UNIVERSITY OF NAPOLI FEDERICO II

Doctorate School in Molecular Medicine

Doctorate Program in
Genetics and Molecular Medicine
Coordinator: Prof. Lucio Nitsch
XXVII Cycle

"Molecular mechanisms underlying the functional multiplicity of human *DKC1* gene"

CANDIDATE: Dr. Nunzia Di Maio
MENTOR: Prof. Maria Furia

Napoli 2015

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LIST OF PUBLICATIONS RELATED TO THE THESIS

Turano M, Angrisani A, Di Maio N, Furia M. Intron retention: a human *DKC1* gene common splicing event. Biochem. Cell Biol 2013; 91: 506-512.

ABSTRACT

Hypomorphic mutations of the *DKC1* gene cause the X-linked dyskeratosis congenita, an inherited multisystemic disorder characterized by a variety of symptoms, including mucocutaneous features, stem cell dysfunction, bone marrow failure, and increased susceptibility to cancer. *DKC1* encodes an evolutionarily conserved protein, called dyskerin, whose activity is essential for cellular vitality. Dyskerin participates in at least two functional ribonucleoprotein (RNP) complexes that play essential roles in the cell. The protein is in fact an essential component of H/ACA snoRNPs, which are involved in the processing and pseudouridylation of rRNAs. In addition, through its binding to the telomerase RNA component (TERC), that contains an H/ACA RNA motif, dyskerin participates in the active complex of telomerase, ensuring telomeres stability and maintenance.

In order to investigate and characterize in more detail the effects triggered by *DKC1* loss of function on human cells, we generated a stable cellular model able to reduce gene expression, upon inducible RNA interference, without affecting the expression of the alternatively spliced cytoplasmic isofom 3.

I focussed my analyses on the effects triggered by dyskerin depletion well before telomere erosion, in order to define the spectrum of telomere-independent outcomes. Intriguingly, I found that dyskerin depletion affected cell adhesion. In fact, dyskerin dowregulation perturbed both cell-cell and cell-substrate adhesion, causing reduced expression of some proteins involved in the tight and adherens junctions, and in the focal adhesions. Moreover, co-immunoprecipitations analyses revealed interactions between dyskerin and some component of focal adhesions, while confocal microscopy indicates that this interaction can occur at level of the plasma membrane. Finally, I observed that dyskerin depletion alter cell morphology and increases cell motility. Altogether, these results could be of valuable help to understanding the puzzling relationship that links pseudouridine synthases loss-of-function to cancer predisposition.

1.BACKGROUND

1.1 Dyskeratosis congenita: symptoms and transmission manner

Dyskeratosis congenita or Zinsser-Cole-Engman syndrome is a rare disorder that embraces a variety of symptoms including hyperpigmentation, skin atrophy, nail dystrophy and mucosal leukoplakia (for a review Kirwan and Dokal 2009).(Fig.1).

Figure1: Main phenotypic traits of DC patients (DC): mucosal leucoplakia (a), ungueal dystrophy (b), anomalous pigmentation of the skin (c), cerebral hypoplasia (d), premature hair graying (e). From Kirwan and Dokal, 2009.

It is now known that this characteristic symptomatology is coupled to a series of additional symptoms such as mental retardation, premature aging and bone marrow failure. The latter represents the main cause of early death, although DC patients are more disposed to the development of lung diseases and malignant neoplasms, which may also be fatal (Mason and Bessler. 2011). The syndrome exhibit genetic heterogeneity, with three kinds of inheritance modes: Autosomal Dominant (AD-DC), Autosomal Recessive (AR-DC) and X linked recessive (X-DC). The X-linked form is due to hypomorphic mutations of *DKC1* gene (Heiss et al. 1998) located on the long arm of the X chromosome at the locus 28. Many mutations affecting the *DKC1* gene have been so far identified, including an *in frame* triplet deletion (201-203 CTT) that causes the lost of the aminoacid leucin 37 (Heiss et al. 1998); a ~2 kb (kilo bases) chromosomal deletion that causes the loss of exon 15 and the use of a cryptic polyadenilation signal (Vulliamy et al. 1999); a single mutation into the promoter region (-141; C→G) that disrupts a binding site of the transcription factor SP1, thus affecting the basal level of the promoter (Knight et al. 1999); a mutation in intron 1 (IVS1+592; C→G), that generates a cryptic donor splice site which induces the incorporation into the mature transcript of 246 intronic

bases that interrupt the CDS and generate a non functional transcript (Knight et al. 2001).
The autosomal dominant forms, which collect about 10% of clinical cases (Cossu et al. 2002), generally show a more attenuated and delayed clinical picture and exhibit genetic "anticipation", a phenomenon in which the phenotype's severity increases in subsequent generations, so that the disease is manifested earlier from generation to generation. Three causative genes have been identified: *TERC*, the telomerase RNA component (Vulliamy et al. 2001); *TINF2*, which encodes for a protein present in the "shelterin" complex involved in telomere maintenance (Savage et al. 2008) and *TERT,* that encodes for the telomerase reverse transcriptase component (Armanios et al. 2005). Finally, the autosomal recessive variant, which is present in 5% of clinical cases (Cossu F. 2002), is caused by mutations in the genes encoding for the nucleolar proteins Nop10 and Nhp2, which associate to telomerase and have an essential role also in the maintenance of snoRNA of H/ACA class and in pre-rRNA maturation. The X-linked form is undoubtedly the most widespread, grouping about 80% of clinical cases (Cossu et al. 2002). The causative gene, *DKC1*, was identified in 1998, when a screening of cDNA from different patients identified mutations in its coding region (Heiss et al. 1998).

1.2 *DKC1* and its orthologs

The human *DKC1* gene encodes a nucleolar protein of 58 kDa, called dyskerin, whose sequence is phylogenetically highly conserved. In fact, dyskerin has orthologs in yeast (Cbf5), rat (Nap57) and Drosophila (NOP60B / minifly). The *Cbf5* yeast gene, originally identified as a binding factor of centromeres and microtubules (Jiang et al. 1993), was the first member of this genes family to be identified. *Cbf5* is an essential gene that encodes a protein containing a domain composed of ten KKE/D repetitions arranged in tandem. These repeats, are homologous to portions of the domain responsible of microtubule binding (Noble et al. 1989). Later it was demonstrated that CBF5 is a pseudouridine synthase responsible to the rRNA pseudouridylation (Lafontaine et al. 1998). In fact, it presents a considerable sequence homology with the pseudouridine synthase TruBp of E. coli and shares sequence motifs conserved "KP" and "XLD" present in the catalytic domains of three distinct families of pseudouridine synthase. In line with the bacterial TruBp, the XDL motif of Cbf5 contains an aspartate at position 95, essential for its pseudouridylation activity and its replacement with an alanine abolishes the rRNA pseudouridylation in vivo (Zebarjadan et al. 1999). In addition, the inactivation of *Cbf5* gene turns out to be lethal because it creates defects in the processing and pseudouridylation of rRNAs precursors (Cardwell et al. 1997; Zebarjadian et al. 1999) Finally, it was also observed a CBF5 involvement in the cell cycle, through its KKE / KKD motif at the C-terminal. In particular, W. Jiang et al. (1993) noted that the complete deletion of the *Cbf5* gene, led to cell cycle arrest in G1 / S phase, whereas cells containing a

C-terminal truncated CBF5 gene, producing a protein containing only three copies of the KKD/E repeat, delay with replicated genomes at the G/M phase of the cell cycle.
The dyskerin mammalian homolog, Nap57, is involved in the nucleus-cytoplasm shuttling of pre-ribosomal structures (Meier and Blobel. 1994). The Drosophila homologue gene, called NOP60B / minifly (Philips et al. 1998) is essential for the rRNAs precursors maturation and pseudouridylation. His absence is lethal, while a reduction of its espression entails pleiotropic effects such as lower body size, feminine infertility and developmental delay (Giordano et al. 1999). Subsequently, studies in primary cells obtained from patients with X-DC and from *DKC1* knock-out or mutant mice (carrying the same mutations found in human patients) unrevealed that the disease is accompanied by a reduction of telomeres length (Mitchell et al. 1999; Vulliamy et al. 2001). However, in transgenic mice the symptoms manifested many generations before the telomeres underwent a real shortening, supporting the idea that reduced levels of protein synthesis and pseudouridylation played a more important role in the pathogenesis of X-DC. He et al. (2002) created two Cre/LoxP transgenic mice carrying two different allelic variants of the *DKC1* gene. In particular, one mouse carried a large deletion that eliminated the 12-15 exon region, and the other one carried a deletion of the only exon 15 designed to produce a truncated dyskerin protein similar to that found in a DC patients. Females heterozygous for both deletions showed a complete embryonic lethality from day 9.5 post conception (d.p.c.). At day 7.5 d.p.c., no male embryo was vital, while female embryos showed degeneration of extra embryonic tissues. Since in these tissues the paternal X chromosome is inactivated, this suggested the occurrence of a maternal effect, so far still unexplained at the molecular level. Moreover, heterozygous females in which the deletion was of paternal origin had a strong tendency to selectively inactivate the mutant chromosome, retaining the wild type chromosome active in all cells. Since X chromosome inactivation is a very early event in embryonic development, this skewed inactivation pattern is likely to result from a “cell competition” mechanism, by which cells that inactivated the wild-type chromosome were strongly disadvantaged, and then competed-out by cells actively expressing the wild type X chromosome. Moreover, recreating the last exon deletion, thus resulting in a truncated protein lacking 21 aa, there was observed a growth rate reduction associated with an accumulation of reactive oxygen species (ROS). An increase in ROS, examined through the phosphorylation of H2AX, an indicator of DNA damage, can result from ribosomal stress caused by the perturbation of ribosome biogenesis (Gu et al. 2013). Once indentified *DKC1* as X-DC responsible gene, it is passed to the description all related orthologs present in plants, fungi, protists and archaea (Watanabe and Gray. 2000; Maceluch et al. 2001; Lin and Momany. 2003). The importance of these proteins for the cell life is demonstrated by the observation that in all examined organisms null mutations are lethal, while

ipomorfic mutations or gene silencing cause a number of phenotypic abnormalities (Giordano et al. 1999; Tortoriello et al. 2010; Zhang et al. 2012).

1.3 *DKC1* and dyskerin structure

The *DKC1* gene consists of 15 exons and hosts two H/ACA snoRNAs (small nucleolar RNAs), named ACA36 and ACA56, in intron 8 and 12, respectively. The snoRNA ACA36 drives the pseudouridylation of U105 and U1244 residue of 18S rRNA, whereas the snoRNA ACA56, allows the U1664 28S rRNA residue pseudouridylation. Moreover, ACA56 is known to act as a micro RNA (miRNA) precursor (Ender et al. 2008). In addition to the canonical transcript encoding dyskerin and the known alternative transcript which directs the synthesis of a truncated dyskerin variant with cytoplasmic localization, called isoform 3 (Angrisani et al. 2011), our research group has identified others 3 alternative transcripts. These new isoform, called isoform 4, 5 and 6, respectively, are characterized by total or partial retention of a specific intron and could encode proteins that lack the PUA domain, essential for the binding to the RNA, or the nuclear localization signals, and consequently would be unable to carry out the dual dyskerin canonical functions (Fig.2).

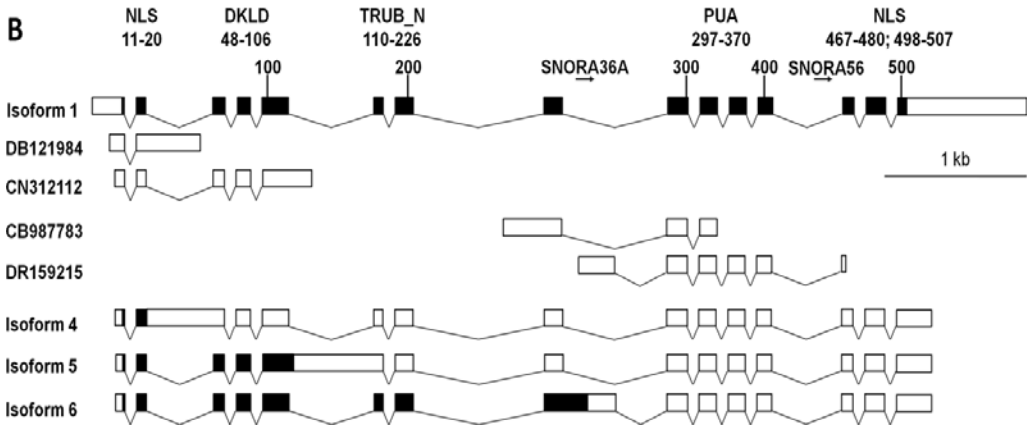


Figure 2. Structure of intron-retaining *hDKC1* transcripts. On the top, the structure of the 2.6 kb *hDKC1* mRNA (iso 1) encoding canonical dyskerin (GenBank ID: NM_001363). Exons are depicted as boxes, coding regions are in black; the position of the main dyskerin functional domains is indicated above, together with that of the intronically encoded SNORA36A and SNORA56. Below, structures of the ESTs marked with # and their related transcripts, as derived by RT-PCR cloning and nucleotide analysis.(From Turano et al.2013)

The transcripts expression was confirmed in both different cell lines and normal tissue with a variation of their expression levels, suggesting that *DKC1* isoforms may contribute to the gene functional diversity across different cell types. Since all new transcripts were characterized by premature stop codons (PTCs), to exclude that the new isoforms could be under the NMD surveillance mechanism which commits such mRNAs to the degradation, the expression of

these transcript was analysed in cells (colorectal adenocarcinoma) treated and not treated with cycloheximide, an inhibitor of protein synthesis that represses NMD. *DKC1* isoforms containing PTC are resulted unaffected by the treatment. Finally, to evaluate the isoforms coding potential, it was checked the accumulation of the RNA isoforms in both the cytosolic and nuclear fractions. We found the isoforms 4 and 5 essentially retained in the nuclei, while isoform 6, together with canonical isoform 1, was efficiently exported in the cytoplasm suggesting a possible translation (Fig.3).(Turano et al. 2013).

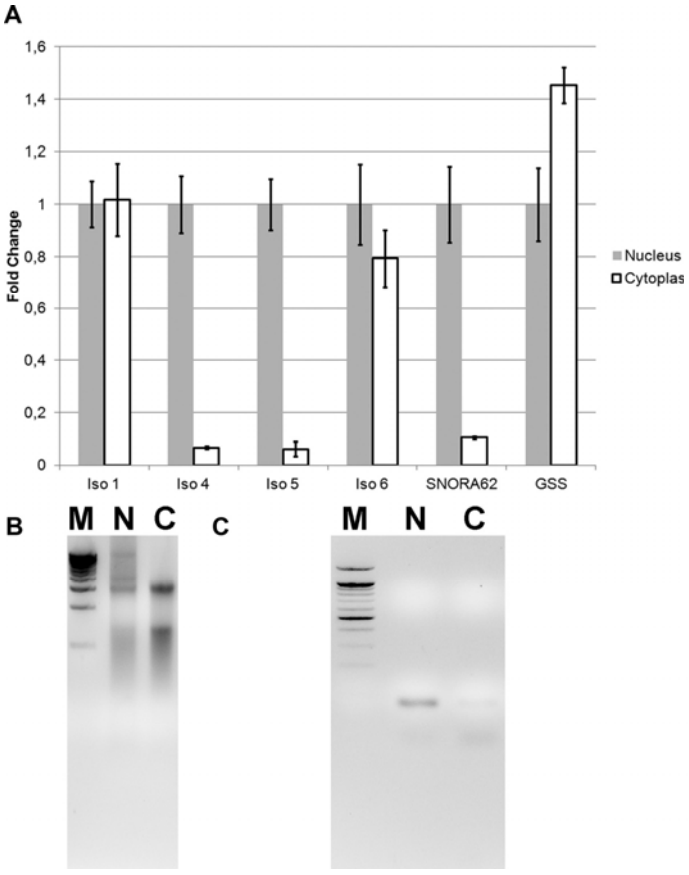


Figure 3.Comparative estimate of the levels reached by each new *hDKC1* isoform in nuclear and cytosolic fractions. (A) Abundance of each isoform was determined by qRT-PCR analysis; the snoRNA *SNORA62* was used as a nuclear marker, while *GSS* mRNA was used as marker of the cytosolic fraction. Expression was normalized with respect to *POLR2A* mRNA. (B) Agarose gel of 800 ng fractionated RNA; M 1 kb DNA ladder(Applichem), N nuclear RNA, C Cytoplasmic RNA. (C) Semiquantitative RT-PCR of *SNORA62* nucleolar RNA; M 100 bp Plus DNA ladder(Applichem), N nuclear, C Cytoplasmic.(From Turano et al.2013).

The sequence alignment of dyskerins from different organisms has revealed the presence of highly conserved regions which reflects their functional conservation. This aspect was directly attested by swapping experiments, which showed that Drosophila and rat dyskerin were both able to rescue yeast Cbf5 mutations (Phillips et al. 1998; Yang et al. 2000). The protein structure in mature form proposed by the Pfam database (DKC1_HUMAN) identifies the presence of three conserved functional domains: the first, known as dyskerin like Domain (DKLD; 48-106 aa), whose function is currently unknown; the second, known as domain TruB_N, (110-226 aa), is the pseudouridine synthase catalytic domain and contains the active site directly involved in the pseudouridylation process; the third, the PUA RNA binding domain, (297-370 aa) is involved in the recognition of the RNA of H/ ACA family (Figure 4).

The protein exhibit also two nuclear localization signals (NLS), characterized by the presence of lysine rich sequences, located at both the N- and C-terminus positions.

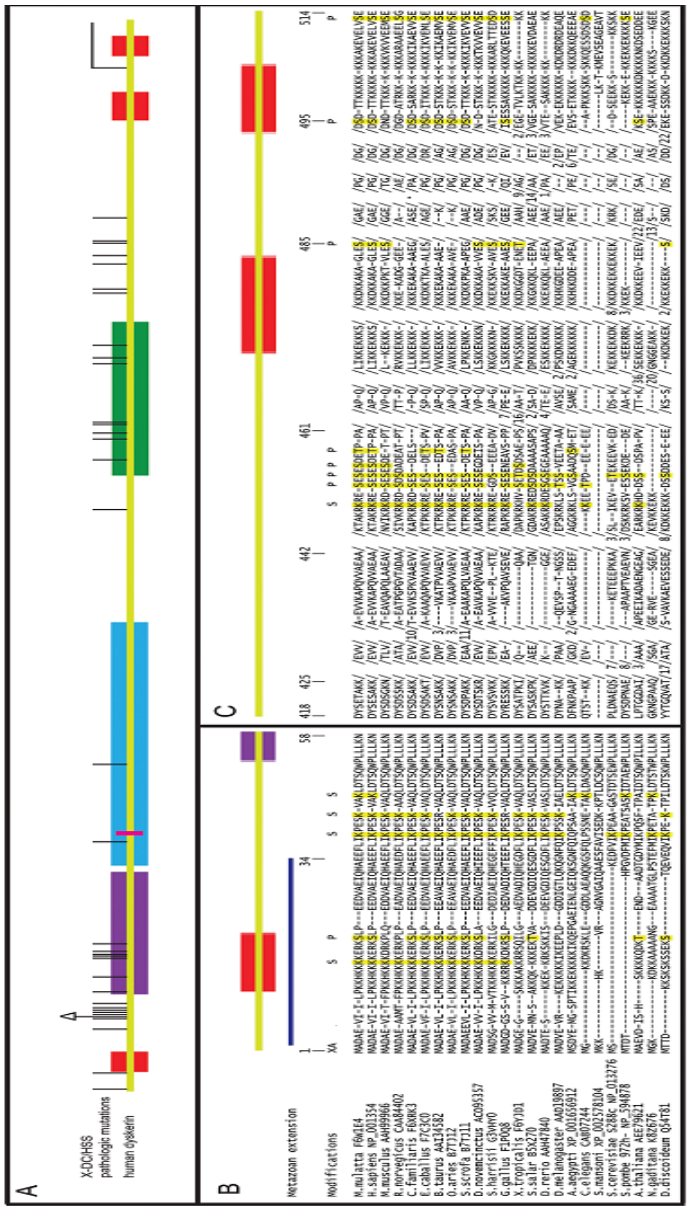


Figure 4. Dyskerin structure and sequence conservation. A) Organization of Dyskerin functional domains. The red boxes at N- and at C terminal ends represents the NLS (nuclear localization signals). The purple box represents the DKCLD domain. The TruB_N e PUA domains are respectively in blu and in green. Multiple allignament of terminal sequence N- **(B)** e C- **(C)** of dyskerin derived from different organisms (Da Angrisani et al., 2014).

Many post translational modification of human dyskerin have been annotated in the UniProt database. These modifications show a different degree of phylogenetic conservation, with the elimination of initiator methionine and N-

acetylation of residue 2, characteristic of eukaryota, with a few exceptions in unicellular organisms, and phosphorylation of serines 21, 451, 453, 455, 485, 494, 513 and of threonine 458, and sumoylation of lysines 16, 39, 46 and 448 evolutively preserved. Dyskerin modification by SUMO1 and SUMO2/3, firstly revealed by high-through put mass spectrometry experiments at four residues (Manza et al. 2004; Blomster et al. 2009; Westman et al. 2010), has recently been directly confirmed by Brault et al. (2013). These authors also provided a direct correlation between pathologic mutations and sumoylation defects. Looking at protein sequence, the divergence main points observed between eukaryotic and archaeal dyskerins are located at the N- and C-terminal, where the eukaryotic proteins have a typical extension (Figure 2B and C). An interesting aspect is that the N-terminal extension length is greater in metazoans; where stands a block retained about 30 amino acids (2-34 in humans), absent in the yeast proteins. Probably, even if it has not been defined yet, this portion has a regulatory function and this hypothesis is supported by the fact that it is subjected to two post-transcriptional modifications: the sumoylation of lysine 16 and the phosphorylation of serine 21 (Angrisani et al. 2014).

1.4 Dyskerin functions

All eukaryotic diskerins described so far have mainly a nuclear localization. Inside the nucleus, dyskerin participates in three essential complexes (Fig. 5): the H/ACA small nucleolar ribonucleoparticles (snoRNPs) (Kiss et al. 2006), the telomerase holoenzyme (Cohen et al. 2007) and the specific Cajal body ribonucleoparticles (scaRNPs).
The H/ACA snoRNPs are hetero-pentameric complexes in which dyskerin is associated with a molecule of small nucleolar RNA (snoRNA) of H/ACA class and three highly conserved proteins: NOP10, NHP2 and GAR1 (Kiss et al. 2006). Nascent H/ACA snoRNAs are mainly intronic and the assembly of the H/ACA snoRNP complexes occurs before the host intron is spliced out from the primary transcript (Richard et al. 2006). While the association of snoRNA with Gar1p, Nop10p, Nhp2p is only transitory, the dyskerin-snoRNA association is irreversible and settled during the snoRNA maturation (Kittur et al. 2006). In addition is known that the Naf1 protein is essential for the biogenesis of H/ACA snoRNP (Dez et al. 2002; Fatica et al. 2002). In fact, in yeast this protein participates in the early steps of the RNPs assembling and it has been found associated with snoRNA genes transcriptionally active, while did not show any interaction with the mature snoRNP particles (Ballarino et al. 2005). Similar results were also highlighted in human cells by Kittur et al. (2006) who proposed that, during the maturation of snoRNP, Naf1 is replaced by Gar1 protein, and this assures that the mature particle is released from the transcription site of snoRNA and transported in the nucleolus.

