, Type IIIa and Anemia, Congenital Dyserythropoietic, Type Ia
MYC	Transcription Cofactor (PC00217)	Burkitt Lymphoma; High-Grade B-Cell Lymphoma Double-Hit/Triple-Hit
MYCN	Transcription Cofactor (PC00217)	Diseases associated with MYCN include Feingold Syndrome 1 and Familial Retinoblastoma
NPM1	Chaperone (PC00072)	AML With Myelodysplasia-Related Features; Leukemia, Acute Myeloid
NR2C2	C4 Zinc Finger Nuclear Receptor (PC00169)	Aging and Premature Aging

Table 1. Cont.

Protein Name	Function	Associated Disease
NTRK1	Transmembrane Signal Receptor (PC00197)	Insensitivity To Pain, Congenital, With Anhidrosis and Thyroid Carcinoma Familial Medullary
OBSL1 *	Cytoskeletal adaptor protein	Three M Syndrome 2; Three M Syndrome 1
PARK2 *	E3 ubiquitin-protein ligase	Parkinson Disease 2, Autosomal Recessive Juvenile and Leprosy 2
PRC1	Non-Motor Microtubule Binding Protein (PC00166)	Bladder Cancer
RC3H1 *	ROQUIN-1	Immune Dysregulation and Systemic Hyperinflammation Syndrome.
RECQL4	DNA Helicase (PC00011)	Baller–Gerold Syndrome; Rapadilino Syndrome
RPL11	Ribosomal protein (PC00202)	Diamond-Blackfan Anemia 7 and Diamond–Blackfan Anemia
SOAT1	Acyltransferase (PC00042)	Atherosclerosis Susceptibility; Adrenal Carcinoma
SRPK1 *	Protein Kinase	Denys–Drash Syndrome

This observation underscores the functional relevance of the relationship between CypA and the core components of both major RNP families and aligns with the broad involvement of CypA in various diseases, wherein it influences different pathophysiological pathways depending on the complexes it engages with [3]. Overall, this remark supported the possibility that CypA could participate, at least transitorily, in common gatherings with these complexes.

Clusterization of interactors common to CypA, fibrillarin, and dyskerin by using the PANTHER classification system (<http://www.pantherdb.org> (accessed on 7 July 2023)) showed that the most abundant protein classes were represented by a protein modifying enzyme (11.8%), cytoskeletal protein (11.8%), and gene-specific transcriptional regulator (11.8%), together accounting for up 35.4% of the total (Figure 4B).

Upon a thorough examination of the known interactors of each H/ACA RNPs core component, dyskerin, Nop10, NHP2, Gar1, and NAF1, which are transiently recruited at the site of snoRNA transcription [29,58], a remarkable observation emerged: most interactors shared a substantial number of common partners with CypA (Figure 4C). Of note, NAF1 and NOP10 displayed the least number of interactions with CypA, possibly due to their involvement in the early stages of RNP assembly. This intriguing finding implies that CypA might preferentially interact with mature and active H/ACA snoRNPs, suggesting a dynamic association that might play a pivotal role in this process.

Discussion

The nucleus serves as a crucial site for the diverse functional roles of CypA, particularly in regulating the assembly and trafficking of large RNP complexes with distinct functions [7]. In this study, we provide new insights into the functional repertoire of this multifunctional chaperone by demonstrating its localization in the nucleoli and its interaction with both the canonical and Iso3 truncated variants of human pseudouridine synthase dyskerin. These findings suggest a potential role for CypA in nucleolar dynamics, which is consistent with previous data from various systems. Notably, CypA has been shown to function as a ribosome biogenesis chaperone in filamentous fungi [60], and its specific involvement in H/ACA RNP dynamics has been observed in *S. cerevisiae* [61]. A previous study identified NHP2, a core component of H/ACA RNPs, as a client of CypA and demonstrated that the cis/trans isomerization of the highly conserved Pro83 residue influenced H/ACA RNP assembly. In our study, we present several lines of evidence suggesting that CypA may also target human dyskerin, indicating that proline isomerization should be

considered among the various post-translational modifications described for this multifunctional protein. Dyskerin, in addition to harboring a significant number of phosphorylated residues [55], is extensively subjected to PARylation and SUMOylation [62,63], two types of post-translational modifications known to contribute to the biogenesis, trafficking, and subnuclear localization of the RNPs and have the ability to modulate liquid–liquid phase separation (LLPS) [64]. Future experiments will elucidate whether dyskerin is involved in the early stages of nucleolar phase separation, as has been established for several key nucleolar proteins such as fibrillarin [21] and GAR1 [18], which, together with dyskerin, form the active tetramer of H/ACA RNPs [27].