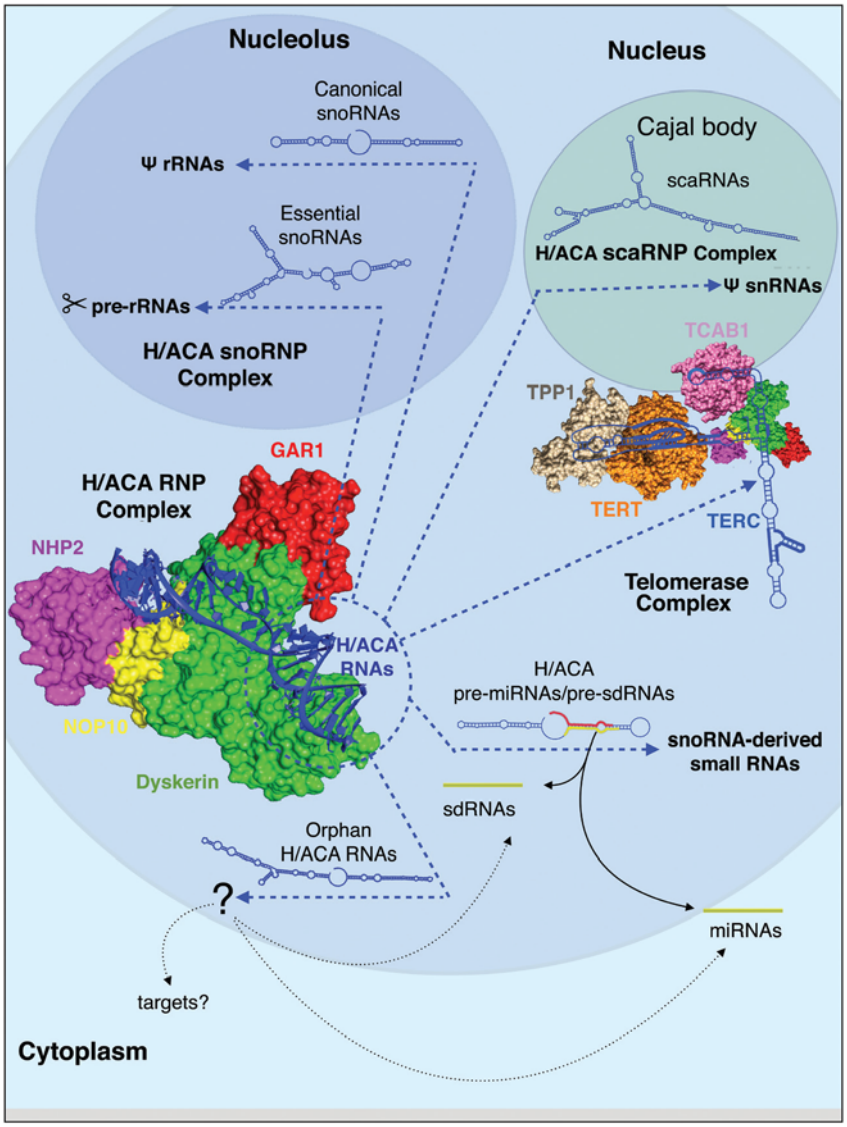


Figure 5: Dyskerin nuclear complex. The three complexes are localized in the nucleus, where they are involved in different functions. While snoRNA complexes direct the re-RNA process, those with canonical snoRNA direct the RNA pseudouridylation. When dyskerin associates with orphans RNA of H/ACA class, with unknown targets,unlike , the result complexes could guide the cell RNA modification or act as precursor of regulator RNA small (miRNA/dsRNA). At least, if it associates with scaRNA, the tetramer dyskerin -NOP10-NHP2-GAR1 forms the scaRNP, localized in the Cajal bodies, where direct the RNA pseudouridylation. (Angrisani et al. 2014).

The typical secondary structure of H/ACA snoRNAs is composed of two hairpins and two short single stranded sequences containing, respectively, the H (ANANNA) and ACA boxes, with the latter always localized 3 nucleotides upstream the snoRNA3'end (Fig.6A). Hairpin structures are interrupted by recognition loops containing the guide sequences; within the region of base pair complementarity to the target RNA, the first unpaired nucleotide represents the uridine that will be modified. The inner ring of single-stranded (often referred to as the pocket pseudouridylation) is always located 14-16

nucleotides upstream the H or the ACA box.(FIG.6B). The most common targets of snoRNPs pseudouridylation are represented by rRNAs and snRNAs, and correct pseudouridylation of these molecules seems to be necessary for their proper functionality and for efficient ribosome biogenesis. The main function of these complexes is to catalyze the pseudouridylation of new synthesized RNA ribosomal.

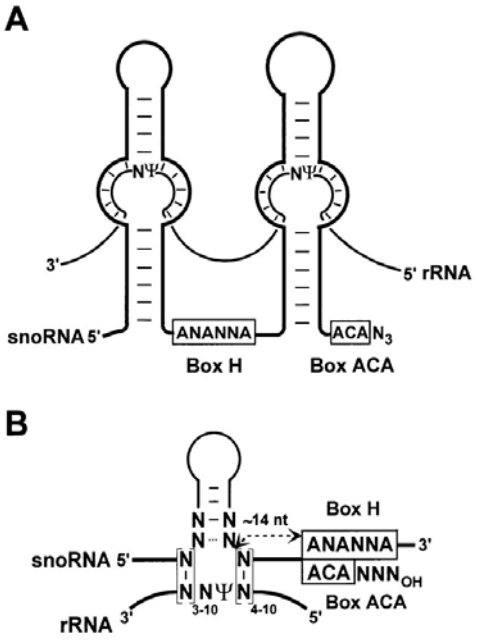


Figure 6: H/ACA snoRNA Structure.
A) Schematic representation of a canonical H/ACA snoRNA molecule
B) Site of pseudouridylation, located 14-16 nt upstream the box

The snoRNA, therefore, acts as a guide selecting, by sequence complementary, the RNA target and the specific residue to be pseudouridilated (Lafontaine and Tollervey. 1998). In this process dyskerin, is the pseudouridine synthase that directs the conversion of uridine into pseudouridine. Since the most common targets of pseudouridylation are stable RNA, that play a key role in basic cellular processes such as rRNA and snRNAs, where residues pseudouridilated are often found in functional highly conserved positions (Maden. 1990; Yu et al. 2011; Ge and Yu. 2013), it was easy to predict that the uridine isomerization into pseudouridine could modulate gene expression in a variety of manners. Since for a growing number of snoRNAs, indicated as "orphan", has not been identified any RNA target, other cellular RNAs could be subject to pseudouridylation. Intriguingly, many snoRNA "orphans" show a tissue specific expression pattern, as it is the case of HBI-36, a snoRNA specifically expressed in the brain, at the level of the choroid plexus (Cavaillé et al. 2000). Although pseudouridylation represents the most common modification found on cellular RNAs, its biological role is still elusive. Pseudouridines are able to form hydrogen bridges with bases other than adenine, and its presence can influence folding and activity of stable RNAs, like tRNAs, rRNAs and snRNAs (Arnez and Steitz. 1994; Newby and Greenbaum 2002), and also affect their interaction with RNA-binding proteins. Unlike for the ribosome structure, it was previously suggested that this modify not only contributes to the rRNA folding and ribosomal subunits assembly, but could also influence the efficiency of the codon translation and recognition (Luzzatto and Karadimitris. 1998). In the ribosomes biogenesis, dyskerin not only plays an important role in the pseudouridylation reaction, but it is also involved in the maturation of pre-rRNA, through nucleolytic cuts guided by the

H/ACA snoRNAs U17 (also called E1), E2 and E3. For the 5' maturation of the18S rRNA is, in fact, request the function, probably as a chaperone, of the snoRNA U17, which in humans present two isoforms, the U17A and U17b. The snoRNA E2 is necessary for the 3 'end maturation of the same rRNA, whereas the snoRNA E3 is required for the 5' maturation of the 5.8S rRNA. The rRNA pseudouridylation is highly conserved during evolution, but it is not the only role played by dyskerin. Due to its ability to bind the RNA component of telomerase (TERC), dyskerin also participates in telomerase holoenzyme assembly in Cajal bodies, playing an important role in maintaining the telomeres integrity. Telomeres are composed of about a thousand copies of a short sequence repeat rich in G (in humans the telomeric sequence is 5'-TTAGGG-3 '), which protrude from the end, forming a single-stranded telomeric and shorten to each replication cycle due to the DNA polymerase inability to replicate the linear DNA molecules ends (Machado-Pinilla et al. 2008). This progressive telomeres erosion is countered by telomerase which mediates the synthesis of telomeric repetitive sequences of DNA as a template using TERC, a snoRNA molecule of H/ACA class. Cohen et al. (2007) identified dyskerin as essential component of the complex. In fact, although there are at least 32 other proteins associated to 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 a telomeres shortening, but both in mouse and in human cells has been observed that they lead to lower TERC levels (Wong and Collins. 2006; Mochizuki et al. 2004). 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. This simple scheme is supported by the fact that the stem cells, in contrast to somatic cells, have an active telomerase and that the DC is a progressive disease that worsens with age .

The tissues most affected in DC, at least in its classical form, tend to be those that require a constant renewal of stem cells, such as blood, skin, nails, etc. (Mason and Bessler. 2011). It is noteworthy that novel functions have been hypothesized for the snoRNP (small nucleolar ribonucleoprotein) complexes in the last years. In fact, several reports indicated that H/ACA snoRNAs can act as precursors of miRNAs, a feature that appears to be largely widespread in eukaryotes. SnoRNA-derived miRNAs have in fact been described so far in Protozoa (Saraiya and Wang. 2008), humans (Ender et al. 2008), mouse, *Arabidopsis*, yeast, chicken (Taft et al. 2009) and Drosophila (Jung et al. 2010). Moreover, the percentage of snoRNAs able to be processed into miRNAs has been estimated to range from 60% in humans and mouse to more than 90% in *Arabidopsis*. Although the snoRNA-miRNA processing mechanism is mostly unclear, it appears to be Dicer-1-dependent but Drosha/DCGR8-independent (Ender et al. 2008; Taft et al. 2009). Considering that miRNAs play a fundamental role in post-transcriptional gene regulation (for a review, see Fabian et al. 2010), it is plausible that pseudouridine synthase

depletion may widely affect developmental processes by inhibiting the snoRNA-derived miRNA regulatory pathway. Indeed, the occurrence of a large variety of developmental defects has recently been described in Drosophila as consequence of pseudouridine synthase gene silencing (Tortoriello et al. 2010). It is thus tempting to suggest that this mechanism might possibly account, at least in part, for the plethora of manifestations displayed by X-DC. Consistent with this hypothesis, dyskerin reduction has been recently shown to impair in an unpredictable way the biogenesis of snoRNA-derived miRNAs, allowing a huge spectrum of possible effects in conditions in which the expression of *DKC1* is perturbed (Alawi and Lin 2010).

1.5 Dyskerin and cancer

The involvement of dyskerin in cell proliferation was discovered in 1992 when Dokal et al. observed that primary skin fibroblasts cultures derived from DC patients were abnormal both in morphology and proliferative rate (doubling time about twice normal) compared to control cells. Since then, it was assumed that the reduced levels of dyskerin perturbs the cell cycle progression and proliferation. In particular it has been observed an arrest in the G2/ M phase in yeast cells with Cbf5 mutated (Jiang et al. 1993), in cells dyskerin depleted (Alawi and Lin. 2011, 2013), in fibroblasts from patients X-DC (Carrillo et al. 2013), and in mouse embryonic fibroblasts characterized by the expression of a catalytically inactive dyskerin (Gu et al. 2013). Therefore dyskerin depletion causes a reduction of cell proliferation, but this is in contrast with the increased predisposition to cancer observed in patients with X-DC. Cumulatively, the incidence of cancer in X-DC patients was reported about 40% -50% by the age of 50 years (Alter. 2009).
The fact that cell proliferation is related to nucleolar functions is demonstrated by the evidence that people affected by abnormal production of ribosomes, have a high predisposition to develop the cancer. Because of the metabolic demand, in fact, the proliferating cells are characterized by a demand increase of protein synthesis and the rate of ribosomes biosynthesis (Montanaro et al. 2010). Consequently, dyskerin mutated cells show ribosomes defects and thus a low degree of proliferation (Derenzini et al. 2005). On the other hand, owing to the nature of DNA replication, the cells in active proliferation need to have telomerase well functioning to prevent the telomeres shortening and, consequently, an activation of DNA damage mechanism (Chan et al. 2004) Since the modification sites are located within specific domains of the ribosome, important for tRNA and mRNA binding, the decrease of the modified uridine residues in the ribosome, may result in an impairment of specific mRNA playing a key role in cancer (Yoon et al. 2006; Bellodi et al. 2010; Montanaro et al. 2010; Rocchi et al. 2013). These mRNAs can be translated through the both classic translational mechanism and an IRES-dependent translation mechanism, such those encoding the p27 apoptotic

protein and the antiapoptotic factors XIAP and Bcl-2. Although active in physiological conditions, IRES-dependent translation plays a key role in the response to apoptotic stimuli and during G0 / G1 cell cycle phase in which the cap-dependent translation is reduced. The authors suggested that the level of rRNA pseudouridylation could affect the ability of the ribosome to recognize highly structured IRESs, a key step for the cap-independent translation.Further information was recently added by Rocchi et al. (2013) who assayed the translation efficiency of a pool of viral and cellular IRESs in DKC1 silenced breast cancer cell lines. These authors observed that p53 and CrPV IRES-mediated translation were reduced, while c-myc, HCV and EMCV IRES-dependent translation were unaffected. Furthermore, VEGF and HSP70 IRES-mediated translation were upregulated. These results lead to conclude that the level of ribosome pseudouridylation can differently modulate IRES-dependent translation: expression of some specific mRNAs could be repressed, while that of others be either unaffected or upregulated .

It is important also to note that the over-expression of dyskerin is involved in certain types of cancer, including colorectal cancer (Turano et al. 2008; Witkowska et al. 2010), breast cancer (Montanaro et al. 2006, 2008), prostate cancer (Sieron et al. 2009), ovarian cancer (Schaner et al. 2003), melanoma (McDonald et al. 2004), neuroblastoma (Westermann et al. 2007), lymphoma (Piva et al. 2006), and hepatocellular carcinoma (Liu et al. 2012), suggesting that the protein could be an important proliferative signal of tumor cells. In fact, several reports have shown a significant correlation between the increase of dyskerin level and advanced clinical stage (Montanaro et al. 2006; Sieron et al. 2009; Witkowska et al. 2010; Liu et al. 2012) , suggesting that the amount of the protein could be used as a prognostic marker, at least in some tumors although the precise mechanisms underlying dyskerin over-expression in tumor progression remains to be fully defined.

1.6 Focal adhesions

The adhesion of cells to the extracellular matrix is the key to the regulation of cell morphology, migration, proliferation, survival and differentiation (Gumbiner et al. 1996). These functions are essential during the development, in the tissues maintenance and in the induction of tissues repair. Integrins are the predominant receptors that mediate cells adhesion to the components of the extracellular matrix (ECM). They are expressed on the cell surface as a heterodimer consisting of α and β subunits. Integrin subunits are type I transmembrane proteins containing both a large extracellular domain responsible for binding to ECM ligands and a cytoplasmic portion (CP) recruiting multiple intracellular proteins (Fig.7). The extracellular domains are responsible for ligands binding, while the cytoplasmic domains form multi-molecular complexes providing a connection to the cytoskeleton (Hynes, 2002). In this way, the exterior and interior of the cell are physically connected,

and this allows the bidirectional transmission of mechanical and biochemical signals across the plasma membrane, and leads to a cooperative regulation of different cellular functions, including adhesion, migration, growth and differentiation (Qin et al. 2004). So each integrin recognizes a distinct ligand on the cell surface and the cytoplasmic portions act as a platform for the recruitment of cellular proteins such as scaffold and signal proteins to the inner surface of the plasma membrane, where they form structures called focal adhesions (FA).

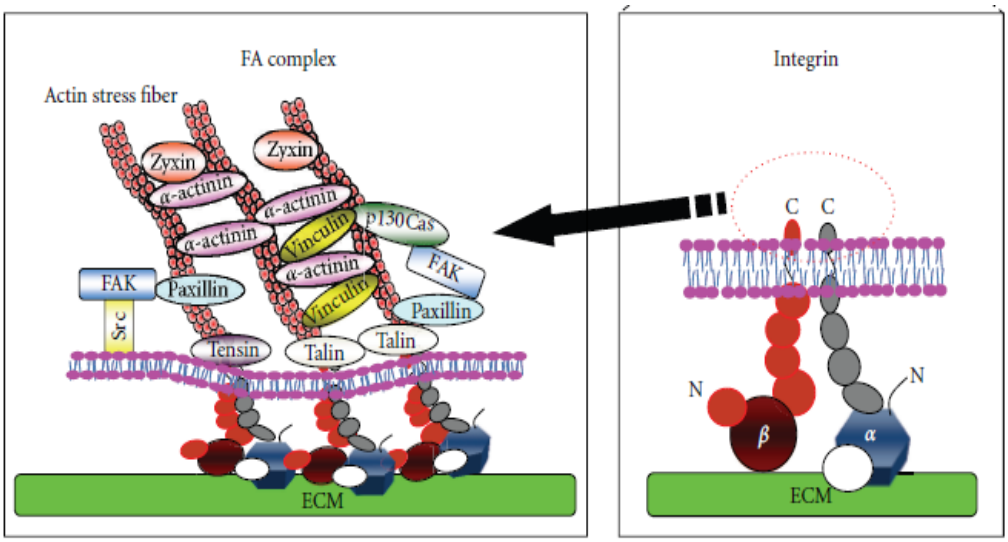


Figure 7: Integrins mediate cell adhesion to the extracellular matrix: They work as hetero dimers α and β; the cytoplasmic portion recruit signaling proteins and the actin cytoskeleton. Adapted from M. Nagano et al; 2011

The protein scaffolds of focal adhesions such as: talin, paxillin,tensin, and the α-actinin, provide strong bonds to the actin cytoskeleton, and thus firmly connect the cells to the ECM (Humphries et al; 2007). This binding allows the generation of the voltage required to change the cell morphology and the tensile force required to move the cell body during the migration. In addition, more signaling proteins, including kinases and phosphatases, are recruited where they transmit signals that control the proliferation, survival and cell migration. In particular, two well characterized tyrosine kinases, such as focal adhesion kinase (FAK) and Src play a central role in signaling cascades mediated by integrin, transmitting signals from the FA to the cellular machinery by phosphorylating multiple proteins associated with integrins (Mitra and Schlaepfer 2006), since integrins have no intrinsic enzymatic activity. Integrins clustering leads to the rapid recruitment of FAK to the focal adhesion complex and its concurrent phosphorylation on tyrosine (Parsons and Parsons, 1997). FAK is "activated" by its phosphorylated on tyrosine 397 (Tyr397), a major site of phosphorylation (Schaller et al. 1994). This correlates with increased catalytic activity of FAK (Calalb et al. 1995; Lipfert et al.

1992), and also creates a high affinity binding site recognized by the SH2 domain of Src family kinases and leads to the recruitment and activation of Src through the formation of a bipartite kinase complex. (Schaller et al.1994). Activation of the FAK-Src complex is central to regulation of down-stream signaling pathways that control cell spreading, cell movement and cell survival. The process of cell adhesion to the ECM has been studied by seeding cells onto an ECM-coated substratum in culture (Vlodavsky ET AL.1980) . These analyses contributed to the elucidation of the process of the initial attachment of cells to the ECM and the formation of integrin-mediated cell adhesion structures. However, cells must also detach from the ECM during migration, and the mechanism and regulation of the disassembly of cell adhesion structures is less well studied. (Vlodavsky, and Gospodarowicz. 1980): The stimulation of the FA complexes formation improves the adhesion of cells to the ECM, giving rise to cells with a flattened morphology. On the contrary, the FA destabilization reduces the adhesion to ECM and gives rise to a spherical shape, non-adherent cells (Fig.8).

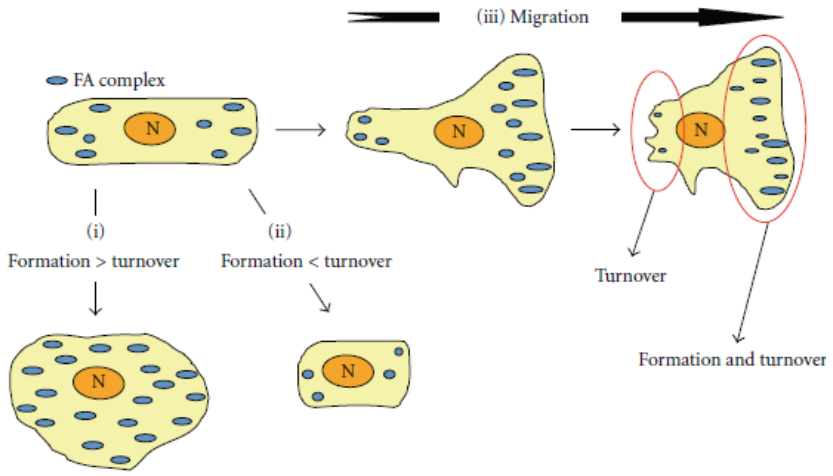


Figure 8:Training and turnover of focal adhesions. Training and turnover is crucial for cell adhesion and cell migration. Adapted from M. Nagano et al; 2012.

During the cells migration on a substrate, the focal adhesions bind strongly the matrix so as to generate the necessary forces to pull the cell body forward. Subsequently, the cells must release the ECM, so as to continue the movement of the cells. As such, the directional migration of the cell requires continuous training of FAs to the front edge of the cell body and the release of this tie in the back. The integrins clustering is the initial step of cell adhesion and is stabilized to form FAs linking actin stress fibers in a process regulated by Rho / ROCK. In contrast, the extension of the microtubules to the FAs triggers their disassembly and induces the subsequent internalization from the cell surface integrin. Therefore, the formation and turnover of FAs are regulated by

different mechanisms. Although the fate of the internalized integrins has not yet been elucidated, several studies have reported that once internalized, integrins are predominantly recycled back to the plasma membrane, although a fraction of α5β1 integrin has been shown to traffic to lysosomes for degradation during migration. Following endocytosis, integrins travel to early endosomes from which they can either be returned directly to the plasma membrane in a Rab4-dependent manner or further trafficked to the perinuclear recycling compartment (PNRC) before recycling through Rab11-dependent mechanis (Sejeong Shin et al. 2011).

Among protein scaffold the paxillin plays an important role in the focal adhesion. Its name, derived from the Latin paxillus, a stake or peg, is consistent with its proposed function in linking actin filaments to integrin cell adhesion sites. Paxillin is one of the first protein that is found in focal adhesions rising to the front edge of the cell, where it is quickly arranged (Digman et al. 2008), suggesting an important role in promoting the assembly of accessions and in defining their molecular composition. Many authors report that during the formation of new focal adhesions paxillin is particularly enriched at the periphery of these small adhesions point (Zimerman et al. 2004) where paxillin seems to interact with vinculin, providing a link with the cortical actin at the leading edge of the cell.

Alpha-actinin is a major component involved in microfilament stabilization and in linking microfilaments to adherens junctions, influencing microfilament organization and/or cell adhesion and motility. In addition to its structural role as a crosslinker of actin filaments, α-actinin is known to serve as a platform for a number of protein-protein interactions within the cytoskeletal organization (reviewed in Otey and Carpen. 2004). Evolutionary analysis, in fact, suggests that this protein best resembles the ancestral proteins of the spectrin superfamily, which is characterized by ubiquitously conserved spectrin repeats (SRs) and found in a large variety of taxa (Virel and Backma. 2004).

Recent study suggest new roles for this protein, played not at focal adhesion or adherens junctions level, but in the nucleus. Zhao X et al. (2015), reported α-actinin 4 involved in the NF-κB transcriptional regulation. In particular, interacting wth p65 and p50 in the nucleus, it is recruited to the promoters of NF-κB target genes regulating the expression of genes encoding pro-inflammatory cytokines such as IL-1β and IL-8,3. chemokines and adhesion molecules In addition, by mass-spectrometry Khotin MG et al, (2009) have detected in nuclear complexes with alpha-actinin-4, protein such β-actin, α- and β-tubulins, ribonucleoprotein which regulates splicing, peroxiredoxin-1, which is involved in oxidative stress, and glycolytic enzyme D-3-phosphoglycerate dehydrogenase, suggesting a possible role in the transcription and splicing processes.

1.7 Tight junctions

Tight junctions (TJ) govern the permeability of epithelial and endothelial cells and are the most typical structures of these cell types (S. Tsukita et al 1999). They are regions where the plasma membrane of adjacent cells forms a series of contacts that appear to completely occlude the extracellular space thus creating an intercellular barrier and an intramembrane diffusion fence (V. Wong, et al. 1997). Briefly, the TJ has a characteristic structure, appearing as discrete sites of fusion between the outer plasma membrane of adjacent cells. TJ completely circumscribe the apices of the cells as a network of intramembrane fibrils appearing as what is generally described as a series of "kissing" points. The molecular components of the TJ have been extensively investigated (A.S. Fanning et al.1999) and could be reasonably separated into 3 regions: (i) the integral transmembrane proteins — occludin, claudins and junctional adhesion molecules (JAM), together with other CTX family members; (ii) the peripheral or plaque anchoring proteins, often containing PDZ motifs — zonula occludens (ZO)-1, -2, -3, MAGI-1 etc.; and (iii) TJ-associated/regulatory proteins — α-catenin, cingulin etc.(fig. 9)

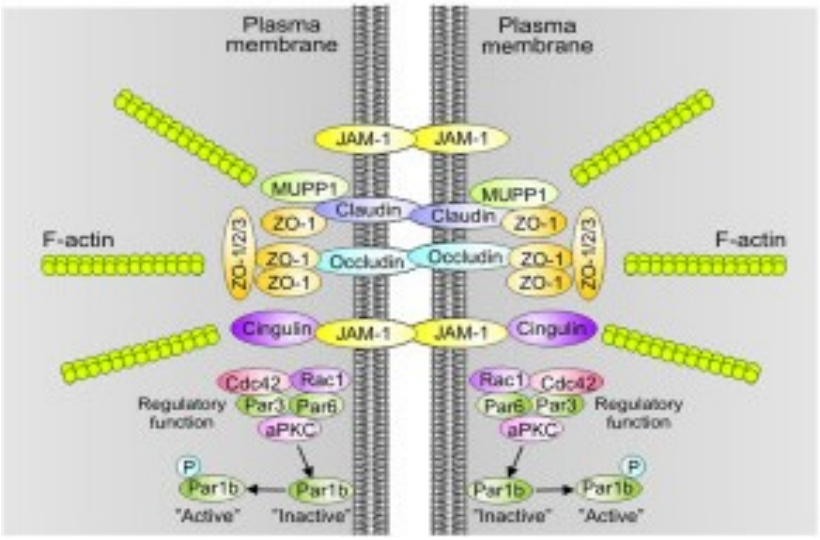


Figure 9:Tight junctions (TJs)structure. TJs contain at least four major groups of transmembrane proteins: the JAMs, claudins, occludin and a number of cytoplasmic peripheral proteins. While the transmembrane proteins mediate cell-to-cell adhesion, the cytosolic TJ complex connects to different factors (e.g. ZO-1/-2/-3, MUPP1 or cingulin) that link the involved transmembrane proteins to the actin-cytoskeleton. (Modified by Backert et al. 2013)

In epithelial cells the TJ prevent cell dissociation (F. Hollande et al. 2001) and their expression may be modulated by growth factors, cytokines, regulatory mechanisms or promoter methylation. Regulatory mechanisms may be via the suggested pathway of the epithelial–mesenchymal-transition (EMT) as the process of acquisition of an invasive phenotype by tumors of epithelial origin

can be regarded as a pathological version EMT (Martin et al. 2005) In, fact TJ determine epithelial cell polarity and disappear during EMT.
The main functions ascribed to the TJ is to seal the intercellular space and to ensure the separation of apical and basolateral fluid compartments of epithelia and endothelia. In this way they help to define apical and basolateral membrane domains of these polarized epithelial and endothelial cells. In addition TJ molecules act as intermediates and transducers in cell signalling, thus playing a role in the processes of cell differentiation, cell growth and proliferation. They act as cell–cell adhesion molecules and function as a barrier to cell migration. Successful assembly and maintenance of the TJ is accomplished by anchorage of the transmembrane proteins by the peripheral or plaque proteins such as ZO-1 which act as a scaffold to bind the raft of TJ molecules together and provide the link to the actin cytoskeleton and the signalling mechanism of the cell
It is becoming increasingly clear that the development of human cancer is frequently associated with the failure of epithelial cells to form TJ and to establish correct apicobasal polarity (I.J. Latorre et al.2005). An increasing number of studies have shown that numerous TJ components are directly or indirectly involved in cancer progression including ZO-1, ZO-2, claudin-7, claudin-1 and occludin. Highly differentiated adenocarcinomas provide an important insight regarding the use of TJ molecules as possible prognostic indicators and future targets for therapy. For example, in colorectal cancer it has been demonstrated that low expression levels of claudin-1 and ZO-1 were directly associated with higher tumor grade (Tracey A. Martin et al. 2009).

1.8 Adherens junctions

Adherens junctions (AJ) perform multiple functions including adhesion of the cell membrane to adjacent cells, anchoring of the cytoskeleton to the plasma membrane, cytoskeletal remodeling, regulation of the local stability of membrane and intracellular trafficking and transcriptional regulation. They are responsible for the formation and maintenance of tissues and organs in multicellular organisms. The core of the AJ includes interactions among transmembrane glycoproteins of the classical cadherin superfamily, such as E-cadherin and N cadherin, and the catenin family members including p120-catenin, β-catenin, and α-catenin. Together, these proteins control the formation, maintenance and function of AJ. Classical cadherins are Ca^{2+}-dependent adhesion proteins and have five characteristic extracellular cadherin (EC) repeat domains, that represent critical components of many intercellular junctions. These domains form trans-cadherin interactions between neighboring cells and initiate weak cell-cell adhesion and formation of the AJ (Halbleib JM et al.2006) (fig.10).

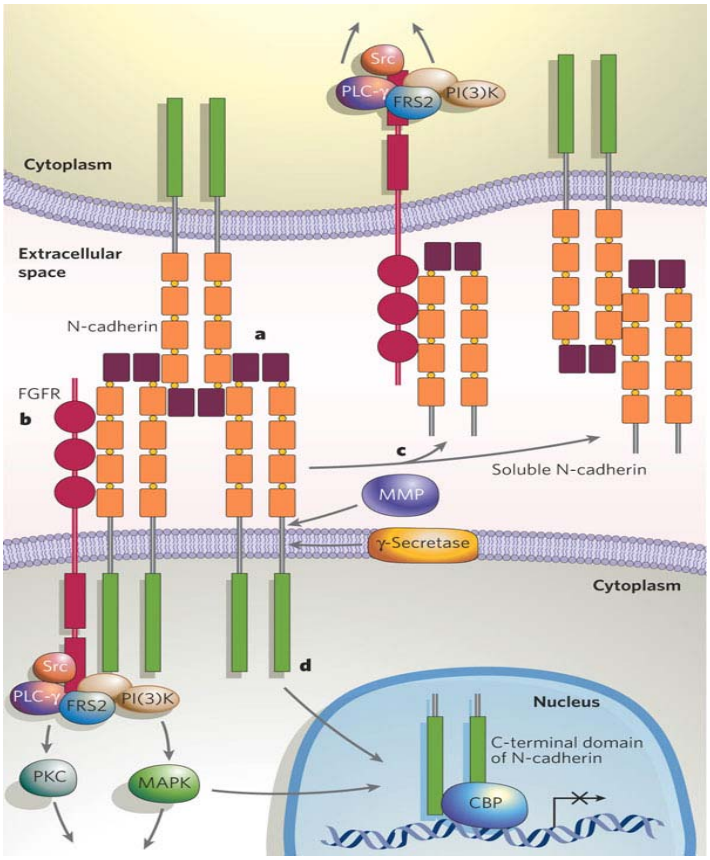


Figure10:N- cadherin in adherens junctions. The cadherin superfamily are characterized by an extracellular domain with multiple, highly five cadherin repeats that represent Ca2+-binding sites, critical components of many intercellular junctions(from Gerhard Christofori 2006)

The binding of Ca^{2+} to each EC domain is required for the correct conformational organization of the cadherin extracellular domain (Pokutta S, et al. 1994) and this association undergoes homophilic interaction with that of cadherins present on the apposed cells. In fact, the interaction of calcium ions with these sequences controls the conformation of the extracellular domain (Pokutta et al. 1994), switching its adhesive function "off" and "on.

AJ are important during development, playing a central role in cell sorting and compartmentalization (Brasch J et al. 2012), and they are also ubiquitously expressed throughout the adult human body. During normal development and in pathological contexts (such as cancer), opportunistic cell–cell contacts either are self-reinforcing, leading to the formation of stable intercellular junctions, or induce cell-contact instability, leading to cell–cell intercalation or even cell migration. Despite their importance, the mechanisms leading to these opposite cell responses are only partially understood.

E cadherin is the main cadherin which induces the formation of stable intercellular adherens type junctions in epithelial cells (Gumbiner et al. 1988; Me`geet al. 1988). However, N-cadherin, which is mostly expressed in neural,

endothelial, cardiac and skeletal muscle cells, in colorectal adenocaricinoma (RKO cells), triggers a wide range of cell responses, as well. The molecular and cellular mechanisms leading to this variety of cell responses are not currently known, but they might involve differential recycling (Kawauchi et al. 2010), anchoring of cadherins to actin networks (Giannone et al. 2009), co-activation of growth factor receptors (Hazan et al. 2004) or other signaling pathways (Ratheesh et al. 2012) that regulate the stability of adhesion complexes, organization of the cytoskeleton and cell polarity.

2. AIM OF STUDY

Hypomorphic mutations of the human *DKC1* gene cause a congenital disease (X-DC), pointing out that the gene is involved in essential functions.

DKC1 is in fact highly conserved and implicated in a wide and disparate spectrum of basic and essential cellular processes, such as ribosome biogenesis, pseudouridylation of cellular RNAs,regulation of IRES-dependent translation,nucleo-cytoplasmic shuttling, DNA repair, stem cell maintenance, telomere integrity and snoRNA-derived miRNAs production. While the list of possible gene functions progressively increased, the underlying mechanisms remained to be fully elucidated.

To characterize in more detail the effects triggered by *DKC1* loss of function, I focussed my research activity on the generation of a stable cellular model, not yet available, that could allow to reduce gene expression upon inducible RNA interference (RNAi). In addition to be stable and reproducible, the system was also aimed at silencing the most abundant dyskerin isoform without affecting the expression of the alternatively spliced isofom 3, which is characterized by retention of intron 12 and by a cytoplasmic localization. Once setted-up the system, I finalize subsequent experiments to characterize the main effects triggered by dyskerin depletion. One of the main challenge to understanding the molecular basis of the X-DC is that of establishing if the disease must be considered mainly a telomerase disorder, or telomere deficiency is just one of the consequences of perturbed H/ACA snoRNP functions. I thus focussed my analyses on the effects triggered by dyskerin depletion well before telomere erosion, in order to define the spectrum of telomere-independent outcomes. Intriguingly, I found that one of the main outcome was that of affecting cell adhesion. This finding pushed me to concentrate subsequent experiments on the role played by this protein at the plasma membrane, so I defined its interactions with component of focal adhesions and of tight and adherens junctions. The results obtained revealed a novel and unexpected biological role played by dyskerin in cell adhesion and motility.

3 MATHERIALS AND METHODS

3.1 Design and cloning of short hairpin RNAs.

shRNAsequences were ordered as oligos (Sigma) and cloned into pLKO-Tet-On (Novagen). The oligos were: Oligo Forward 5' CCGGTATGTTGACTACAGTGAGTCTCTCGAGAGACTCACTGTAGTC AACATATTTTT3'Oligo Reverse 5' AATTAAAAATATGTTGACTACAGTGAGTCTCTCGAGAGACTCACTG TAGTCAACATA 3' Oligos were annealed and ligated in pLKO-Tet-On digested with AgeI and EcoRI enzymes (promega). Then competent Stbl4 E. coli cells (Invitrogen) were transformed with ligation reaction products. Plasmidic DNA from some positive clones was sequenced to confirm the identity of shRNA construct.

3.2 Cell culture, transfection, and selection.

To generate a stable cell line, transfections of RKO cells were performed using 3µg pLKO-Tet-On-shDKC1 and 12 µl of Metafectene Pro (Biontex), following manufacturer's instructions. Following a 20-day puromycin selection (750 ng/ml, Sigma) some clones were collected and grown in DMEM (Dulbecco's Modified Eagles Medium) supplemented with FBS 10% (Clontech, tetracycline free) 2 mM L-glutamine, penicillin 100 U/ml, streptomycin 100 µg/ml and 0,75µg/mlpuromycin at 37°C 5% CO_2 in a humidified chamber.

3.3 Growth curve of doxycycline treated and untreated cells.

To measure cell growth, equal number of dox- and dox+ cells (10^5/dish) were seeded in triplicates in 35mm plates and incubated for various periods of time. Cells were harvested every 24 h up to 4 days and counted by the Trypan blue dye exclusion method.

3.4 Cell proliferation rate assay using MTT.

RKO cells dox+ and dox- were seeded in triplicate at the density of 5×10^3 cells/well in 96-well plates. The day after, cell culture medium was aspirated and, after a washing in PBS, replaced with 100 µL of 0.5 mg/ml MTT solution per well. After 4h of incubation at 37°C in a 5% CO2 incubator, the medium was removed and the precipitated formazan was dissolved in 100 µl of acidic isopropanol. The absorbance was quantified by spectrophotometry at 570 nm using a microplate-reader.

3.5 FACS analysis

Control and doxycycline treated cells at the indicated time were trypsinized, counted, washed three time in phosphate-buffered saline (PBS) and fixed in ice-cold methanol at -20 °C overnight. Cells were then washed twice with cold PBS, counted and rehydrated at density of 10^6 cells/ml by incubation in PBS for 30 min on ice. Subsequently, cells were resuspended in hypotonic solution 0.1% Na-Citrate, 50 μg/mL RNAse, 50 microg/ml propidium iodide and incubated for 30 min in the dark at R.T. The DNA content of the labeled cells was measured using a Becton Dickinson FACS Calibur flow cytometer and data were analyzed using the Cell Quest Pro and ModFit 3.0 softwares.

3.6 Protein extraction and quantification

For protein extraction, cells were washed twice with ice cold PBS, detached from dish by a cell scraper and harvested in 4 ml of PBS. Cells then were pelleted by centrifugation for 5 minutes 1400xg at 4°C. The PBS was eliminated and cells resuspended by gentle vortex in 100 μl of RIPA lysis buffer. After 30 minutes of incubation in ice, the cell lysates were transferred in micro centrifuge tubes and centrifuged for 15 minutes 14000 rpm at 4°C to eliminate insoluble material. The supernatants were transferred in new tubes and stored at -80°C. Protein concentration of extracts was determined by the BioRad reagent following manufacturer's instructions.

3.7 Western blot analysis

Samples were run on a SDS-PAGE under reducing conditions, transferred onto 0.2 mm nitrocellulose filters (Ge Healthcare), and blocked with 5% dried milk protein in TBST buffer. Then membranes were incubated with anti-dyskerin (sc-48794), anti-paxillin (sc-365379), anti-GAPDH (TA 30884), anti-profilin (sc-137236), anti-P21 (TA307018), anti-procaspase 3 (sc-7148), anti-caspase 3(04-439), anti- β-tubulin (E7-s) anti-PARP 1 (sc-7150) anti- integrin β1 (MAB1965) anti-FAK (MA-5644), anti α-actinin (sc-166524) anti-vinculin (ab18058) anti-dyskerin N-terminal (SAB2104539) for 2 hours at RT. The membranes were washed twice for 5 minutes with TBS-t-; then they were exposed to anti-rabbit or anti-mouse (Bethyl) in TBS-t-m for 1 hour. Membranes were washed again in same conditions and exposed to ECL-Plus (Advasta) for 1 minute. The excess of ECL-Plus was eliminated and chemioluminescence was visualized exposing the membrane into the ChemiDoc (BioRad) and images were saved on a PC.

3.8 Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR).

The protocol employed for total RNA extraction and the preparation of first strand cDNA were as previously described (Turano et al. 2008) qRT-PCR experiments were performed using the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) under the following conditions: an initial denaturation step of 95 °C for 10 min, followed by 40 two-step cycles at 95 °C for 10 s and at 60 °C for 60 s. Each 15-µl PCR reaction was conducted in triplicate using 5 ng of cDNA, 6 pmol of each primer and the iTaq Universal SYBR Green Supermix(Bio-Rad). The comparative CT method was used to calculate the gene expression levels relative to the GSH housekeeping gene. PCR oligo primers were selected using Primer3 software (http://frodo.wi.mit.edu/primer3/) and their sequences, were designed on two different exons, in order to avoid amplification of genomic DNA contamination.

DKC1	CTCGGAAGTGGGGTTTAGGT	ACCACTTCAGCAACCACCTC
GSH	GGACTGGCCCTGGGAATT	CCTTCTCTTGAGCAATCAGTA

3.9 Scratch Wound Assay

RKO cells dox+ and dox- were cultured as triplicate in 35 mm plates until reaching confluence. The cells were wounded with a 200 µl sterile pipette tip. To eliminate detached cells, culture medium was removed and wells were washed with PBS. Dox+ and dox- cells were putted on a time lapse support and scratch area was monitored over 24h using live-cell imaging system (Leica).

3.10 Transwell migration assay

We assayed the migration ability of RKO cells using 24-well Millipore transwell chambers with 8 µm membranes (Corning, USA). $2x10^5$ cells were seeded into the upper insert of each chamber in a serum-free medium and 10% FBS culture medium was added to the bottom chambers. After 24 hours of incubation, cells were washed with PBS three times and fixed with 4% paraformaldehyde. Non-migrated cells remained on the upper side of the filter were scraped off with a cotton swab. Cells attached to the bottom side of the membranes were stained with 0.5% crystal violet for 20 min, photographed and counted.

3.11 Adhesion Assay.

Control and doxycycline treated cells were detached using PBS containing1 mM EDTA and were resuspended in a DMEM

adhesion medium containing 0.5% BSA. They were next plated to 30 min in triplicates ($2.0 \bullet 10^5$ cells/well) onto matrigel -coated wells, previously blocked for 60 min with 1% BSA/DMEM. Cells were allowed to attach without spreading (6–7 min). The adhesion medium was discarded, and the attached cells were quantify by MTT assay.

3.12 Immunofluorescence Assay

RKO transfected cells were seeded on glass coverslips in 6-well plates and treated for 48 and 72 h with doxycycline. The cells were fixed with 3,7% paraformaldehyde for 10 minutes, permeabilized in 0.5% Triton X-100 for 15 minutes and blocked in phosphate-buffered saline buffer (PBS) supplemented with 3% bovine serum albumin (BSA) for 30 minutes. After each step, the cells were rinsed in PBS. The cells were then incubated at RT for 1 h with antibodies anti-dyskerin (sc-48794), anti-paxillin (sc-365379), anti-integrin β1(MAB1965) anti-FAK (MA-5644), anti α-actinin (sc-166524), anti-pFAK Y397 (SC-81493), anti-N-caderin (4061S), anti-Zo1(5406S), anti-vimentin (NCL-L-VIM-V9) followed by incubation with Cy3-conjugated goat anti-mouse IgG (Bethyl) or FITC-conjugated sheep anti-rabbit IgG secondary antibodies for 30 minutes at RT. Finally, the coverslips were mounted with Hoechst solutions on glass slides and examined under a fluorescence microscope (Nikon Eclipse E1000) and/or under a confocal microscope (Leica TCS SP5) with the same exposure settings.

3.13 Wheat Germ Agglutinin (WGA) and phalloidin staining

To phalloidin staining, cells were fixed with 3,7% paraformaldehyde for 10 minutes, permeabilized in 0.5% Triton X-100 for 15 minutes and blocked in phosphate-buffered saline buffer (PBS) supplemented with 3% bovine serum albumin (BSA) for 30 minutes. After each step, the cells were rinsed in PBS. The cells were then incubated at RT for 30 min with (phalloidin DyLight 550 *21835*).Finally, the coverslips were mounted with Hoechst solutions on glass slides and examined under a confocal microscope (Leica TCS SP5) with the same exposure settings.
To WGA staining, cells were incubated at RT for 10 min with (wheat germ agglutinin, texas red- conjugate W21405), fixed with 3,7% paraformaldehyde for 10 minutes. Finally, the coverslips were mounted with Hoechst solutions on glass slides and examined under a fluorescence microscope.

3.14 Z-stack analysis

All captured pictures (in RAW format) have been analyzed and processed with ImageJ v1.440 software Z-stack analysis was performed by using STACK>ZProjection and STACK>Othogonal views ImageJ plug-in.

3.15 Coimmunoprecipitation assay

1mg of protein extract was incubated overnight at 4°C with 5 µg of anti-pFAKY397 (SC-81493), anti-dyskerin (sc-48794), anti α-actinin (sc-166524), anti-FAK (MA-5644), antibody preferably under gentle agitation or rotation, before addition of protein G-Sepharose (SIGMA) for 3 h. Beads were washed, resuspended in 40µl of loading buffer, and boiled for 10 min before SDS-PAGE.

4 RESULTS.

4.1 Generation and characterization of stable shDKC1-inducible cell lines

My PhD project has been focussed on the generation of a stable cellular model, not yet available, in which it could be possible to reduce inducibly the expression of the *DKC1* gene, miming the pathological conditions of X-DC patients. For this reason, I cloned in the PLKO-Tet On plasmid a sequence for a short hairpin RNA directed against *DKC1* Isoform 1 mRNA. This vector contains all the necessary components for the inducible expression of shRNA in target cells: a puromycin-resistant gene to permit the selection of transfected clones; sequences encoding the tetracycline repressor protein (TetR) downstream of the constitutive polymerase II hPGK promoter; the sequence encoding a shRNA targeted to *DKC1*. In the absence of tetracycline or its synthetic derivative doxycycline, shRNA expression is repressed by constitutively-expressed TetR protein bound at the Tet-responsive element within the H1 promoter. Upon the addition of tetracycline/ doxycycline to the growth media, shRNA expression is triggered and results in target gene silencing (Wiederschain et al. 2009). After have tested the silencing efficiency of the vector in transient transfection, the RKO colon rectal carcinoma cell line was used for obtain stably transfected clones. This cell line was chosen mainly because patients often develop tumors of the colon and rectum. Following a 20-day of puromycin selection (750 ng/ml, Sigma), independent clones were collected and maintained in media supplemented with tetracycline-free FBS (Clontech) and puromycin. Then cells were stimulated for 24h with different doxycycline concentration and the optimal doxycycline dose of stimulation was determined, by western blot analyses, to be of 400ng/ml. In most experiments, stable inducible RKO clones were cultured in the absence or presence of doxycycline for 96 hours; in some cases, the induction was maintained up to two weeks. Dyskerin silencing was evaluated at both mRNA and protein levels. As shown in Figure 11, the mRNA level, as assayed by quantitative real-time PCR was significantly reduced starting from 24 hrs of induction. Dyskerin protein levels were then evaluated by either immunofluorescence and western blot assays (11 B and C), and a decrease in dyskerin expression of about 50% was observed already after 24h of treatment. Confocal microscopy analyses of RKO cells (control and doxycycline treated) showed disappearance of dyskerin nucleolar accumulation upon antibiotic administration, thus confirming the efficiency of gene silencing.

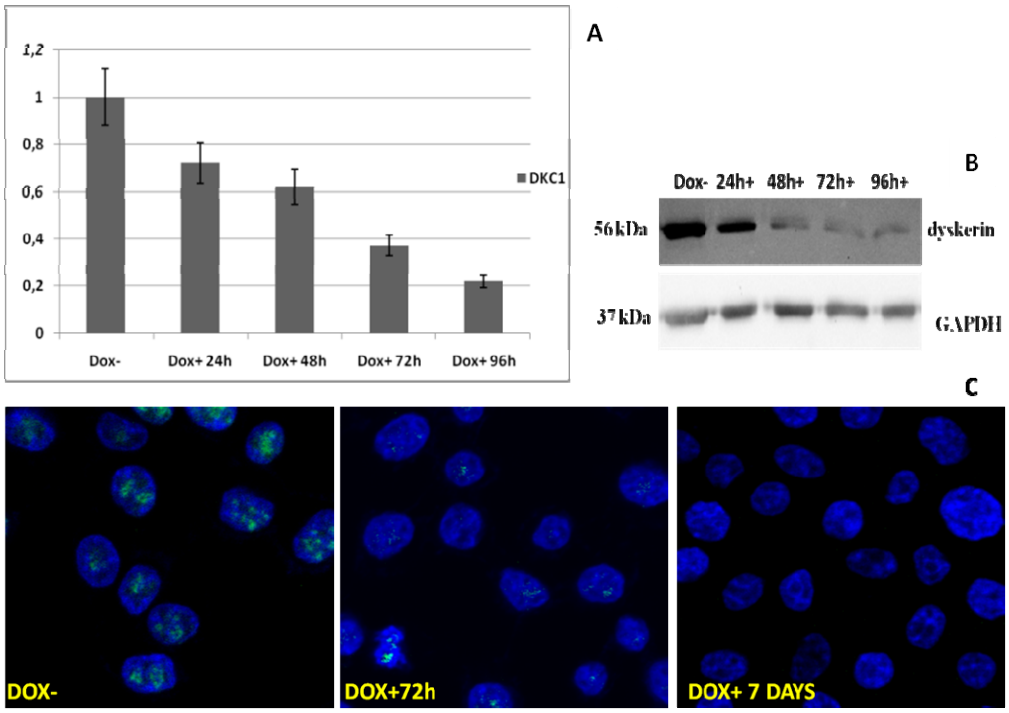


Figure 11:Efficiency of the inducible *DKC1* silencing system. (A) Real time RT-PCR assay shows a reduction of *DKC1* mRNA accumulation following doxycycline administration.(B) Western blot and immunofluorescence analyses (C) confirm a decrease of dyskerin level and its progressive loss from nucleoli.