Addressing the CypA-dyskerin interaction, the observation that it persists and can be modulated in response to stress signals, even at cytoplasmic stress granules (SGs), highlights the functional potential of this interaction. This is particularly noteworthy considering that pseudouridylation affects the entire transcriptome and can be modulated by environmental signals [64].

Moreover, considering that the PPLase activity of CypA may play a critical role in influencing the local and flanking protein structures, it is plausible that this modification could impact the broad range of non-canonical dyskerin functions, which currently include redox metabolism [39], vesicular trafficking [65], and its ability to act as a co-transcriptional factor [66]. For that reason, the discovery of the CypA-dyskerin interaction holds significant relevance for patients affected by Dyskeratosis Congenita X-linked (X-DC). X-DC is a rare genetic disorder caused by mutations in the dyskerin-encoding gene *hDKC1*. Dyskerin is

a key component of both telomerase and the snoRNA H/ACA complex [54]. Dyskerin functions as a pseudouridine synthase within the snoRNA H/ACA complex, guiding modifications in ribosomal RNA and other classes of RNAs essential for cellular processes. These modifications play important roles in RNA stability, function, and processing, contributing to vital cellular functions such as translation, pre-mRNA splicing, and ribosome biogenesis.

While telomerase dysfunction is central to X-DC, the pathogenesis of the disease is influenced by additional factors, including dyskerin telomerase-independent functions. The contribution of the telomerase-independent roles of dyskerin to the etiology of X-DC is further supported by studies on both animal and cellular models [39,45,65,67–69].

This multifaceted role highlights the complexity of X-DC, emphasizing the need to comprehensively understand dyskerin various cellular functions for potential therapeutic strategies. The identification of CypA-dyskerin interactions unveils an unknown dimension of dyskerin's regulation, shedding light on potential disease-contributing pathways. Notably, this discovery holds several key points of relevance: 1. limited treatment options for X-DC necessitate new therapeutic targets. The interaction between dyskerin and CypA presents an avenue to explore modulating dyskerin's activity to potentially alleviate symptoms or slow disease progression; 2. understanding how CypA influences dyskerin's function may unravel the intricate regulation of pseudouridine activity, with implications beyond X-DC, including in cancer research; 3. as a member of the peptidyl–prolyl cis–trans isomerase (PPLase) family, CypA's interaction with dyskerin suggests a role in ensuring proper folding and stability, contributing to a broader understanding of protein homeostasis and chaperone-mediated processes; 4. insights from this interaction may lead to specific biomarkers aiding in X-DC diagnosis and prognosis; 5. the interaction between dyskerin and CypA could explain phenotypic variations observed in affected individuals, informing personalized treatment approaches tailored to molecular defects; 6. the discovery opens avenues for therapeutic interventions targeting cellular senescence, stress response, and immune dysfunction associated with dyskeratosis congenita; and 7. dyskerin's roles in stem cell maintenance and tissue regeneration may have implications for regenerative medicine in conditions where telomerase dysfunction affects tissue repair and renewal.

This discovery enhances our understanding of dyskerin's functions and offers potential opportunities to address the complexities of dyskeratosis Congenita X-linked and related cellular processes in various disease contexts.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/genes14091766/s1>. S1: list of interactors associated with dyskerin, CypA, NAF1, Fibrillarin, Nop10, NHP2 and Gar1; S2: R code for the Upset plot.

Author Contributions: Conceptualization, M.F. and M.T.; investigation, D.M., V.B. and C.D.L.; resources, M.T.; data curation, R.V.; writing—original draft preparation, D.M., V.B. and M.F.; writing—review and editing, M.F., R.V., M.T., D.M. and V.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within article/supplementary materials.

Conflicts of Interest: The authors declare no conflict of interest.

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The third and final part of the project

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