As previously mentioned in the background section, our research group examined in detail the *DKC1* transcriptional activity, with the aim to establish whether alternatively spliced isoforms could contribute to its functional complexity. In addition of a truncated isoform (isoform 3; Angrisani et al. 2010) we found that an additional alternative transcript,named isoform 6, was efficiently exported into the cytoplasm (Turano et al. 2013). I thus checked the specificity of the silencing system by determining the expression levels of these alternative isoforms by western blots. In these experiments I used an antibody that recognizes an N-terminal dyskerin epitope, and thus is able to identify dyskerin isoforms 1, 3 and 6. As shown in Fig.12, the shRNA silencing RNA (targeted on exons 12-13junction), cannot silence isoform 3; in contrast, both isoforms 1 and 6 are efficiently down-regulated upon doxycycline treatment.

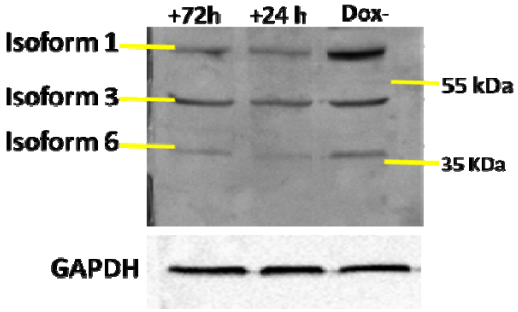


Figure 12: Expression of dyskerin alternative isoforms upon silencing. As expected, isoforms 1 and 6 are significantly reduced after Dox-induced silencing; in contrast, expression of isoform 3 is unaffected. GAPDH levels are checked as internal control.

Once validated the silencing system, I checked whether the nucleolar localization of some components of H/ACA and C/D complexes (such as NOP10, fibrillarin), and that of nucleophosmin, a nucleolar phoshoprotein involved in the ribosome biogenesis, was perturbed upon *DKC1* silencing. As shown in Fig. 13, no significant differences in the localization of the tested proteins was observed between control and doxycycline treated cells, leading to exclude the occurrence of obvious alterations in the nucleolar architecture.

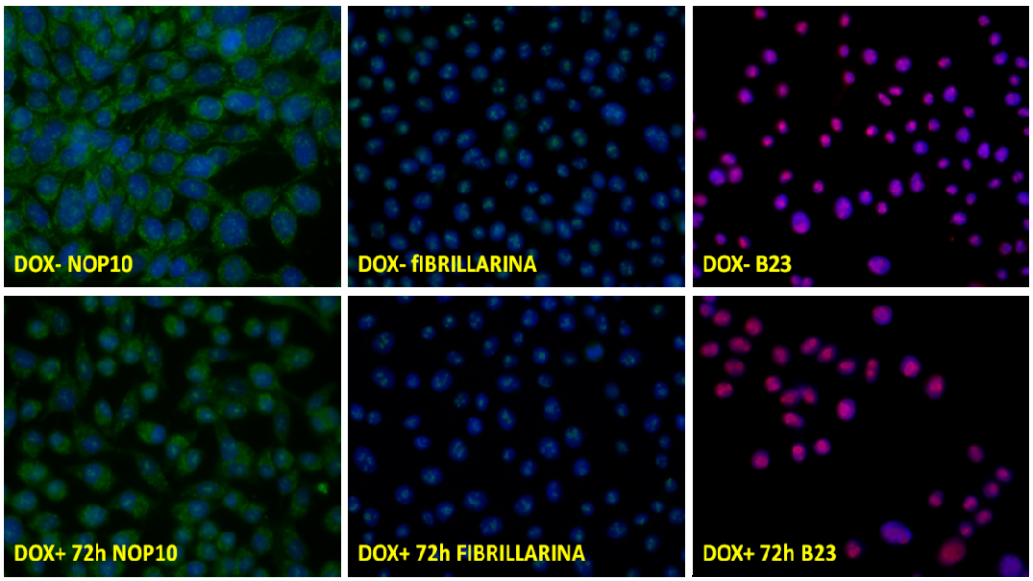


Figure 13:**Immunolocalization of NOP 10, fibrillarin and nucleophosmin in control and *DKC1* silenced cells.** No significant variation is observed between doxycycline-treated and untreated cells.

4.2 Dyskerin depletion alters cell proliferation and cell cycle progression

I thus checked whether gene silencing perturbed cell proliferation, an effect already described upon shRNA transient transfection of prostate cancer cell lines 22Rv1 and Du145 (Sieron et al. 2009) and human osteosarcoma (U2OS) and cervical carcinoma (HeLa) cell lines (Alawi and Lin 2011). I followed cell proliferation by both direct counting of viable cells or by measuring the mitochondrial metabolic rate using MTT[3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide], that indirectly reflects viable cell number. Proliferation of doxycycline-treated cells progressively decreased upon time, with the highest level of growth inhibition observed at 96 hours of silencing and resulting in an about 34% of decrease (see Fig. 14).

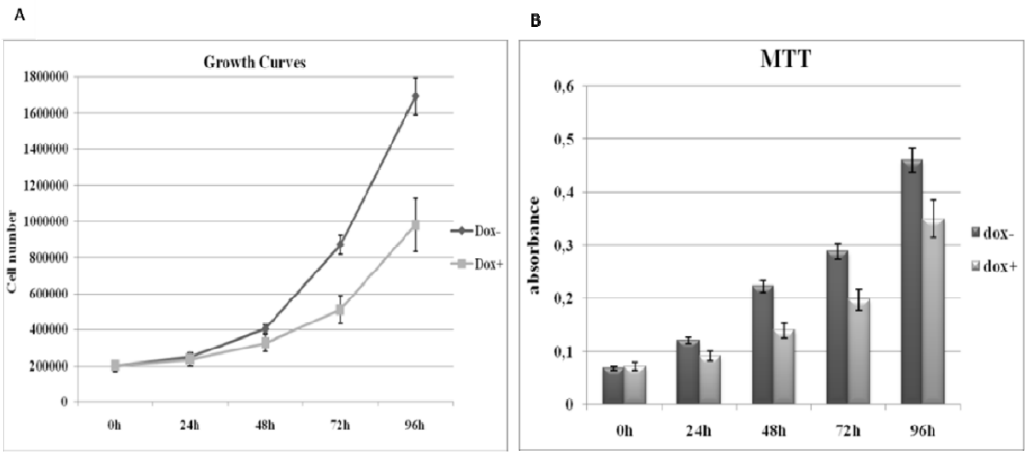


Figure 14:Cell proliferation following doxycycline treatment. Growths curves (A) and MTT assays (B) showthat a decrease of cell proliferation occurs after various time of silencing induction.

Since in literature is reported that U2OS dyskerin-depleted cells and mouse embryonic fibroblasts characterized by the expression of catalytically inactive dyskerin undergo cell cycle arrest at G2-M phase (Alawi and Ping Lin. 2011; Gu et al. 2013), I checked by FACS analysis if the RKO silenced cells were similarly subjected to a G2-M block. As evidenced in Fig.15, I instead found that RKO doxycycline-treated cells show a significant increase in the G1 percentage (42.4% compared to 33, 5% of control cells) after 72h of treatment. This increase is accompanied by a slight reduction in the S-phase percentage (42.8% compared to 46.9% of the control cells) and a consequent reduction in the G2 phase percentage (14.4 % compared to 17.4% of control cells).

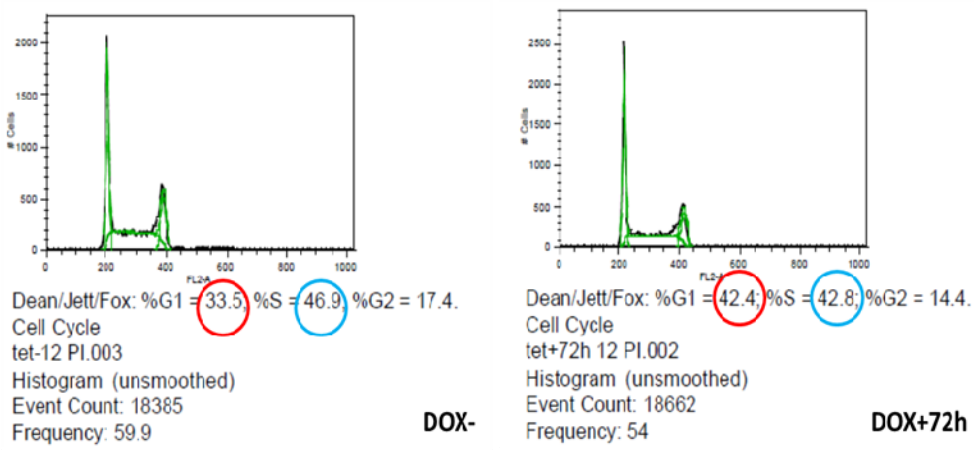


Figure 15: F.A.C.S analysis of control and *DKC1*silenced cells. Dyskerin depleted cells (on the right) tend to accumulate at G1; a reduced number of S phase cells is also observed respect to control (on the left).

To further check this aspect, I tested by Western blotting analysis the expression level of p21, a protein that plays a key role in G1/S cell cycle arrest (Rowland and Peeper. 2006; Abbas and Dutta. 2009). p21 binds cyclin E-CDK2, preventing proliferation of DNA-damaged cells and causing a delay/arrest in the G1 phase (Kang et al.2005). In keeping with FACS results, an increase of p21 levels was observed in dyskerin-depleted cells (Fig. 16).

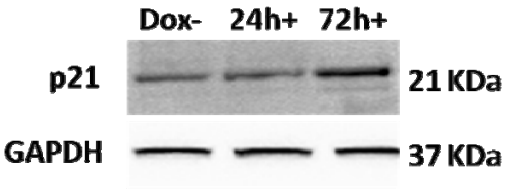
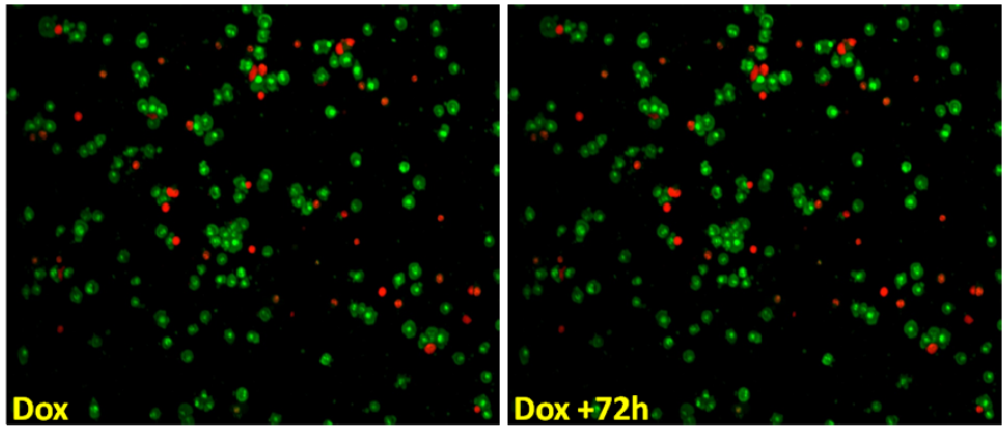


Figure 16. p21 expression upon *DKC1* silencing. Western blot analysis indicating an increase in p21 expression upon *DKC1*silencing.

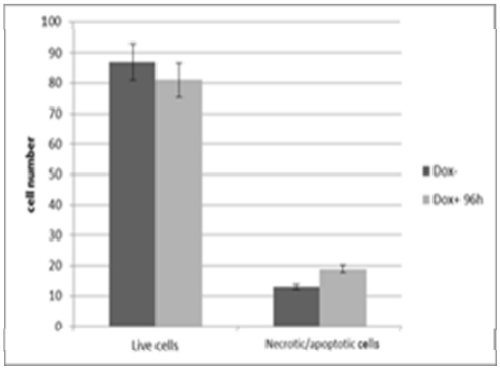
Since cells can enter in an irreversible growth-arrested stage called replicative senescence (Campisi, 2005), I perfomed a β-galactosidase staining, an assay routinely used to detect senescent and aging cells (data not shown) to evaluate this possibility. Cells after 96h of silencing were then stained by β-galactosidase. The results obtained (not shown) ruled out the possibility that senescence induction is a precocious effect of gene silencing.

4.3 Dyskerin depletion does not induce apoptosis

I then wondered whether the observed decrease in cell proliferation could be due to the onset of apoptosis or necrosis processes. These aspects were first followed by acridine orange and ethidium bromide (AO-EB) staining, in order to reveal nuclear changes and apoptotic bodies formation (Ribble et al. 2005). Acridine orange (AO) is a vital dye that penetrates both living and death cells and stains specifically nucleic acids (DNA/RNA). Once bound, its fluorescence has a maximum emission at 525 nm (green emission). In contrast, Ethidium Bromide (EB) penetrates only in cells with damaged membrane. After AO-EB double staining, living cells are colored uniformly in green, while apoptotic or necrotic cells have incorporated EB and thus are colored in orange; in turn, orange apoptotic cells can be distinguished from those necrotic because of the presence of condensed chromatin (Attari et al.2009).This staining revealed that no significant increase in the number of apoptotic/necrotic and living cells. (Figs. 17A e 17B) occurred immediately upon *DKC1* silencing.



17(A)



17(B)

Figure 17: Acridine orange and ethidium bromide staining of *DKC1* silenced cells. A:After staining of Dox-treated and untreated cells**,** green and orange cells were counted . B: Statistical analysis indicates that no significant difference in the percentage of apoptotic/necrotic and living cells occurs between control and silenced cells.

In order to confirm this aspect, I checked by Western Blot analysis the expression of Poly ADP-ribose polymerase-1 (PARP-1) and Caspase 3 (Cas3), two proteins routinely used as apoptotic markers. PARP-1 is a nuclear protein essential for cell function and survival (Cotter TG. 2009; Bhaskara VK et al. 2009) that in apoptotic cells is processed by caspases 7 and 3. This cut results in PARP-1 inactivation and in the completion of the apoptotic process. As showed in Fig.18, no PARP-1 fragment was observed in the silenced cells after 72 hrs of silencing. Similarly, I dind't observed any activation of pro-Caspase 3 (Fig.18), and the same results were obtained after 10 days of Dox induction (not shown). In contrast, in cells induced to apoptosis by puromycin treatment (used as positive control) both PARP-1 and pro-Caspase 3 were efficiently cleaved (Fig. 18). Taken together, these data indicated that the decrease in cell number observed after Dox treatment is not due to apoptosis.

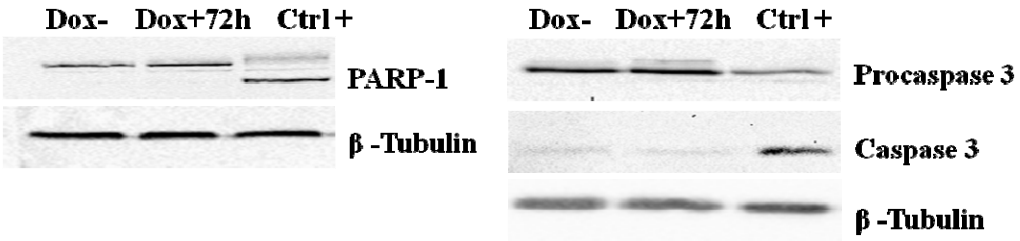


Figure 18: Western blot analysis of apoptotic markers in dyskerin-depleted cells. Cleavage of either PARP-1 and Pro-Cas3 was not observed after 72 hrs of Dox-induction; in contrast, in puromycin-treated apoptotic cells (Ctrl+) PARP-1 and ProCaspase 3 were efficiently cleaved.

4.4 Dyskerin depletion perturbs cell morphology and adhesion.

Following doxycycline treatment, I noticed that some silenced cells start losing their shape**,** becoming round and detaching from the surface, while control cells maintained an epithelial-like, non refractile morphology (Fig.19). To exclude that this effect could depend on doxycycline administration, untransfected RKO cells were treated with increasing doxycycline doses; however, no changes in morphology or cell adhesion was noticed, indicating that the observed effect were strictly related to *DKC1* silencing. Since about 15% of the silenced cells detached from the plate, I collected and stained these cells with trypan blue, used to stain selectively dead tissues or cells, to further exclude the possibility that their detachment was due to cell death. However, these cells proved to be fully viable and, when seeded in Dox- culture medium, rescued normal growth and morphological properties.

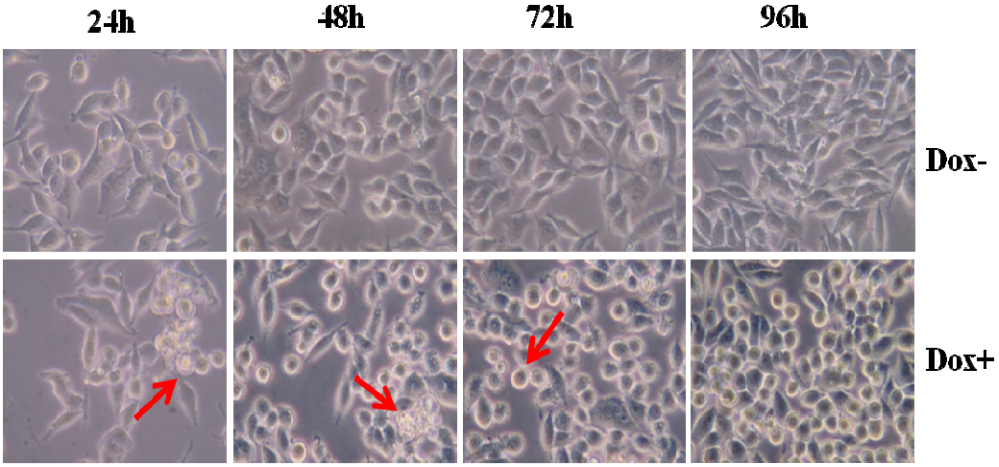


Figure19: *DKC1*silencing induceschanges in cell morphology. Following doxycycline treatment, some *DKC1* silenced cells cells assume a round-shape morphology (see red arrows).

I then stained silenced and control cells with the antibody against Wheat Germ Agglutinin (WGA), a carbohydrate-binding protein that selectively recognizes the predominant sugar residues found on membranes, and with Phalloidin, a dye that binds F-actin, in order to get a preliminary view on cell structure. As shown in Fig. 20, WGA perfectly delineated the cell boundaries and highlighted the round shape of dyskerin-silenced cells, most of which remained isolated, unlike control cells that adhered each other like in a monolayer. Phalloidin staining suggested that reduced actin polymerization took place within the restricted cytoplasmic areas of the silenced cells, implying the occurrence of cytosketal rearrangements. Since the overall picture suggested a possible role of dyskerin incell adhesion, I focused subsequent experiments on cell-cell and cell-substrate adhesion properties of the silenced cells.

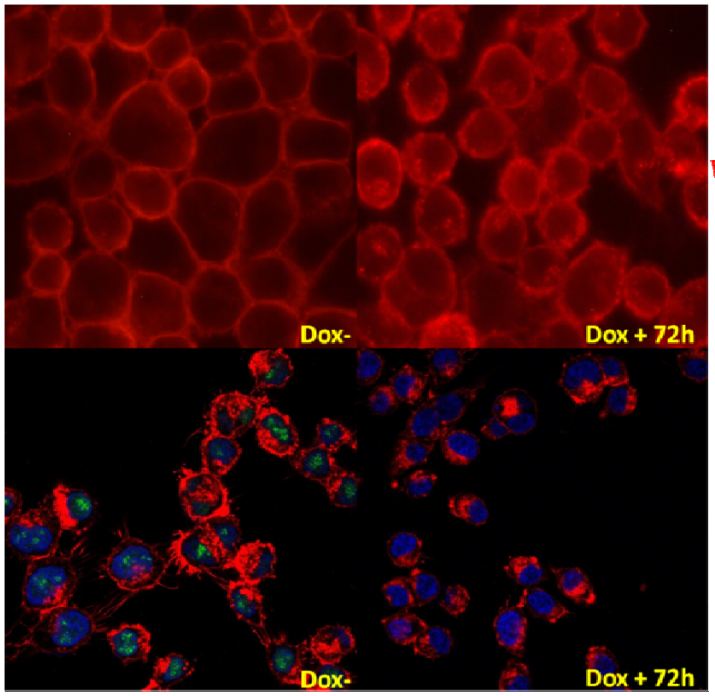


Figure 20: Phalloidin and WGA staining of control and dyskerin-depleted cells. Following *DKC1* silencing, cells acquire a round shape and exhibit a reduced accumulation of F-actin.

4.5 Dyskerin depletion alters cell-substrate adhesion

To check the cell-substrate adhesion properties of *DKC1* silenced cells, an adhesion assay was performed. Silenced and control cells were seeded in plates coated with matrigel (a mixture of laminin, collagen IV and proteoglycan that mimics the basement membrane) or in plates coated with BSA, used as control. After 30 min of incubation, adherent cells were quantified by MTT assay. As shown in Fig.21, the results show that the silenced cells show lower adhesion to extracellular matrix than control cells.

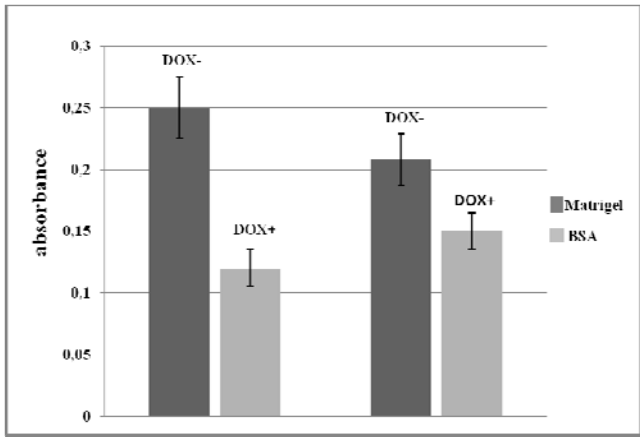


Figure 21: Cell-substrate adhesion assay. In the assay, control and silenced cells were quantified by MTT. The histogram shows that *DKC1* silenced cells exhibit reduced adhesion to the extracellular matrix**.**

Adhesion of cells to the extracellular matrix (ECM) is a key parameter able to regulate cell morphology, migration, proliferation, survival and differentiation

(Gumbiner et al. 1996).Adhesion to ECM is mediated mainly by integrins. Each integrin acts as receptor that recognizes a distinct ligand on the cell surface, while its cytoplasmic domain acts as a platform for the recruitment of scaffold and signaling proteins at the inner surface of the plasma membrane, where structures that mediates tight adhesion to the ECM, called Focal Adhesions (FA), are composed (Hynes, 2002).
I thus analyzed the expression of integrin β1 monomer in the silenced cells. As shown in Fig.22, integrin β1 expression is reduced, particularly at cell baseline. Moreover, I noticed that the protein accumulates in the perinuclear region of dyskerin-depleted cells. This region might correspond to the "PeriNuclear Recycling Compartment" (PNRC), where integrins, once internalized, can be recycled by members of Rab 11 protein family.

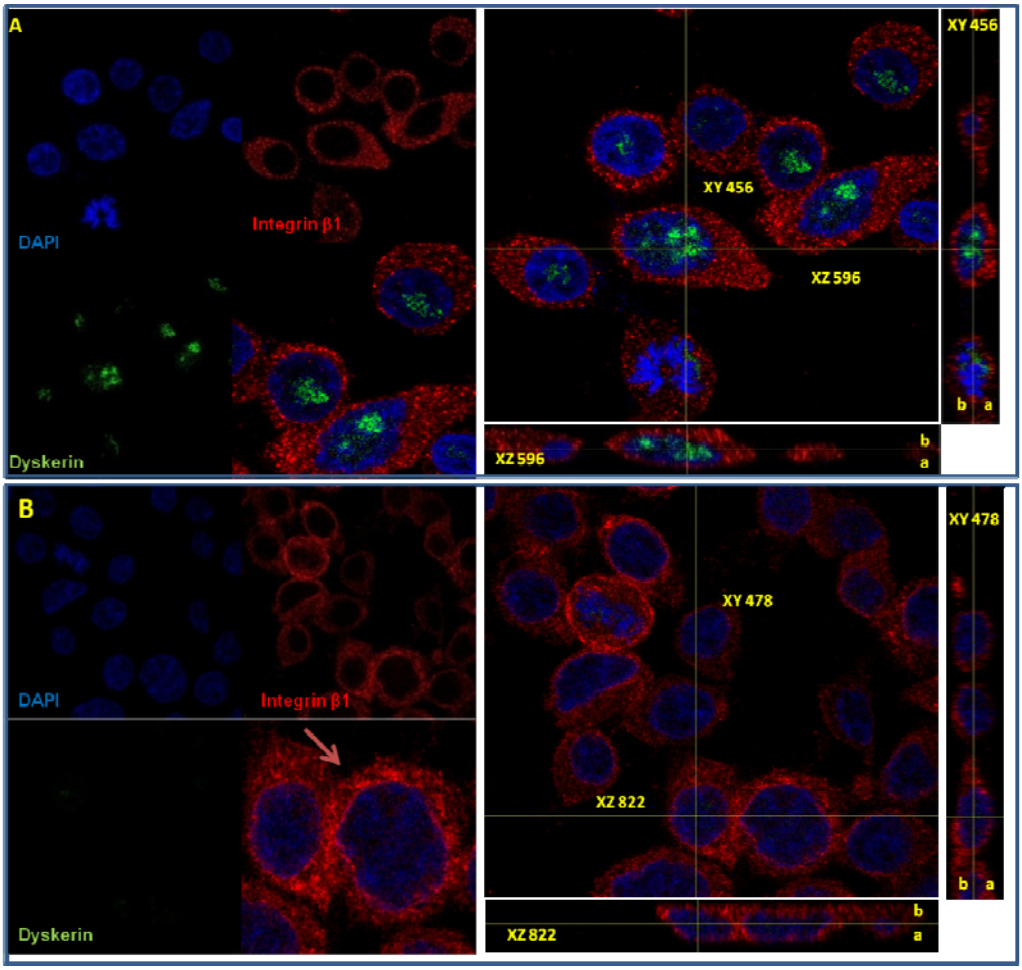


Figure 22: Expression and basal-apical distribution of integrin β1 in control (A) and silenced cells (B).

In keeping with this observation, western blot analysis confirmed that a slight reduction of integrin β1 expression level occurs upon dyskerin depletion (Fig. 23).These results are compatible with a destabilization of Fas, that would

consequently reduce adhesion to ECM and possibly account for the presence of spherical,non adherent cells.

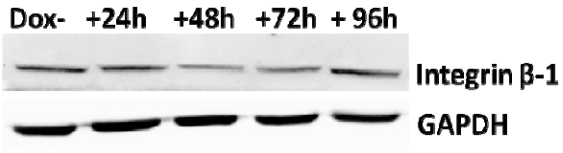


Figure 23: Expression of integrin β1 in the silenced cells. Western blot analyses indicates that integrin β1 accumulation is slightly reduced upon *DKC1* silencing.

To gain further support to this view, I analyzed the expression of the focal adhesion tyrosine kinase (FAK). FAK acts as molecular switch, playing a central role in the signaling cascades mediated by integrins (Mitra and Schlaepfer, 2006).In effect, FAK total levels decreased significantly upon dyskerin depletion (Fig.24).

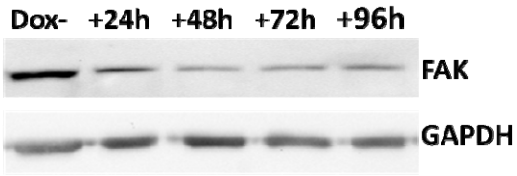


Figure 24: Expression of FAK in the silenced cells. Western blot analyses indicates that FAK accumulation is significantly reduced upon *DKC1* silencing.

Confocal microscope analyses of cells after 48h of *DKC1*silencing confirmed the strong decrease of FAK expression (Fig.25). This reduction may affect normal localization of FAK at the focal contact sites.

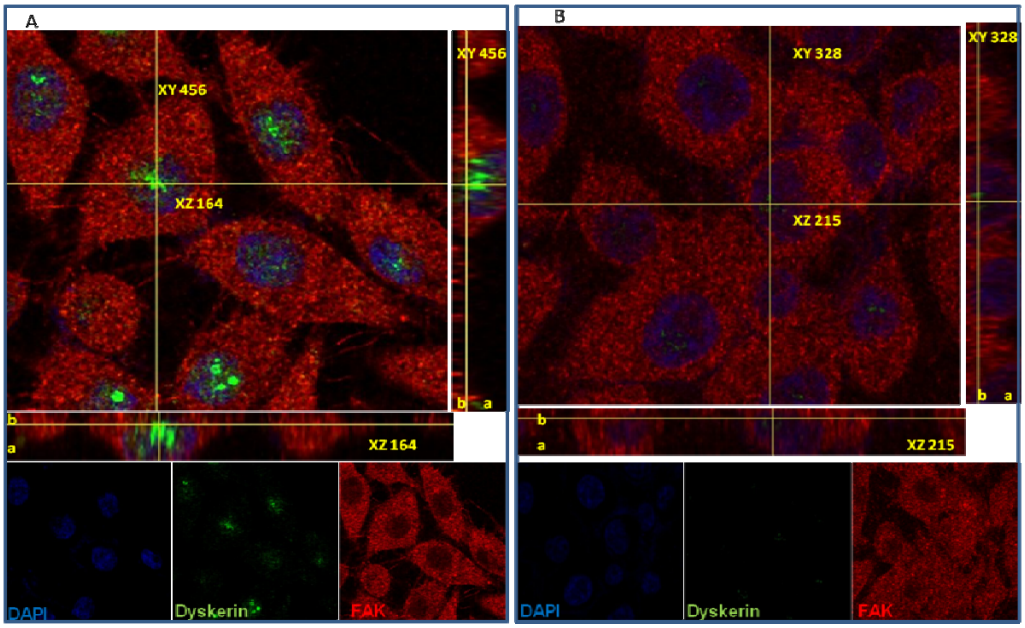


Figure 25: FAK immunofluorescence analysis. Confocal microscopy analysis shows a decrease of FAK expression in dyskerin-depleted cells (B) respect to control cells (A)

Finally,I analyzed the expression of paxillin, an adaptorprotein that integrates adhesion and growth factor-related signals at the interface between membrane and the actin cytoskeleton (Digman et al. 2008).Silenced cells show a decrease of paxillin levels, mainly at the basal level (Fig. 26).

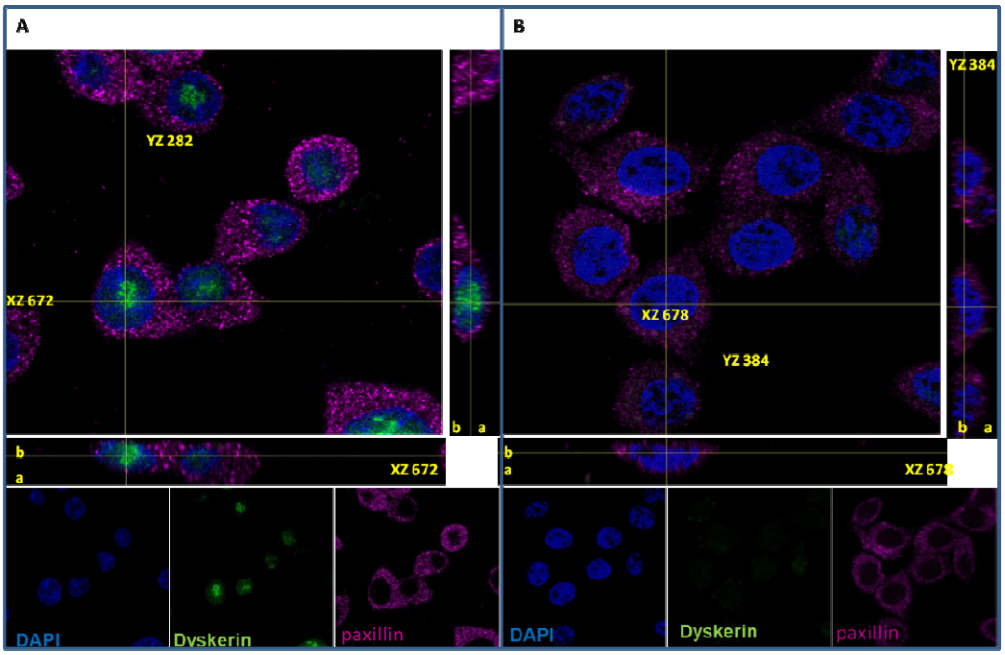


Figure 26.Confocal microscopy analysis of paxillin expression. Immunofluorescence analyses show a decrease of paxillin expression in dyskerin depleted cells (B) respect to control (A).

As shown in Fig. 27, western blot analyses confirmed the reduction of paxillin and its phosphorylated form.

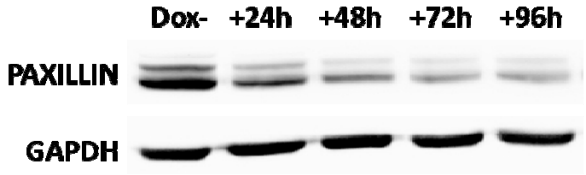


Figure 27: Western blot analysis of paxillin expression in dyskerin depleted cells. Levels of both paxillin and its phosphorylated form are markedly reduced upon *DKC1* silencing

4.6 Dyskerin interacts directly with FAs components

Finally, I asked whether dyskerin could interact directly with FAs components, stabilizing or recruiting them at FA sites. To check this hypothesis, I first perfomed co-immunoprecipitation experiments, analyzing the possible interaction of dyskerin with FAK, and more specifically with its tyrosine 397 posphorylated form. In this approach, I then used not only the previously mentioned antibody able to recognize all FAK isoforms, but also an

antibody that specifically reacts with the tyrosine 397 posphorylated FAK isoform. Intriguingly, co-immunoprecipitation essays showed direct interaction between dyskerin and both the unphosphorylated and phosphorylated FAK isoform(Fig.28)

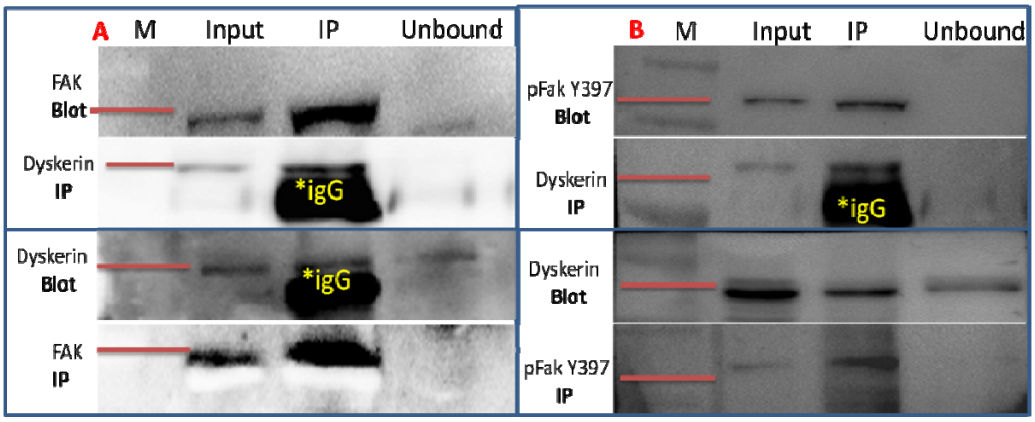


Figure 28:Dyskerin- FAK direct interaction. Co-imunoprecipitation experiments reveal direct interaction between FAK and dyskerin (A), as well as of Y397 p-FAK and dyskerin (B).

Since auto phosphorylation of FAK at residue Tyr-397 occurs upon integrin clustering at the plasma membrane, (Ben-Zion Katz et al. 2003) this result suggested a new and so far unpredicted intracellular localization of dyskerin. Since the dyskerin isoform 1 has been so far considered to be localized exclusively into the nuclei, I wished to further investigate FAK-dyskerin interactions by checking their immuno-colocalization by confocal microscopy.

A

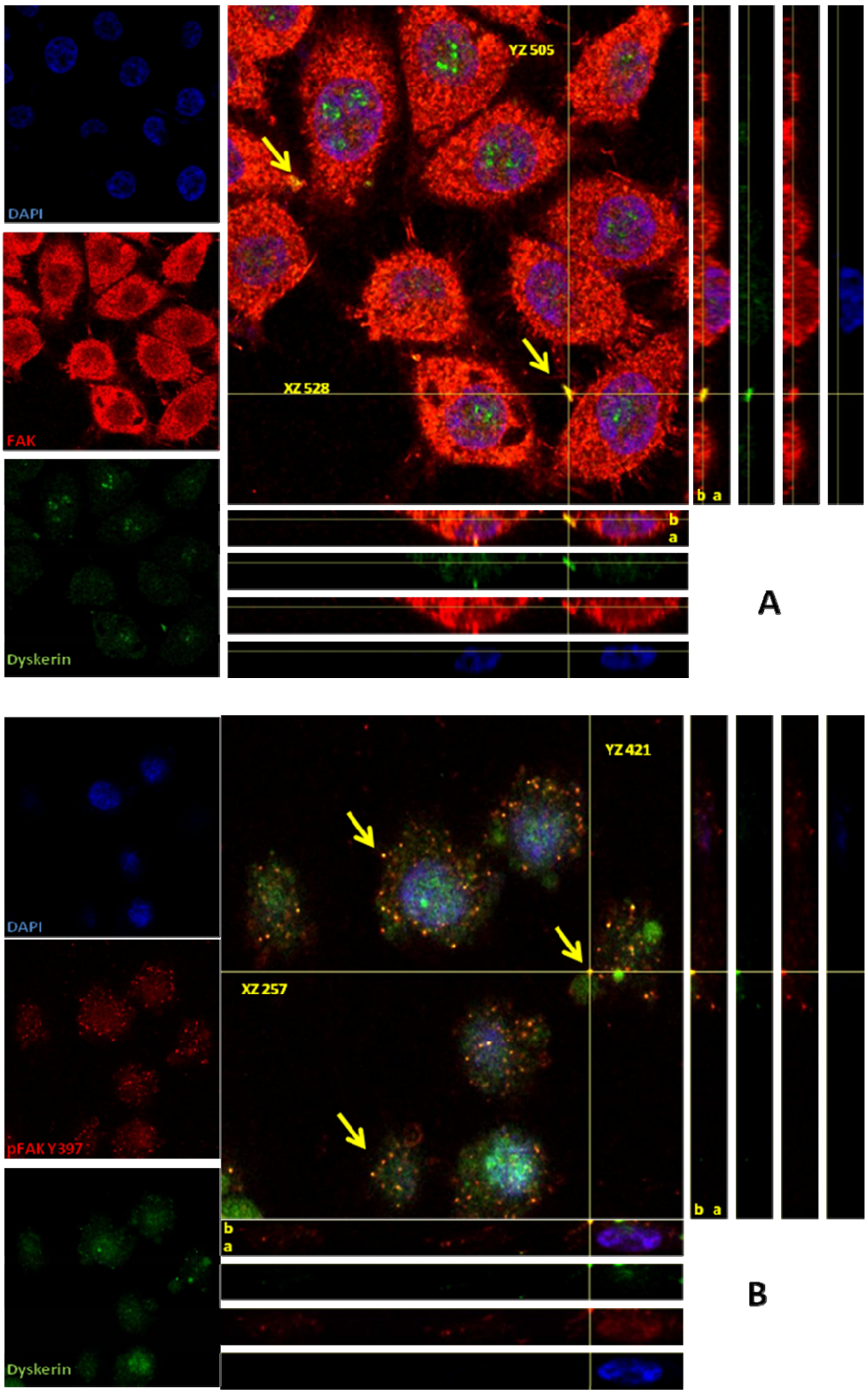


Figure 29: Co-localization between FAK(A), p-FAK and dyskerin (B). Cofocal analysis show that Dyskerin interacts with both FAK isoforms at the plasma membrane.

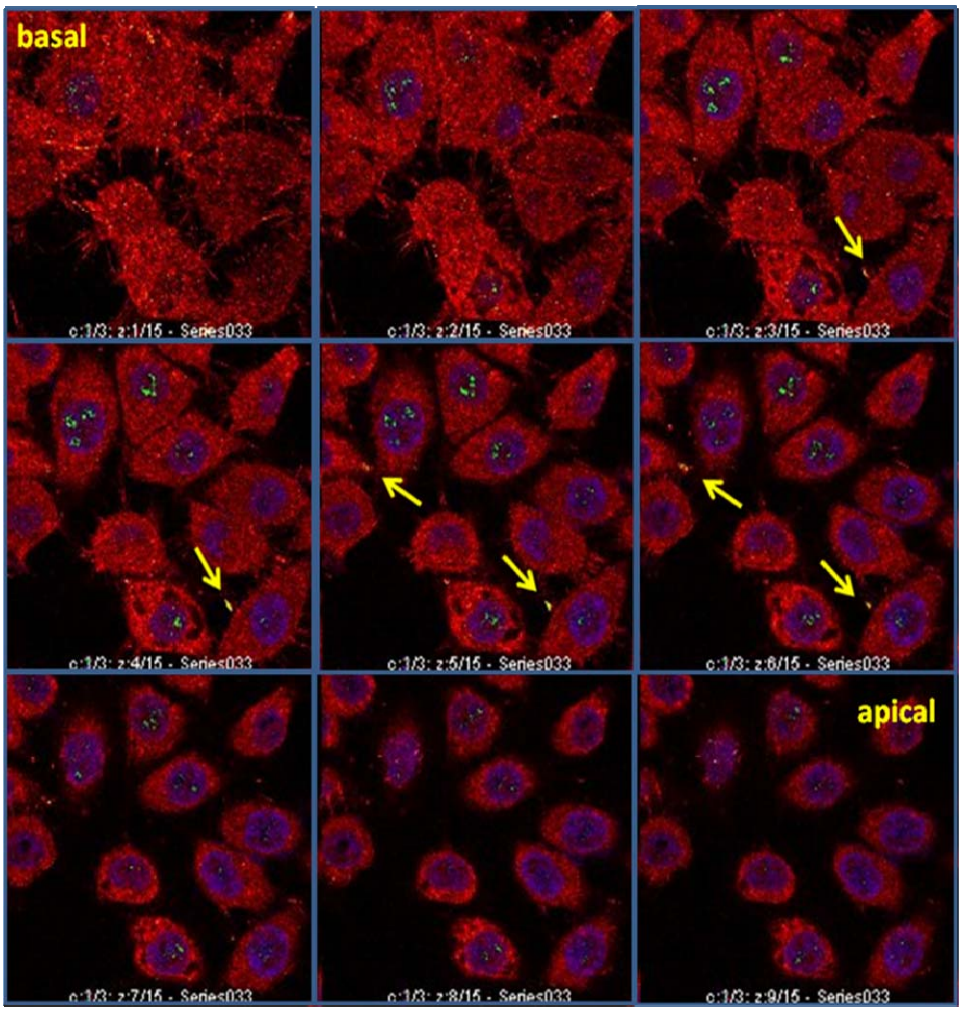


Figure 30:Co-localization of dyskerin and FAK. Detailed view images acquired at different focal planes conferming that the co-localization of dyskerin and FAK(yellow arows)occurs mainly at the basal planes.

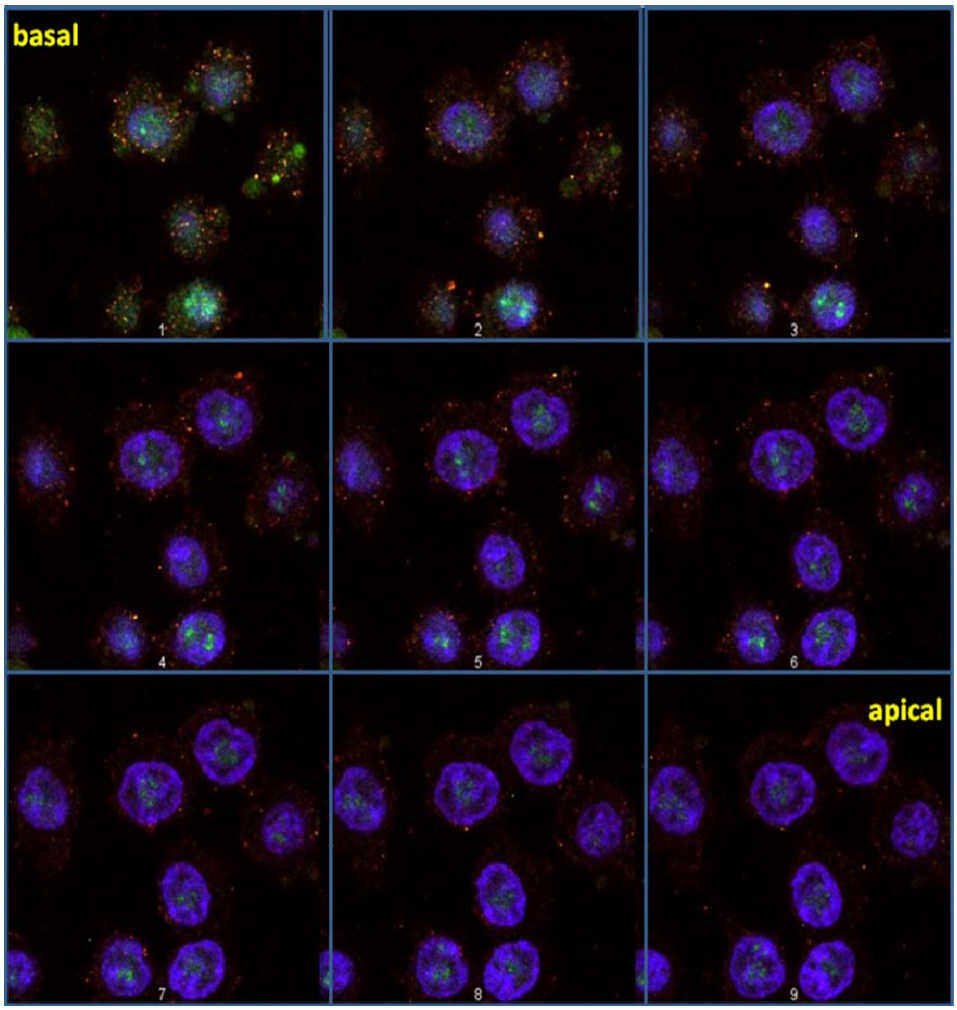
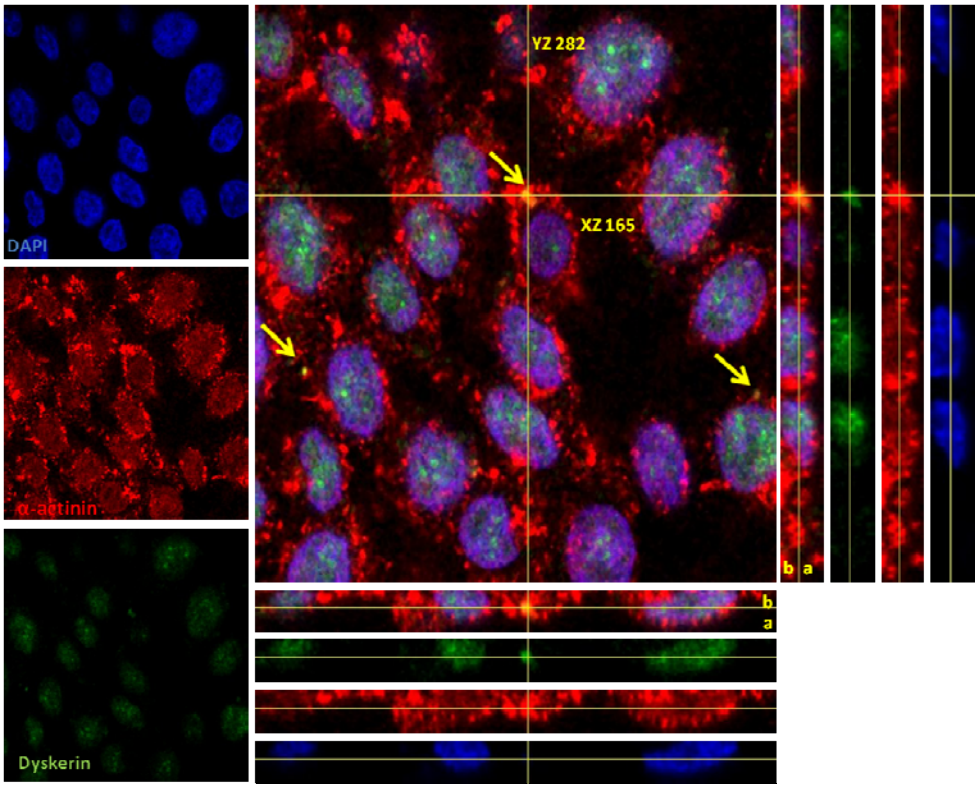


Figure 31:Co-localization of dyskerin and pFAK. Detailed view images acquired at different focal planes conferming that the co-localization of dyskerin and p-FAKoccurs mainly at the basal planes.

Indeed, this analysis confirmed that dyskerin colocalizes with FAK and its specific phosphorylated form on Y397 at cell plasma membrane, at the FAs level.

I also performed co-immunoprecipitation and co-localization experiments in order to check dyskerin/α-actinin potential interaction.Also in this case, both approaches indicate the occurrence of a direct interaction between the two proteins (Fig.32).



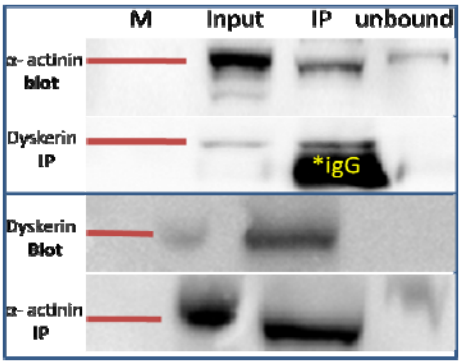


Figure 32:**Co-localization and co-immunoprecipitation of dyskerin and α-actinin**. Both approaches indicate the occurrence of a direct interaction between the two proteins.

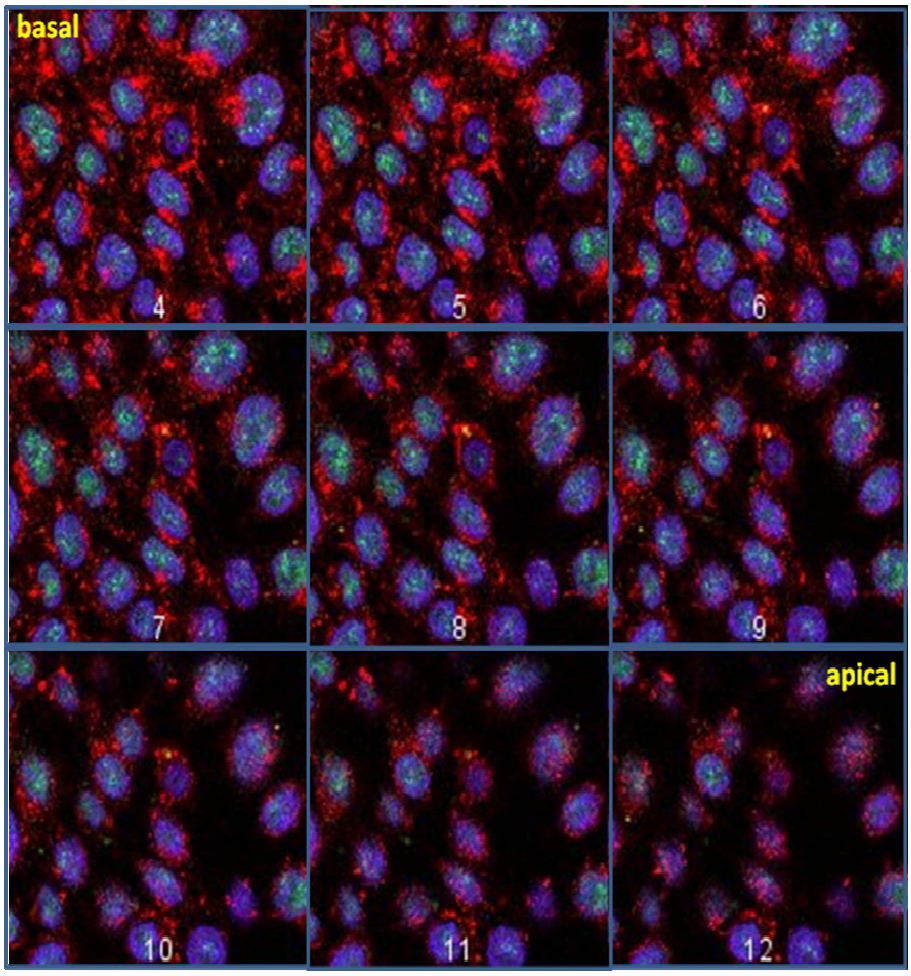


Figure 33: Co-localization of dyskerin and α-actinin .A detailed view of images acquired at different focal planes conferming co-localization of dyskerin and α- actinin at different planes

Conversely, I was unable to evidentiate any interaction between dyskerin and paxillin(data not shown).

4.7 Dyskerin depletion perturbs cell-cell adhesion.

I subsequently focussed on the effects of dyskerin depletion in cell-cell adhesion. RKO cells, although of epithelial origin, express N-cadherin (a marker typically expressed in neural, endothelial, cardiac and skeletal muscle cells) instead of E-cadherin in their adherens junctions (Buck et al. 2007). I thus analyzed the expression of N-cadherin and of ZO1, a protein involved in the formation of thetight junctions of polarized epithelia. Immunofluorescence analysis showed a decrease of N-cadherin at adherens junctions of the silenced cells (Fig. 34); moreover, the protein showed a vesicular-like pattern. The expression of ZO-1,a protein localized at apical cell-cell contact regions (Tsukita et al 1999) similarly decreased upon gene

silencing. However, ZO1 levels fluctuate among the silenced cells, with some of them remaining completely unstained; moreover, the protein often does not mark distinctly the cell boundaries (Fig. 35).

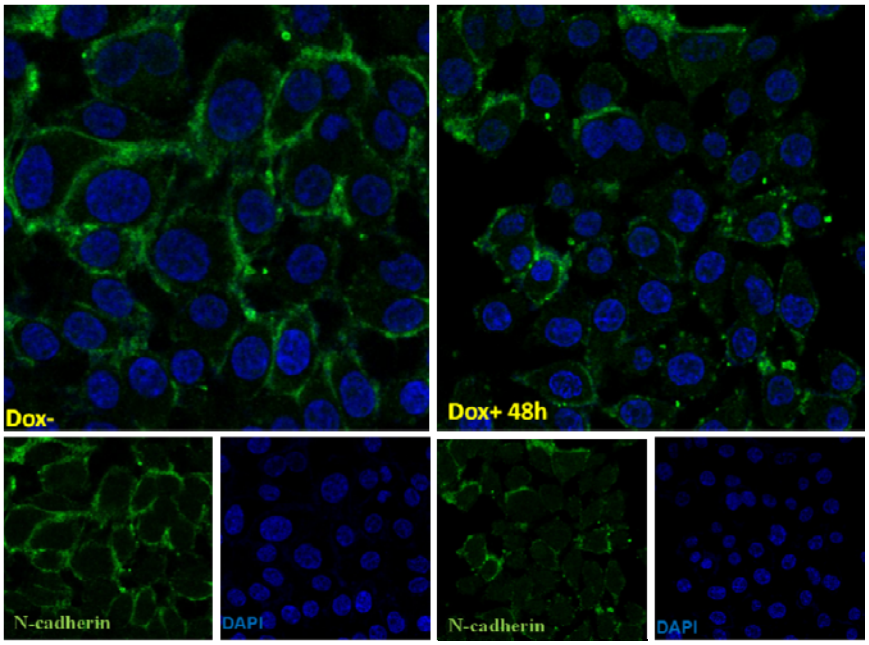


Figure 34: Confocal microscopy analysis of N-cadherin expression in dyskerin-depleted cells. Note that N-cadherin expression is reduced and exhibits a vesicular-like pattern

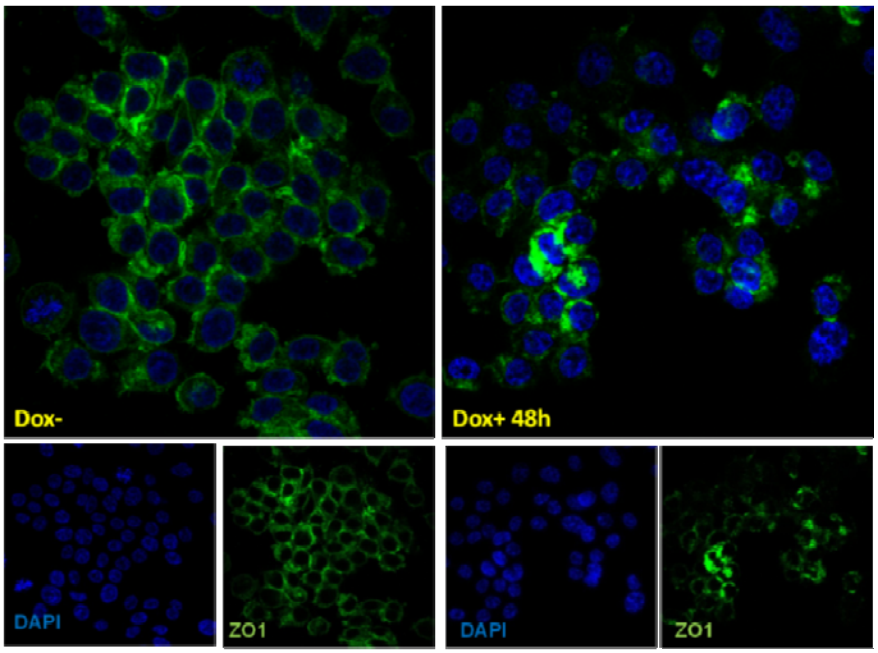


Figure 35: Immunoflorescence analysis of ZO1in dyskerin depleted cells. Note that ZO1 expression is irregularly reduced and it is often absent from cell boundaries.

Finally, I analyzed the expression of α-actinin, a major component involved in microfilament stabilization and in the link of microfilaments to adherens junction (AJs) and FAs components (reviewed by OteyandCarpen. 2004; Sjoblom et al. 2008).Four isoforms of α -actinin are expressed in a tissue-specific manner in human cells. Actinin-2 and -3 are specifically expressed in muscle cells, whereas actinin-1 and -4 are ubiquitously expressed (Beggs et al. 1992; Millake et al. 1989; Youssoufian et al. 1990). Although α-actinin1 and 4 are approximately 84% identical and share 90% similarity in their amino acid sequences,only the isoform 4 is reported to have a nuclear localization (Khotin MG et al.2009; Zhao et al. 2015).

After 48h of *DKC1* gene silencing, I observed by immunofluorescence a neat decrease of α-actinin level at the adherens junctions and an increase of its diffuse localization in the nuclei. In keeping with these results, western blot for analysis of nucleus/cytoplasm fraction confirmed this observation (Fig 36). Since the antibody used in my experiments recognizes both actinin-1/-4 isoforms, its likely that it followed the nuclear accumulation of actinin-4, as observed in several cancer cell lines upon actin depolymerization (Honda et al. 1998).

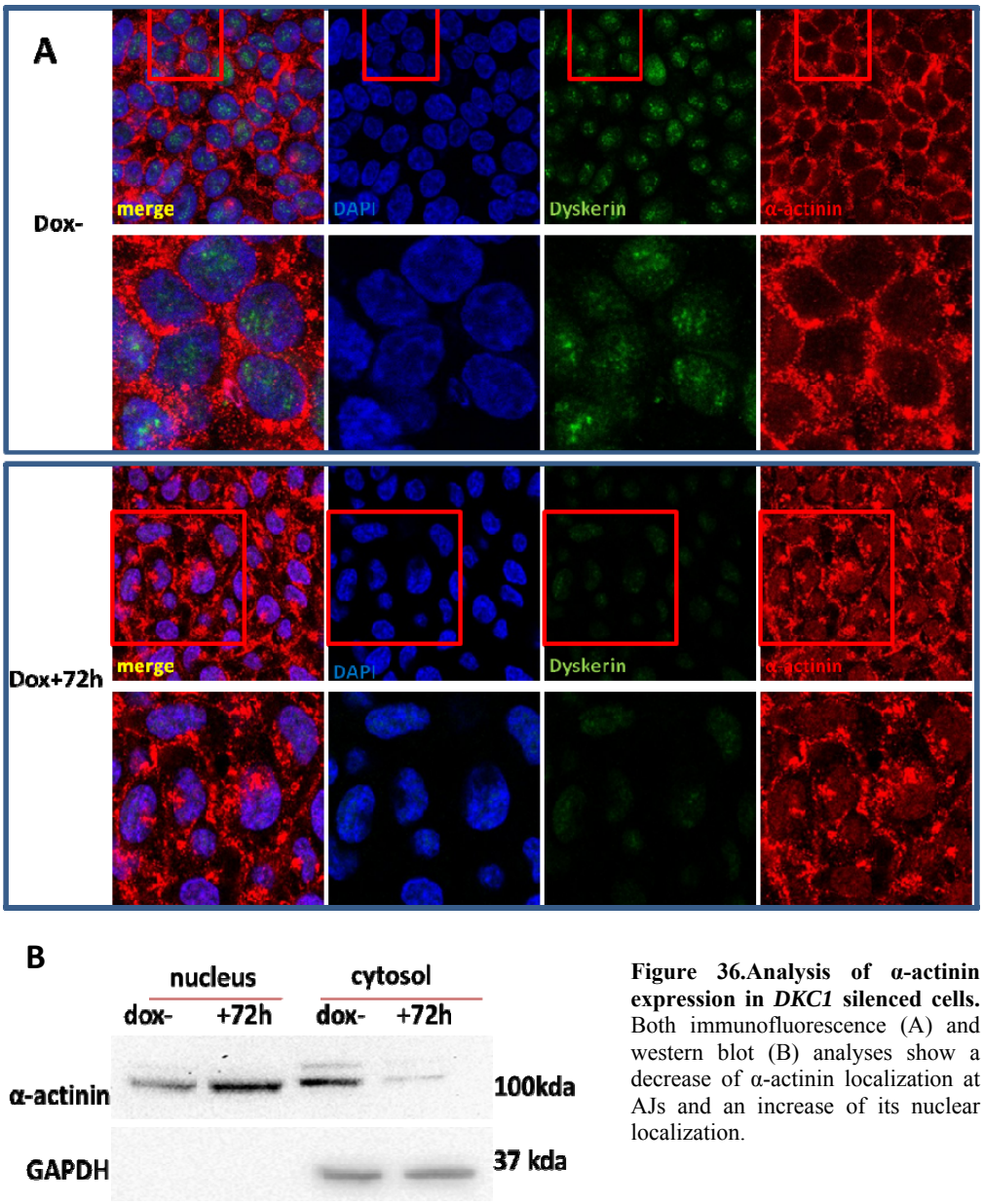


Figure 36.Analysis of α-actinin expression in *DKC1* silenced cells. Both immunofluorescence (A) and western blot (B) analyses show a decrease of α-actinin localization at AJs and an increase of its nuclear localization.

Because of the important role played by actin-binding proteins in the stabilitation of AJs and FAs, I also checked the expression of other actin-binding proteins, such as vinculin, a cytoskeletal protein involved in anchoring F-actin to the membrane (Gingras A et al. 2006) and profilin, a protein involved in the dynamic turnover and restructuring of the actin cytoskeleton (Bae YH at al. 2010). However, the expression level of these proteins remained unvaried following dyskerin depletion (Fig. 37).

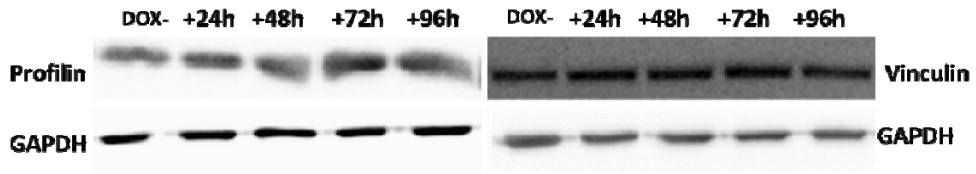


Figure 37. Expression analysis of profilin and vinculin in dyskerin-depleted cells. Western blot assays did not show any significant variation in the level of profilin and vinculin accumulation between control and silenced cells.

4.8 Dyskerin depletion promotes a cell migratory behaviour

A specific characteristic of epithelial cells is their propensity to grow as a layer of extensively communicating cells, which express, at cellular junctions sites, specific epithelial proteins such as cadherin, occludins, claudins, all involved in maintaining of the epithelial layers integrity (Baum and Georgiou, 2011; Dusek and Attardi. 2011; Turksen and Troy. 2011). Epithelial cells are typically polarized, with a basal side attached to extracellular matrix, lateral sides attached to adjacent cells, and an apical side facing the lumen of a vessel. Maintenance of this apico-basal polarity is achieved by the tight junctions, which prevent diffusion of apical membrane proteins (Yeaman et al.1999).Detachment of epithelial cells from their matrix triggers a cellular reprogramming that can result in Epithelia-Measenchimal Transition (EMT). The defining characteristics of EMT are: change of cell morphology, mainly due to loss of cell adhesion molecules;loss of epithelial-specific genes and gain of mesenchymal-specific genes; enhanced migratory capacity of cells (Matise et al. 2012). Since dyskerin depletion induces changes in cell morphology and causes a reductionof cell adhesion, I wished to check also the migratory properties of the silenced cells, I thus performed a migration assay based on the use of a Boyden chamber. This assay consists of a cylindrical cell culture insert, with a defined pore size, nested inside the well of a cell culture plate. Cells are seeded in the top of the insert in serum-free media, while serum is placed in the well below. Migratory cells, that move through the pores toward the chemoattractant below, are then stained and counted. I observed a greater number of cells that cross the insert in the silenced sample compared to control cells, suggesting an increase of cell migration(Fig. 38).

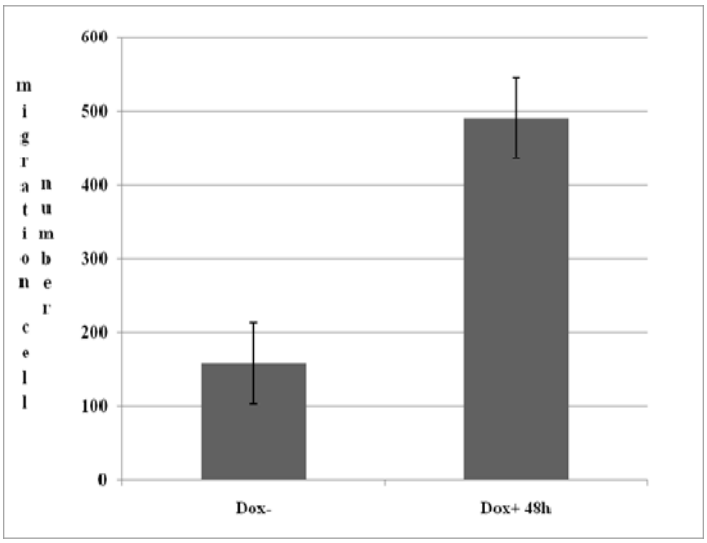


Figure 38: Boyden chamber assay. The migration assay shows an increase in cell migration of about 50% for the silenced cells. The experiment was performed in triplicate; deviation standard of cell counts is indicated by bars.

To confirm the increase in cell migration, I also performed a scratch assay in time lapse, which allowed us to evaluate the cell ability to heal a cut made inside a confluent plate. Migration was followed for 24 hours, i.e. the replication time of RKO cells, to minimize any influence due to variability in cell proliferation. I observed that in the selected time control cells don't cross the cut, while advance at both sides evenly. Instead, the silenced cells cross the cut and advance disorderly (Fig. 39).

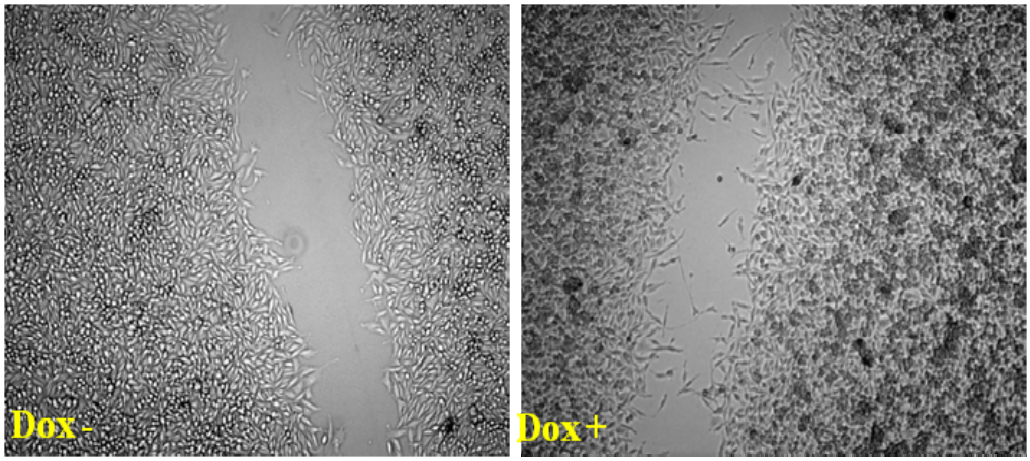


Figure 39: Scratch assay in time lapse. Doxycycline- treated cells cross the cut more rapidly, suggesting an enhanced migratory behavior.

To further investigate the possibility that depletion of human dyskerin could promote EMT, as recently shown in Drosophila (Vicidomini et al. 2015) I preliminarily analyzed the expression of vimentin, a protein that composes intermediate filaments and represents the major cytoskeletal component of

mesenchymal cells.As shown in Fig. 40 ,an increase of this mesenchymal marker noticed in the silenced cells, supporting the view that dyskerin depletion may promote EMT. Worth noting, short filamentous structures termed “vimentin squiggles”are frequently visible in the silenced cells. While vimentin network extend to the cell pheriphery in non-motile cells, squiggles are short fibril that act as motile structures able to altering cell shape and promoting cell motility(Yoon et al.1998).

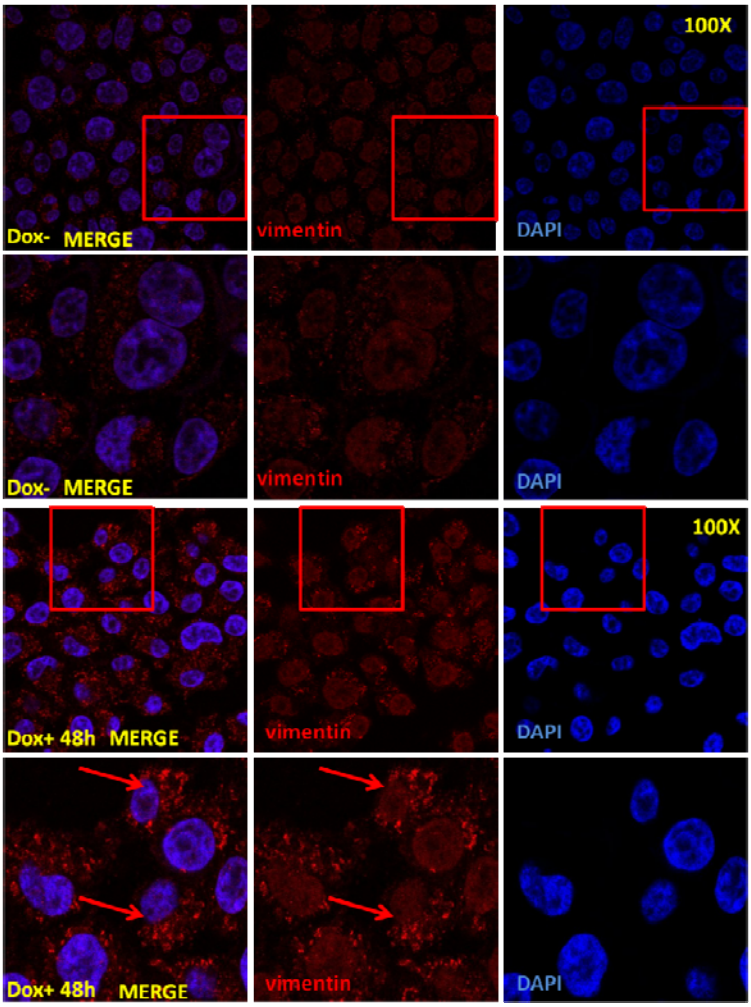


Figure 40: Analysis of vimentin expression. Confocal microscopy analysis shows an increase of vimentin, a mesenchymal marker, in *DKC1*silenced cells. Note that in these cells vimentin is frequently organized in squiggles, short motile filamentous structures.

5. DISCUSSION

In humans, loss of functions mutations in the *DKC1* gene cause the X-linked dyskeratosis congenita, an inherited multisystemic disorder characterized by mucocutaneous features, bone marrow failure, premature aging, loss of stemness and a puzzling predisposition to cancer (Kirwan at al. 2009). *DKC1* encodes an evolutionarily conserved protein, called dyskerin, whose activity is essential for vitality (Heiss et al.1998). Dyskerin is a nucleolar protein with at least two main nuclear functions. First, the protein is a core component of H/ACA small nucleolar ribonucleoprotein particles (H/ACA snoRNPs) involved in rRNA processing (Kiss et al. 2006). Within H/ACA snoRNPs, dyskerin also acts as pseudouridine synthase, promoting the conversion of specific uridine residues to pseudo-uridines. In addition, dyskerin stabilizes the telomerase enzymatic complex through its binding to the H/ACA domain of the telomerase RNA component (hTERC) (Cohen et al. 2007). Moreover, dyskerin has been also involved in a variety of additional functions, such as internal ribosomal entry site (IRES)-dependent translation, DNA repair and stem cell renewal, even though the underlining mechanisms still remain to be fully understood and characterized.

Data available on the effects of *DKC1* gene silencing have so far been obtained by transient silencing. To get more detailed information, I instead planned to establish a cellular model able to stably silence *DKC1* expression. The creation of a stable cell line provides many advantages over transient gene silencing, such as avoiding the need for repeated transfections, establishing a more uniform and reproducible gene silencing, and providing an approach to the study of long-term outcomes. The silencing system was established in the RKO cell line and it was engineered to avoid the silencing of the alternatively spliced dyskerin isofom 3, characterized by retention of the intron 12, and by a cytoplasmic localization. Once obtained and validated the system, I firstly tested whether cell proliferation was reduced in the silenced cells, as previously observed for other human cell lines upon *DKC1* transient silencing (Sieron et al. 2009; Alawi and Lin 2010). Direct counting of viable cells and MTT assays confirmed that dyskerin-depleted cells show a decrease in cell proliferation .The reduced proliferative capacity typical of X-DC patients and of *DKC1* silenced cells is in striking contrast with the increase of tumour susceptibility, that in fact represents one of the most puzzling feature of the disease.

In my experiments, the decrease of cell proliferation was due to a G1/S delay, in keeping with the data reported by Jiang et al. (1993) for yeast cells depleted for CBF5p, the dyskerin homologue protein. However, I noticed that Alawi and Ping Lin (2011) reported that dyskerin-depleted cells have a G2/M delay; the same result was obtained in mouse embryonic fibroblasts characterized by the expression of a catalytically inactive dyskerin by Gu et al. (2013).To further check this point, I thus followed the expression of p21, a protein that binds cyclin E-CDK2 and prevents the cell cycle progression (Rowland and Peeper.

2006; Abbas and Dutta 2009; Kumari et al.2014). Indeed, p21 expression increased in RKO cells upon *DKC1* silencing, thus supporting the occurrence of a G1/S delay.

I then checked whether the proliferative decline could be due to cell death. To this end, I stained the cells with acridine orange and ethidium bromide (AO/EB staining), and followed the activation of PARP-1 (poly ADP-ribose polymerase) and caspase 3. Both approaches lead to negative results, thus indicating that the observed decrease in cell number was not due to cell death.

Indeed, the most marked effect observed in the RKO dyskerin-depleted cells was a morphological change of cell shape. Although previously noticed by two authors (Alawi and Lin. 2010;Sieron et al. 2009), this aspect still remained to be explained.

As concerning RKO cells, I noticed that upon *DKC1* silencing they acquired a round shape, and a subset of them (about 10-20%) detached from the plate. Moreover, phalloidin staining evidenced a reduced degree of F-actin polymerization in the narrow cytoplasmic area of dyskerin silenced cells, suggesting the occurrence of a cytoskeletal rearrangement. The detached cells are still stained by the trypan blue vital dye and, if replated in a culture medium without doxycycline, retain the ability to reattach and proliferate normally. These observations strongly suggested the possibility that dyskerin can promote cell adhesion, thus pushing me to perform cell-substrate adhesion assays. Intriguingly, these assays confirmed that silenced cells showed a lower adhesion to extracellular matrix components than control cells.

Cells attach to a surface by mean of specialized structures called focal adhesions (FAs). These cell-matrix attachment sites play essential roles in many vital cellular processes, including proliferation, survival andmotility (Sastry and Burridge 2000). At FAs sites, clusters of transmembrane integrin receptors bind to matrix molecules and associate with a large protein complex consisting of a variety of anchor and signaling proteins bound to actin filaments. Because of the importance of integrins in the regulation of cell-matrix adhesion process, I tested the expression level of integrin- β1,and found it was decreased at the surface of the dyskerin-depleted cells. Worth noting, in some silenced cells integrin-β1 accumulates irregularly at the "perinuclear recycling compartment" (PNRC), where the internalized integrins can be recycled by Rab 11 proteins. Following endocytosis, integrins travel in fact to early endosomes to be either returned directly to the plasma membrane in a Rab4-dependent manner, or moved to the PNRC tobe recycled by a Rab11-dependent mechanism (Sejeong Shin et al. 2011). However, although integrins are predominantly recycled back to the plasma membrane, during cell migration they are in part transferred to lysosomes for degradation. The irregular accumulation of integrin β1 in the PNRC thus suggests an altered vesicular trafficking.

I then followed the expression levels oftwo other FAs components: paxillin, that links actin filaments to integrin at the cell adhesion sites, and the focal adhesion kinase FAK, that plays a central role in signaling cascades mediated

by integrins, (Mitra and Schlaepfer 2006). Intriguingly, the expression of both proteins, resulted reduced in the silenced cells. These findings are all compatible with a destabilization of FAs and a consequent reduction of adhesion to the ECM, and can thus account for the appearance of spherical, non-adherent cells.

I then analyzed the expression of a subset of actin-binding proteins, such as profilin, vinculin and α-actinin. Among these proteins, only α-actinin underwent a variation in its expression upon *DKC1* silencing. This protein represents a major component involved in microfilament stabilization and in linking microfilaments to adherens junctions and focal adhesion components (reviewed by Oteyand Carpen. 2004; Sjoblom et al. 2008). Noteworthy, I noticed that in the silenced cells α-actinin level decreases at the AJ, while increased in the nuclei. So far,only α-actinin 4 has been shown to havea nuclear localization; this protein has in fact been observed in the nuclei of several cancer cell lines upon actin depolymerization (Honda et al., 1998).Although α-actinin nuclear functions are still unknown, recent studies reported a role in the NF-κB transcriptional activation, indicating that the protein is recruited at promoters of NF-κB target genes, such as IL-1β and IL-8(Zhao et al. 2015). In addition, a role of α-actinin 4 has been evoked in splicing and oxidative stress (Khotin MG et al.2009).

Considering the bulk of above mentioned data, I wondered whether dyskerin could directly interact with some of the examined adhesion molecules. To this aim, I checked by co-immunoprecipitation experiments the possible interaction of dyskerin with FAK, Y397pFAK, α-actinin and paxillin. Intriguingly, FAK, Y397pFAK, α-actinin were actually found able to bind specifically dyskerin. Confocal microscopy analyses confirmed these interactions at the plasma membrane, unrevealing a so far undescribed dyskerin function.

Considering that FAK molecules phosphorylated at residue Tyr-397 are located close to the plasma membrane (Ben-Zion Katz et al. 2003), it is possible to speculate that depletion of dyskerin could destabilize FAs, this way contributing to weakening cell-substrate adhesion. This hypothesis is supported by the work of de Hoog. et al. (2004),who reported the assembly of transitory ribonucleoprotein complexes in the early stages of cell spreading at the so called “spreading initiation centers” (SICs). SICs are distinct from FAs, and their components include some RNA binding proteins, such as hnRNP K, hnRNP E1, and FUS/TLS, all functionally involved in controlling the rate of cell spreading. Since RNA is present, it seems possible that some of these mRNAsare locally translated into proteins, perhaps to fuel the assembly of FAs. As observed by de Hooget al. (2004) “SICs always seem to form directly above classical focal adhesions where they could be local protein factories producing the required components of focal adhesions”. Results reported here raise the intriguing possibilty that dyskerin can be a so far undetected SIC component. Worth noting, the interaction of dyskerin and α-actinin and their co-localization at cell apical membrane is further supported by the work of

Khotin et al. (2009) who identified by mass-spectrometry, dyskerin as a direct interactor of with α-actinin-4.

Taken together, all results I obtained suggested that *DKC1* silencing could promote a migratory cell behavior. During normal development and in pathological contexts (such as cancer), cell-cell contacts are modulated in order to be self-reinforced, in order to establish stable intercellular junctions, or to be weakend, in order to allow cell-cell intercalation or migration. The development of human cancers is frequently associated with the failure of epithelial cells to form TJ and to establish apicobasal polarity (Latorre et al.2005). Moreover,a number of TJ components, including ZO-1,are directly or indirectly involved in cancer progression. For example, in colorectal cancer it has been demonstrated that reduced expression of ZO-1 is directly associated with higher tumor grade (Tracey A. Martin et al. 2009).The TJ loosening, as the removal of epithelial cells from the matrix, can induce a cell reprogramming that allows cellular survival, a process known as epithelial–mesenchymal-transition (EMT). This process involves morphological changes, loss of cell adhesion, loss of epithelium-specific markers, gain of mesenchymal specific markers and enhanced migratory properties of cells (Matise et al. 2012).

It is intriguing to note that the bulk of data reprted here suggests that dyskerin depletion induces many of EMT-specific features in human cells, as recently noted in Drosophila (Vicidomini et al., 2015). The reduced expression of N-cadherin, which ensures the formation of adherent junctions and of ZO1, that is involved in the tight junctions of polarized epithelia., adds further support to this view.

6 CONCLUSIONS

In order to better characterize the effects triggered by dyskerin depletion in human cells, I generated and validated a stable and inducible cellular system able to silence the *DKC1* gene without affecting the expression of its alternatively spliced isofom 3, that is characterized by a cytoplasmic localization. This system proved to work efficiently and, compared to transient gene silencing, has the advantages of not requiring repeated transfections, triggering gene silencing more reproducibly, and allowing to check long-term effects.

Once established the system, I firstly focussed my analyses on the effects exherted by dyskerin depletion well before telomere erosion, in order to define the spectrum of telomere-independent outcomes. Intriguingly, I found that dyskerin depletion perturbs cell-cell and cell-substrate adhesion, causing reduced expression of a subset of proteins involved in tight and adherens junctions and in focal adhesions. In parallel, the silenced cells change their morphology, acquiring a round shape.

To shed more light on the role played by dyskerin in cell adhesion I then checked whether dyskerin could interact directly with focal adhesion components. Indeed, co-immunoprecipitation and co-localization experiments converged to demonstrate that dyskerin is able to interact with FAK, Y397 pFAK and α-actinin. These data point out that dyskerin plays an unpredicted, non-canonical role of in cell adhesion, that was further supported by the results obtained by migration assays.

These data add unforeseen information that can be of valuable help in understanding the puzzling relationship that links pseudouridine synthases loss-of-function to cancer predisposition.

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7 AKNOWLEDGMENTS

I thank the prof Maria. Furia for constructive discussions and for her help in experimental planning. I also to thank the doctor Mimmo Turano for his essential contribution to this work, and the PhDs Alberto Angrisani and Rosario Vicidomi for their support and constructive comments. I am grateful to Valeria Costabile and Valentina Morra for the wonderful work days spent together.

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ORIGINAL PAPERS

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ARTICLE

Intron retention: a human *DKC1* gene common splicing event

Mimmo Turano, Alberto Angrisani, Nunzia Di Maio, and Maria Furia

Abstract: Identification of alternatively spliced transcripts produced by a gene is a crucial step in deciphering the bulk of its biological roles and the overall processes that regulate its activity. By using a combination of bioinformatic and molecular approaches we identified, cloned, and characterized 3 novel alternative splice isoforms derived from human dyskeratosis congenita 1 (*hDKC1*), an essential human gene causative of the X-linked dyskeratosis congenita disease and involved in multiple functions related to cell growth, proliferation, and telomere maintenance. Expression of the new isoforms, all characterized by intron retention, was confirmed by RT-PCR in a panel of diverse cell lines and normal human tissues, and despite the presence of premature termination codons, was not down-regulated by the mechanism of nonsense-mediated decay. Accumulation of these transcripts fluctuated distinctly in the diverse tissues and during in vitro differentiation of Caco2 cells, suggesting that their ratio may contribute to the gene functional diversity across different cell types. Intriguingly, the structure of one isoform leads to exonize an intronically encoded small nucleolar RNA (snoRNA), highlighting an additional layer of complexity that can contribute to overall gene regulation.

Key words: alternative splicing, dyskerin, DKC1, pseudouridine synthase, snoRNP.

Résumé : L'identification de transcrits géniques sujets à l'épissage alternatif est une étape clé afin de décrypter l'essentiel de leurs rôles biologiques et les processus globaux qui régulent leur activité. En utilisant une combinaison d'approches bioinformatiques et moléculaires, nous avons identifié, cloné et caractérisé trois nouvelles isoformes épissées de manière alternative issues du gène *hDKC1* (*human dyskeratosis congenita* 1), un gène essentiel qui cause la dyskératose congénitale liée au chromosome X et qui impliqué dans plusieurs fonctions reliées à la croissance cellulaire, à la prolifération et au maintien des telomeres. L'expression des nouvelles isoformes, toutes caractérisées par une rétention d'intron, a été confirmée par RT-PCR dans une série de lignées cellulaires différentes et de tissus humains normaux et, malgré la présence de codons de terminaison prématurés, cette expression n'était pas régulée à la baisse par un mécanisme de dégradation d'ARNm non-sens. L'accumulation de ces transcrits fluctuait distinctement dans les différents tissus et pendant la différenciation *in vitro* des cellules Caco2, suggérant que leur rapport peut contribuer à la diversité génique fonctionnelle parmi les différents types cellulaires. Fait intéressant, la structure d'une isoforme mène à l'exonisation d'un petit ARN nucléolaire (snoARN) codé dans un intron, mettant en lumière un niveau additionnel de complexité qui peut contribuer à la régulation génique générale. [Traduit par la Rédaction]

Mots-clés : épissage alternatif, dyskérine, DKC1, pseudouridine synthase, snoARN.

Introduction

In the human transcriptome, current analyses pointed out that 92%–94% of human multi-exon genes undergo alternative splicing (AS) (Pan et al. 2006; Wang et al. 2008). Among diverse types of AS, intron retention (IR) has been observed for at least 41% of human genes, and it has been suggested that it may represent a specific hallmark of primate genomes (Mollet et al. 2010). Here we focused on the repertory of IR transcripts derived from *hDKC1*, an essential and ubiquitously expressed human gene involved in multiple functions related to cell growth and proliferation. *hDKC1* loss of function mutations cause X-linked dyskeratosis congenita, a syndrome characterized by a plethora of manifestations that includes bone marrow failure, stem cell dysfunction, premature aging, and increased susceptibility to cancer (Kirwan and Dokal 2009). An ubiquitous nucleolar protein, named dyskerin (Heiss et al. 1998), and a truncated dyskerin variant localized in the cytoplasm (Angrisani et al. 2011) are known to be encoded by this gene. Dyskerin participates in two essential nuclear ribonucleoprotein complexes. Through its PUA domain, which recognizes the H/ACA RNA motif of human telomerase RNA component, the protein takes part in the active telomerase complex and plays a key role in telomere maintenance (Kirwan and Dokal 2008). The same PUA domain allows dyskerin to recognize snoRNAs containing H/ACA sequences, and its early association to these nascent molecules stabilizes them throughout their biogenesis and assembly into H/ACA small nucleolar RNA ribonucleoproteins (snoRNPs), upon subsequent interaction with additional core proteins (Kiss et al. 2010; Alawi and Lin 2010). This chaperoning role is acquiring growing importance since snoRNAs, besides acting in RNA modification processes, can be processed to generate microRNAs (Ender et al. 2008) or short regulatory RNAs able to modulate alternative splicing (Khanna and Stamm 2010). H/ACA snoRNPs are involved in both processing and pseudouridylation of rRNA, and thus play an essential role in ribosome biogenesis. Within these complexes, dyskerin acts as pseudouridine synthase and catalyses the conversion of specific uridine residues to pseudouridines on rRNAs and snRNAs (Kiss et al. 2010). Pseudouridylation represents the most abundant type of RNA modification occurring in eukaryotic cells and is known to be required for the translation fidelity of the ribosome (Jack et al. 2011) and efficient activity of the spliceosome (Yu et al. 2011).

Received 15 May 2013. Revision received 23 July 2013. Accepted 28 August 2013.

M. Turano,* A. Angrisani,* N. Di Maio, and M. Furia. Dipartimento di Biologia, Complesso Universitario di Monte S. Angelo, Università di Napoli "Federico II", via Cinthia, 80126 Napoli, Italia.

Corresponding author: Mimmo Turano (e-mail: mimmo.turano@unina.it).

*These authors contributed equally to this work.

Published at www.nrcresearchpress.com/bcb on 3 September 2013.

In addition to the above mentioned dual canonical functions, *hDKC1* is thought to be involved in a variety of diverse cellular processes, such as nucleo-cytoplasmic shuttling (Meier and Blobel 1994), DNA damage response (Gu et al. 2009), and cell adhesion (Sieron et al. 2009; Alawi and Lin 2011; Angrisani et al. 2011), although the exact role played in each of them remains to be elucidated. The functional versatility of this gene is further underlined by its coding non-coding interlaced organization, a feature that is shared by its mouse (http://genome.ucsc.edu/) and Drosophila (Riccardo et al. 2007) orthologues. Specifically, *hDKC1* hosts two snoRNAs of the H/ACA class, named SNORA36A and SNORA56, in introns 8 and 12, respectively. Both these snoRNAs have verified targets on rRNAs (http://www-snorna.biotoul.fr/); in addition, SNORA56 acts as precursor of a microRNA with a so far undetermined function (Ender et al. 2008). This complex structural organization suggested that AS events could also modulate the expression of the intronically hosted snoRNAs, adding further interest to the definition of the full repertory of gene transcriptional outputs.

Materials and methods

Bioinformatic analyses

The human EST databases at the NCBI were searched by the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) entering the *hDKC1* human genomic sequence (GenBank ID: NC_000023). ClustalW (http://align.genome.jp/) was used to verify positive matches and putative CDSs were evaluated by the translate tool at the ExPASy web site (http://web.expasy.org/translate/).

Cell culture and treatment

Colorectal adenocarcinoma (Caco2), cervical carcinoma (HeLa), osteosarcoma (U-2 OS), breast adenocarcinoma (MCF7), and liver carcinoma (Hep G2) human cell lines were obtained from ATCC and cultured following the suggested conditions. To check nonsense-mediated decay (NMD) susceptibility, translation was blocked by treating cells with cycloheximide (100 μg/mL) up to 4 h, as described in (Cuccurese et al. 2005). All media and supplements were purchased from Sigma-Aldrich (St. Luis, Mo., USA). For gene silencing, MCF7 cells were transiently transfected using 3 μg of shRNA (*hDKC1*) lentiviral plasmid pLKO.1-puro (Sigma) or pLKO.1-puro empty vector, and 30 μL of Cellfectin (Invitrogen), following manufacturer's instructions. Selected shRNA (TATGTTGACTACAGTGAGTCTCTCGAG AGACTCACTGTAGTCAACATA) targeted *hDKC1* exon 12–13 junction, which is shared by isoform 1 and all newly described isoforms.

RNA extraction and analysis

RNA isolation, cDNA synthesis, RT-PCR fragments cloning, and semiquantitative RT-PCR analyses of human tissues and cell lines were carried out as previously described (Angrisani et al. 2011). Poly (A)+ fraction was obtained using Genelute mRNA miniprep kit (Sigma-Aldrich, St. Luis, Mo., USA) following manufacturer's instructions. To obtain nuclear and cytoplasmic RNAs, cellular pellet was lysed in 20 mmol/L TrisHCl pH 8.0, 85 mmol/L KCl, 0.5% NP40; after incubation on ice (15 min), nuclei were separated from cytoplasmic fraction by centrifuging at 3000*g* for 5 min and RNA extracted from each fraction. To avoid gDNA amplification, RNAs were subjected to treatment with RQ1 DNase (Promega, Madison, Wis., USA). The cycle numbers of semiquantitative analysis were: 28 for Iso 1 and largest subunit of RNA polymerase II, 32 for Iso 5, and 37 for Iso 4 and Iso 6. qRT-PCR experiments were performed as previously described (Turano et al. 2008); the data were normalized to the expression of the human glutathione synthetase housekeeping gene (De Rosa et al. 2007). After normalization, the data are presented as fold change relative to the reference point. To determine the absolute quantity of *hDKC1* isoforms, standard curves were obtained by preparing 10-fold serial dilutions of plasmids (pGEM T-Easy Vector, Amersham) containing cDNA sequences of each isoform. Sequences of all the primers are listed in Supplementary Fig. 1.[1]

Results and discussion

Identification and cloning of *hDKC1* intron-retaining transcripts

To identify IR transcripts derived from the *hDKC1* gene, we deepened the BLAST analysis of the human EST database, using as query all gene introns. This analysis revealed 4 EST clusters characterized by total or partial retention of a specific intron (Fig. 1A). To rule out the possibility that these ESTs represented partially spliced intermediates, we first confirmed their actual expression by RT-PCR analysis of total RNA obtained from epithelial Caco2 cells. Each EST type was found be actively expressed, so we attempted to extend their upstream and downstream regions by a double PCR reaction on cDNA (schematized in Supplementary Fig. 1)[1]. In the first PCR reactions, a forward primer annealing on the gene 5′ UTR was coupled to reverse primers annealing on the intron specifically retained by the annotated EST; in the second PCR reactions, divergent forward primers annealing on the specific intron were coupled to a reverse primer annealing on *hDKC1* last exon. Following this approach, we failed to obtain any RT-PCR extended product representative of intron 7-retaining transcript, although ESTs of this type have been annotated from at least 6 different tissue sources. Moreover, we were able to reproducibly amplify short RT-PCR fragments retaining this specific intron, indicating that a related transcript was effectively produced in Caco2 cells. The inability to obtain extended amplification products thus remains unclear and might be related to a more complex and unexpected organization of the associated transcript. Productive amplification was instead obtained for each other EST type, so that cloning and sequencing of the obtained fragments led to define the structure of 3 new AS transcripts, hereafter named isoforms 4, 5, and 6, all characterized by retention of a specific intron within an otherwise fully spliced RNA (GenBank Accession numbers: KC954523, KC954524, KC954525, respectively; see Fig. 1B). It has been observed that human transcripts characterized by IR derive preferentially from highly and broadly expressed genes (Sakabe and de Souza 2007), and that the majority of them (78%) retain a single intron within mature RNAs (Mollet et al. 2010); the *hDKC1* new isoforms all conform to these general features. Isoforms 4 and 5 wholly retained introns 2 and 5, respectively, while isoform 6 originated from activation of an intron 8-internal cryptic donor splice site (Fig. 1B). This event leads to exonize SNORA36A sequence, linking the expression of this transcript to the biogenesis of this snoRNA. Since the previously described *hDKC1* isoform 3 led to exonization of SNORA56 sequence (Angrisani et al. 2011), it is plausible that IR events can have a general role in coupling the regulation of *hDKC1* expression to that of its hosted snoRNAs. This view is further supported by the finding that a significant subset of human retained introns hosts snoRNAs, or other small non-coding RNAs, and transcripts retaining these introns were found also in the cytoplasm (Mollet et al. 2010). Consistent with these observations, isoform 6 was detected in the poly(A)+ fraction and enriched in preparations of cytoplasmic RNA (see Fig. 2), despite that the IR event was expected to affect its nuclear export. It has been suggested that regulation of splicing and biogenesis of small intronic non-coding RNAs could be interconnected (Mollet et al. 2010), although it is not yet clear whether cytoplasmic transcripts retaining intronic non-coding RNAs can act as precursors, or conversely, their expression could antagonize the biogenesis of these molecules. As concerning intronic H/ACA snoRNAs, the view that splice site se-

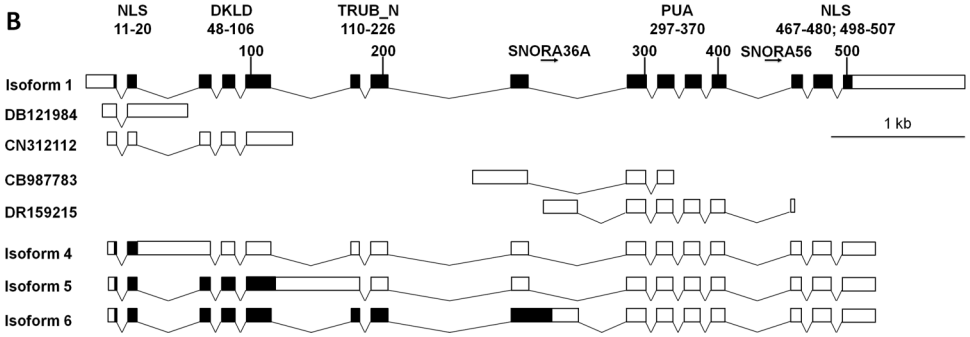
[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/bcb-2013-0047.

Fig. 1. Structure of intron-retaining *hDKC1* transcripts. (A) List of annotated ESTs representative of new *hDKC1* splice isoforms. The accession number (http://www.ncbi.nlm.nih.gov/nucest/) of each annotated EST is indicated; # marks the longest EST of each group, whose structures are depicted in Fig. 1B; * indicates that intron 8, whose whole length is 734 nt, is only partially retained (370 nts) by this isoform. (B) On the top, the structure of the 2.6 kb *hDKC1* mRNA (iso 1) encoding canonical dyskerin (GenBank ID: NM_001363). Exons are depicted as boxes, coding regions are in black; the position of the main dyskerin functional domains is indicated above, together with that of the intronically encoded SNORA36A and SNORA56. Below, structures of the ESTs marked with # and their related transcripts, as derived by RT-PCR cloning (see Supplementary Fig. 1)[1] and nucleotide analysis.

A

Retained intron (nt)	Isoform	EST	Tissue/cell type
Intron 2 (468)	Isoform 4	DB014473	Esophageal, tumor tissue
		DA918152	Small intestine
		DB121984#	Thymus
Intron 5 (596)	Isoform 5	CN312112#	Embryonic stem cells, cell lines h1, h7, and h9
		DA609157	Neuroblastoma
Intron 8 (370*)	Isoform 6	DR159215#	Human embryonic stem cells differentiated to an early endodermal cell type
		BE280855	Choriocarcinoma
		BE732485	Choriocarcinoma
		BI014283	Lung tumor
		BE925811	Breast normal
Intron 7 (919)		CB987783#	Pooled-Glandular
		BE150622	Head neck
		BU958453	Pool of 40 RNAs
		BE925811	Breast normal
		BI090493	Cervix
		CN312109	Embryonic stem cells, DMSO-treated H9 cell line

B



lection and snoRNA recognition are concomitant and possibly interlaced events was first suggested by the observation that recruitment of both H/ACA snoRNP proteins and splicing factors occur cotranscriptionally on pre-mRNAs (Richard et al. 2006).

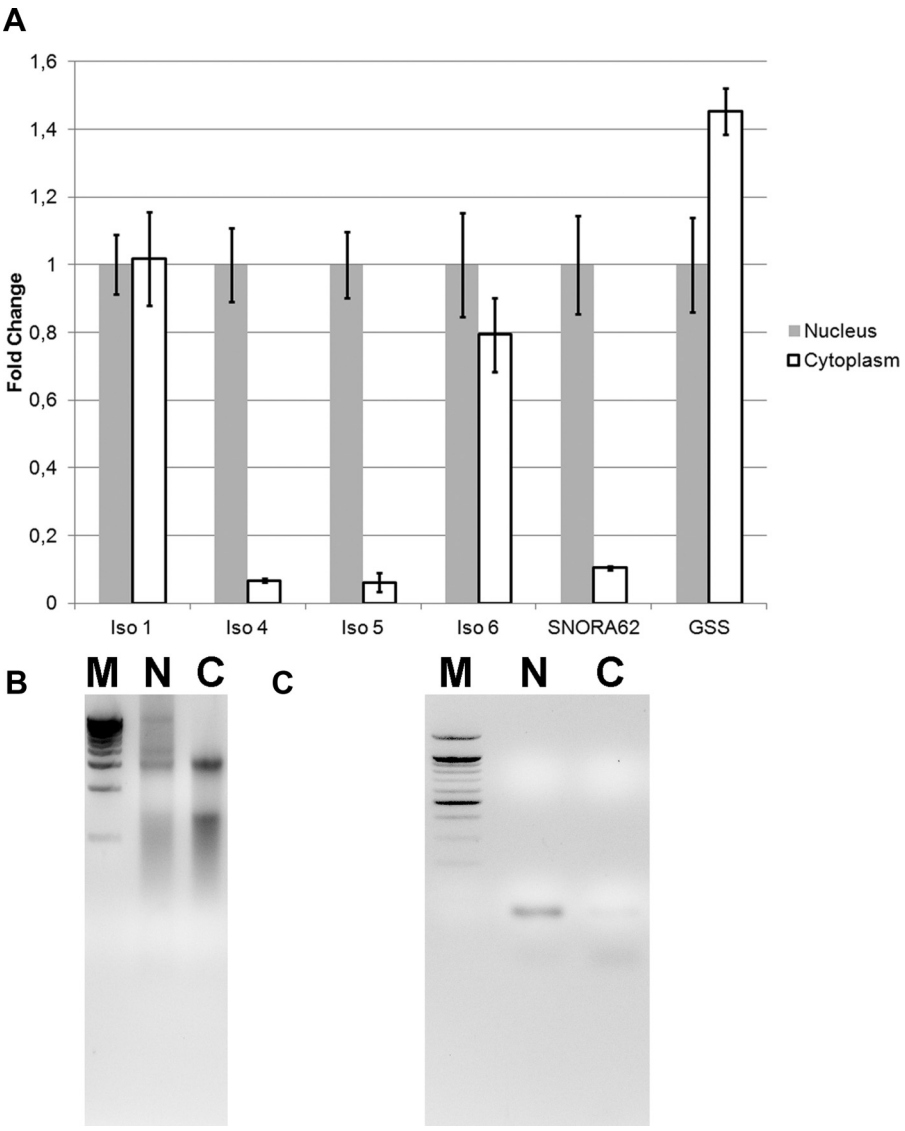
A main question is whether the new *hDKC1* isoforms, all detected in the poly(A) + fraction, represent long, translationally unproductive non-coding RNAs, or can be translated in variant proteins with possible biological functions. Looking at their coding properties, we noticed that these transcripts were characterized by the presence of premature stop codons (PTCs) that interrupted dyskerin CDS prematurely (Fig. 1B). The coding potential of isoform 4 was very low, with dyskerin CDS precociously interrupted within intron 2; isoforms 5 and 6 could instead be able to encode putative proteins of 159 aa (18 kDa) and 313 aa (35.1 kDa), respectively. However, both proteins would lack the PUA domain, which is essential for human telomerase RNA component and snoRNA recognition, and the C-terminal nuclear localisation signal, which is required for snoRNP assembly (Machado-Pinilla et al. 2008) and preeminent nuclear localization (Angrisani et al. 2011); consequently, they would be unable to carry out the dual dyskerin canonical functions. However, an alternative functional role played by these truncated proteins might be based on their ability to encode an internal dyskerin peptide, named GSE24-2 (90–144 aa). Expression of this peptide was reported to be able to increase telomerase activity, block senescence, and recover the dividing capacity of X-linked dyskeratosis congenita cells. Intriguingly, these effects were not reproduced by full-length dyskerin (Machado-Pinilla et al. 2008). Since it is plausible that other dyskerin regions could block GSE24-2 activity, we considered the possibility that truncated dyskerin variants may be able to direct synthesis of this peptide within an active protein context. To investigate this aspect, we checked whether splice isoforms were enriched in cytosolic RNA preparations. As shown in Fig. 2, we found that isoforms 4 and 5 were essentially retained in the nuclei, while isoform 6, together with canonical isoform 1, was efficiently exported in the cytoplasm. Active expression of this isoform was further confirmed by gene silencing experiments; in fact, when cells were transiently transfected with a plasmid bearing a shRNA targeting sequences common to isoforms 1 and 6 (see Methods section), both transcripts were silenced at a comparable degree (see Supplementary Fig. 2)[1].

It is worth noting that the putative isoform 6-encoded protein shares only its first 257 aa with dyskerin but exhibits an unique, highly hydrophobic C-terminal tail of 56 aa (see Supplementary Fig. 3)[1] that might be involved in novel protein–protein interactions. Remarkably, the overall structure of this protein is highly reminiscent of that previously described for a variant protein encoded by *mfl/Nop60b*, the *hDKC1* Drosophila orthologue (Riccardo et al. 2007), which is similarly characterized by a unique, hydrophobic C-terminal domain (see Supplementary Fig. 3)[1].

hDKC1 intron-retaining transcripts are actively expressed in diverse cell lines and normal tissues and are differently regulated during in vitro Caco2 cell differentiation

Expression profiling of new IR transcripts was defined by semi-quantitative RT-PCR analysis of a panel of human cell lines and normal tissues. All *hDKC1* isoforms were detected, although at variable levels, in each tested cell line (Fig. 3A). Absolute quantitation of each isoform was determined in Caco2 cells (Supplementary Table 1)[1] and showed that abundance of these rare variants ranged from about 20- to 70-fold less than that of canonical isoform 1. In vivo expression was also confirmed in a panel of 20 diverse tissues (Fig. 3B), including placenta, where the GSE24-2 peptide was identified upon screening of a library enriched for low-abundance mRNAs (Machado-Pinilla et al. 2008). The expression levels fluctuate distinctly in the diverse tissues, suggesting that *hDKC1* multiple isoforms may contribute to the gene functional diversity across differentiated cell types. Quantitative den-

Fig. 2. Comparative estimate of the levels reached by each new *hDKC1* isoform in nuclear and cytosolic fractions. (A) Abundance of each isoform was determined by qRT-PCR analysis; the snoRNA *SNORA62* was used as a nuclear marker, while *GSS* mRNA was used as marker of the cytosolic fraction. Expression was normalized with respect to *POLR2A* mRNA. (B) Agarose gel of 800 ng fractionated RNA; M 1 kb DNA ladder (Applichem), N nuclear RNA, C Cytoplasmic RNA. (C) Semiquantitative RT-PCR of *SNORA62* nucleolar RNA; M 100 bp Plus DNA ladder (Applichem), N nuclear, C Cytoplasmic.



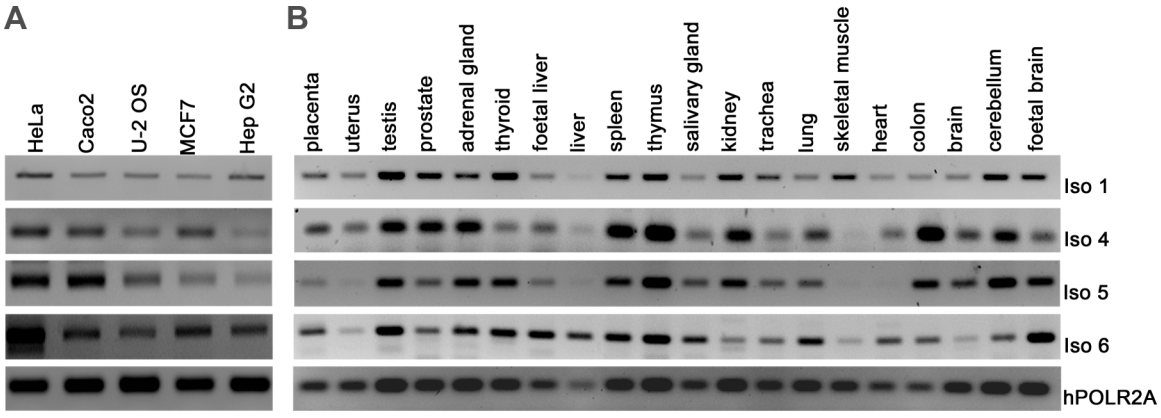
sitometric analyses (Fig. 3C) indicated that the various transcripts reached their maximum level of expression in different tissues, with the canonical isoform 1 being up-regulated in thyroid, isoforms 4 and 5 in colon, and isoform 6 in foetal brain.

Since regulation of AS plays critical roles in cell differentiation (Dujardin et al. 2013), we analysed by qRT-PCR the expression of various *hDKC1* transcripts, including that of the previously described isoform 3 (Angrisani et al. 2011), during in vitro differentiation of Caco2 cells. These cells undergo in long term culture a process of spontaneous differentiation that leads to the expression of several morphological, functional and biochemical characteristics of small intestinal enterocytes. Differentiation starts as soon as confluence is reached (Pinto et al. 1983) and markedly influences the accumulation levels of mRNAs encoded by human apolipoprotein Al and human sucrose isomaltase genes, whose quantification provides a sensitive and reliable molecular marker of the process (Pinto et al. 1983; Reisher et al. 1993). RNA was then extracted on day 3 (taken as reference point for undifferentiated cells) and day 14 (taken as reference point for fully differentiated cells) of growth, and the levels of human apolipoprotein Al and human sucrose isomaltase mRNAs were used as markers of enterocyte-like differentiation (Fig. 4A). As shown in Fig. 4B, expression of the various isoforms was differently modulated upon differentiation, with isoforms 1 and 4 undergoing a reduction, and isoforms 3, 5, and 6 increasing their expression in fully differentiated cells. These data are consistent with the diverse profiling observed in the different tissues, and support the view that differentiation processes can influence the expression of *hDKC1* multiple transcripts.

hDKC1 multiple isoforms escape the NMD-triggered decay

Alternative splicing plays a fundamental role in increasing transcriptome complexity, not only by changing function, abundance, and localization of the encoded products (Kelemen et al. 2013), but also generating nonfunctional transcripts that can determine a global down-regulation of gene function, or act as long noncoding RNAs with diverse regulatory roles (Rinn and Chang 2012). Since all newly identified transcripts, similarly to the previously

Fig. 3. Widespread expression of intron-retaining *hDKC1* transcripts. Expression of various isoforms was checked by semiquantitative RT-PCR analysis in panels of 5 different human cell lines (A). Active expression was also observed in a panel of 20 human tissues (B), together with that of the 2.6 kb transcript encoding canonical dyskerin (Iso 1). A comparative estimate of the levels reached by each *hDKC1* isoform in the diverse tissues was obtained by densitometric analyses (C). The table lists the values obtained for each isoform in a specific tissue; (below) these values were normalized respect to that obtained in the same tissue for the *hPOLR2A* gene, used as internal control. Normalised data were used to calculate a mean value (taken as representative of the average level of each specific isoform). Variations from the mean value more than 2 sigma limits were considered significant and taken as indexes of specific peaks of expression; for each isoform, peak values are marked in italic bold.

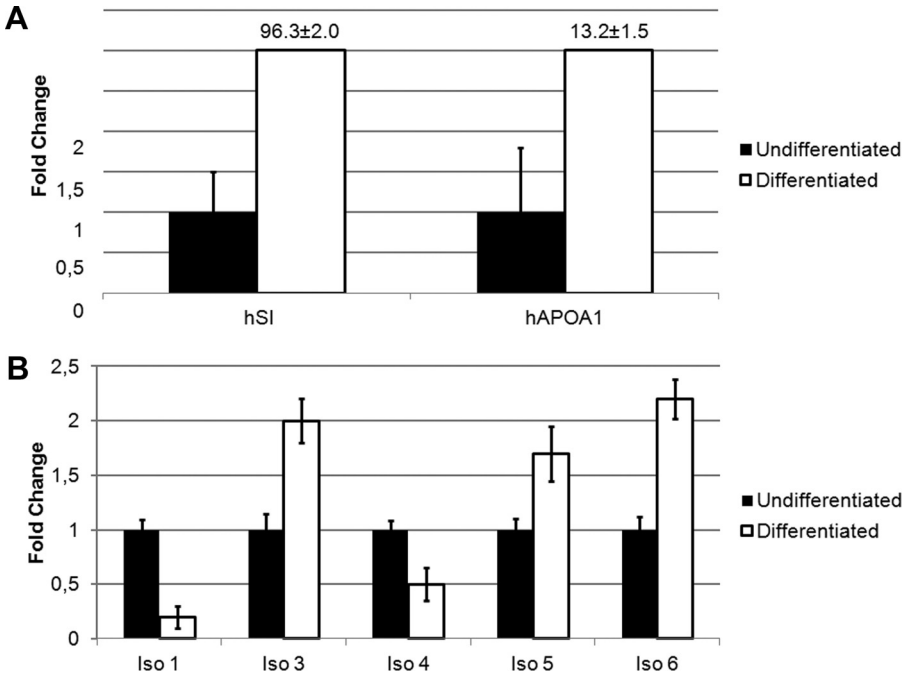


C

Placenta	Uterus	Testis	Prostate	Adrenal gland	Thyroid	Fetal liver	Liver	Spleen	Thymus	Salivary gland	Kideny	Trachea	Lung	Skeletal muscle	Heart	Colon	Brain	Cerebellum	Fetal Brain	
0,9657	0,8626	0,9566	0,9449	0,8086	***1,1083***	0,9216	0,9158	0,9003	0,8301	0,8836	0,8534	0,9560	0,7857	0,9897	0,9128	0,9594	0,7571	0,8415	0,7548	Iso1/POLR2A
0,6011	0,5550	0,5001	0,6209	0,5928	0,4858	0,6311	0,6350	0,7375	0,7727	0,6188	0,5878	0,5651	0,5732	0,4718	0,6356	***1,0914***	0,5568	0,5394	0,4306	Iso4/POLR2A
0,5225	0,4388	0,5663	0,5119	0,6328	0,6713	0,5841	0,6028	0,6712	0,8871	0,6952	0,6501	0,6097	0,5578	0,4395	0,4985	***0,9236***	0,6130	0,8806	0,6400	Iso5/POLR2A
0,5031	0,2622	0,6294	0,3533	0,4397	0,5581	0,7022	0,6193	0,5592	0,6626	0,5376	0,2861	0,3998	0,5007	0,2936	0,5019	0,5009	0,2728	0,3326	***0,8325***	Iso6/POLR2A

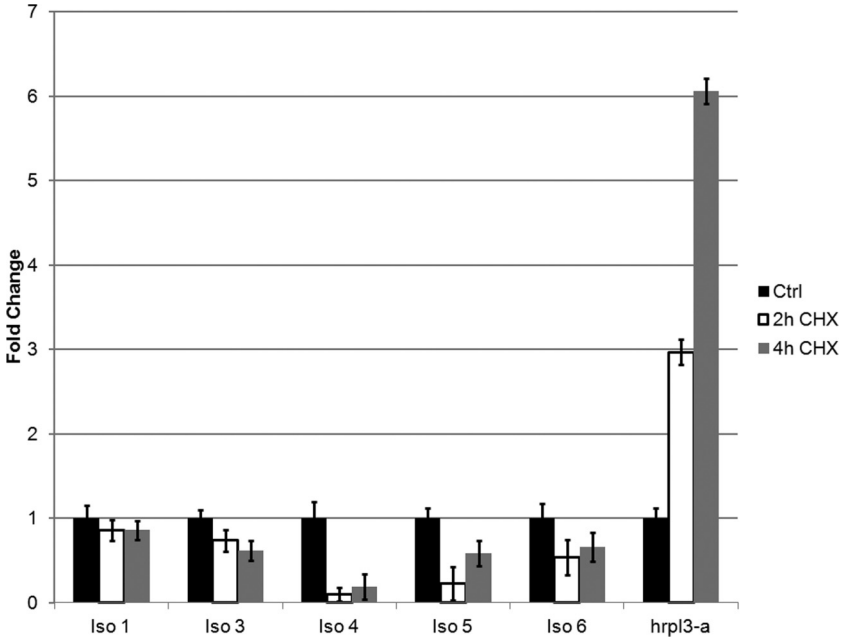
	Iso1/POLR2A	Iso4/POLR2A	Iso5/POLR2A	Iso6/POLR2A
Mean	0,8954	0,6101	0,6298	0,4874
SD	0,0846	0,1361	0,1326	0,1539

Fig. 4. Expression of *hDKC1* intron-retaining isoforms during in vitro differentiation of Caco2 cells. (A) Human apolipoprotein Al (hAPOA1) and human sucrose isomaltase (hSI) mRNA levels were used as markers of enterocyte-like differentiation. (B) Total RNA was extracted and analysed by qRT-PCRs on days 3 (undifferentiated cells) and 14 (differentiated cells) of cell growth. The expression of the canonical dyskerin-coding transcript (Iso 1) is also reported. Data are presented as means ± SD from 3 independent experiments, each performed in triplicate.



described isoform 3 (Angrisani et al. 2011), were characterized by PTCs residing beyond 50 nt 5′ to an exon junction, the NMD surveillance mechanism was expected to commit them to a rapid degradation (Gardner 2010). To assess whether *hDKC1* isoforms could be down-regulated by NMD, we analysed by qRT-PCR the total RNA obtained from Caco2 cells untreated or treated with cycloheximide, an inhibitor of protein synthesis that inactivates translationally active ribosomes and represses NMD. RNA samples were collected at 2 time points from the treatment and normalized with respect to the level of the human glutathione synthetase

Fig. 5. *hDKC1* intron-retaining isoforms are not down-regulated by NMD. qRT-PCR analysis of *hDKC1* alternative transcripts produced by Caco2 cells untreated (Ctrl) or treated with cycloheximide (CHX) at the indicated times. hrpL3-a expression was employed as positive control of the NMD block. Data are presented as means ± SD from 3 independent experiments, each performed in triplicate.



housekeeping mRNA; the expression of hrpL3-a, an alternatively spliced form of the *hrpL3* gene that is known to be under the control of NMD (Cuccurese et al. 2005), was employed as positive control of the NMD block. While hrpL3-a mRNA accumulated efficiently in cells exposed to cycloheximide, as expected for an NMD-regulated transcript, the levels of all *hDKC1* isoforms, irrespective of the presence of PTCs, were found to be unaffected by the treatment, showing that also the cytoplasmic isoform 6 is immune to this surveillance mechanism (Fig. 5). Several PTC-containing mRNAs are immune to the NMD machinery and encode proteins with biological activity (Pan et al. 2006), including *hDKC1* isoform 3 (Angrisani et al. 2011), supporting the possibility that also this new *hDKC1* isoform may be translated.

Conclusions

Among the diverse types of AS events, IR has been suggested to represent a specific hallmark of primate genomes (Mollet et al. 2010). Focusing on *hDKC1,* the essential and highly conserved gene causative of X-linked dyskeratosis congenita, we found that IR events characterize 3 new transcripts derived from this gene. While 2 of them are essentially retained in the nucleus, one is efficiently exported in the cytosol, is immune to NMD, and potentially encodes a truncated protein able to play a novel interesting biological role. As observed for another previously described intron-retaining gene transcript (Angrisani et al. 2011), this new isoform leads to exonize an intronic snoRNA, highlighting the potential of IR events to link *hDKC1* expression with that of its hosted snoRNAs. Finally, expression of all *hDKC1* multiple isoforms was found sensitive to differentiative stimuli, consistent with the observation that IR can be specifically regulated (Mollet et al. 2010). Considering that all new transcripts retain the ability to encode for the canonical dyskerin N-terminal region, which harbors a large group of pathogenic mutations, it is possible that their expression might contribute to the clinical manifestations of the disease and affect its phenotypic heterogeneity.

Acknowledgements

This work was supported by University of Naples Federico II and by P.O.R. Campania FSE 2007–2013, Project CREMe. Alberto Angrisani Postdoctoral Fellowship was supported by Compagnia di San Paolo, Progetto FORGIARE. Nunzia Di Maio doctoral Fellowship was supported by P.O.R. Campania FSE 2007–2013, Project MODO.

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