ALMA MATER STUDIORUM – UNIVERSITA' DI BOLOGNA

DOTTORATO DI RICERCA IN

ONCOLOGIA, EMATOLOGIA E PATOLOGIA

Ciclo XXXII

Settore Concorsuale: 06/A2

Settore Scientifico Disciplinare: MED/05

Evaluation of the role of DKC1 overexpression in breast cancer

Presentata da: Ania Naila Guerrieri

Coordinatore Dottorato — **Prof. Pierluigi Lollini**

Supervisore — **Prof. Lorenzo Montanaro**

Esame finale anno 2020

Summary

ABSTRACT

Dyskerin is a nucleolar protein involved in several cellular processes. In particular, it is part of the pseudouridylation complex and catalyzes the isomerization of specific uridines on ribosomal RNA (rRNA) into pseudouridines, guided by small nucleolar RNAs (snoRNAs). Dyskerin is also part of the telomerase complex stabilizing the telomerase RNA component (hTR). Loss of function mutations in DKC1 cause X-linked Dyskeratosis Congenita (X-DC), a ribosomopathy characterized by failure of proliferating tissues and increased susceptibility to cancer. However, some human tumors like breast, prostate, liver and lung cancers show increased dyskerin expression and worse prognosis. Besides the role of dyskerin as tumor suppressor, literature lacks studies analyzing the function of its increased expression in tumors. In this work, we generated stable DKC1 overexpression cell lines and studied both the behavioral and the molecular effects of higher dyskerin expression. Our findings demonstrated that increasing dyskerin levels confers a more aggressive phenotype and increased translational efficiency in untransformed mammary epithelial cells (MCF10A). Interestingly, also the activity of highly purified ribosomes from MCF10A DCK1 overexpressing cells is significantly increased independently on the translation initiation modality. Furthermore, DKC1 overexpression lead to an up-regulation of the snoRNAs pool, without causing any changes in the global pseudouridylation level of rRNA. Among the snoRNAs pool, the three significantly up-regulated snoRNAs are known to target uridines on rRNA. Thus, we quantified the percentage of pseudouridines (Ψ) through a LC/MS based method on U1492 on 18S rRNA, U4975 and U1445 on 28S rRNA respectively. Our results show no significant changes in pseudouridine levels in these sites, although basing on the *in vitro* translation results, a biological role of the slight changes we detected cannot be excluded. Finally, in line with our *in vitro* findings, we observed

that patients harboring tumors with higher dyskerin expression have worse prognosis, lower disease-free survival and advanced lymph node status respect of patients expressing low dyskerin levels. In addition, in the same tumors with higher dyskerin expression, levels of SNORA64, SNORA70 and SNORA67 are significantly increased. In conclusion, our work indicates for the first time that dyskerin may act as an oncogene in breast cancer, promoting neoplastic transformation from early stage and providing ribosomes with a major translation efficiency. These effects could possible depend either to the modification of U1445 and/or U1492 on rRNA to the up-regulation of SNORA64, SNORA67 and SNORA70 induced by dyskerin overexpression.

INTRODUCTION

Ribosome biogenesis

Ribosome biogenesis is a highly regulated cellular process that lead to the production of ribosomes, ribonucleoproteic complexes aimed to carry out protein synthesis. The process of ribosomes production starts in the nucleolus from the transcription of a unique ribosomal RNA (rRNA) precursor, the 45S rRNA, by RNA Polymerase I (RNA PolI) from ribosomal DNA (rDNA) genes. rDNA genes are present in approximately 400-tandem repeated copies in human genome, localized in the Nucleolar Organizing Region (NOR)[1] and transcribed in different moments through epigenetic regulation[2]. RNA PolI starts the transcription of the long 45S rRNA precursor by binding to the Upstream Binding Factor 1 (UBF1), followed by the Selectivity Factor 1 (SL1) forming the Pre-Initiation Complex (PIC). In the 45S rRNA precursor, each rRNA is interposed by two Internal Transcribed Spacer (ITS1 and ITS2) and enclosed by two External Transcribed Spacer (5'-ETS and 3'-ETS). rRNAs maturation process occurs through cuts, rearrangements and post-transcriptional modifications mediated by specific enzymes leading to the formation of mature 18S rRNA, 28S rRNA and 5.8S rRNA. The 5S rRNA is transcribed in the nucleoplasm by RNA Polymerase III (RNA PolIII) and then imported in the nucleolus[3], as for ribosomal proteins (RPs) which genes are transcribed in the nucleoplasm by RNA polymerase II (RNA PolII) and imported in nucleolus after being translated[4]. Ribosomal biogenesis continues with the production of the major ribosomal subunit or 60S, where S stands for Svedberg as the unit of measure of sedimentation coefficient, formed by the 28S, 5.8S and 5S rRNAs and 47 RPs, and of a minor ribosomal subunit or 40S formed by 18S rRNA and 32 RPs. Both 60S and 40S subunits migrate in the cytoplasm to give birth to the mature 80S ribosome[5,6] (Figure 1). Once

in the cytoplasm, after the removal of the accessories assembly factors, ribosomal functionality is tested assuring that mature ribosomes are competent for translation[7–9].

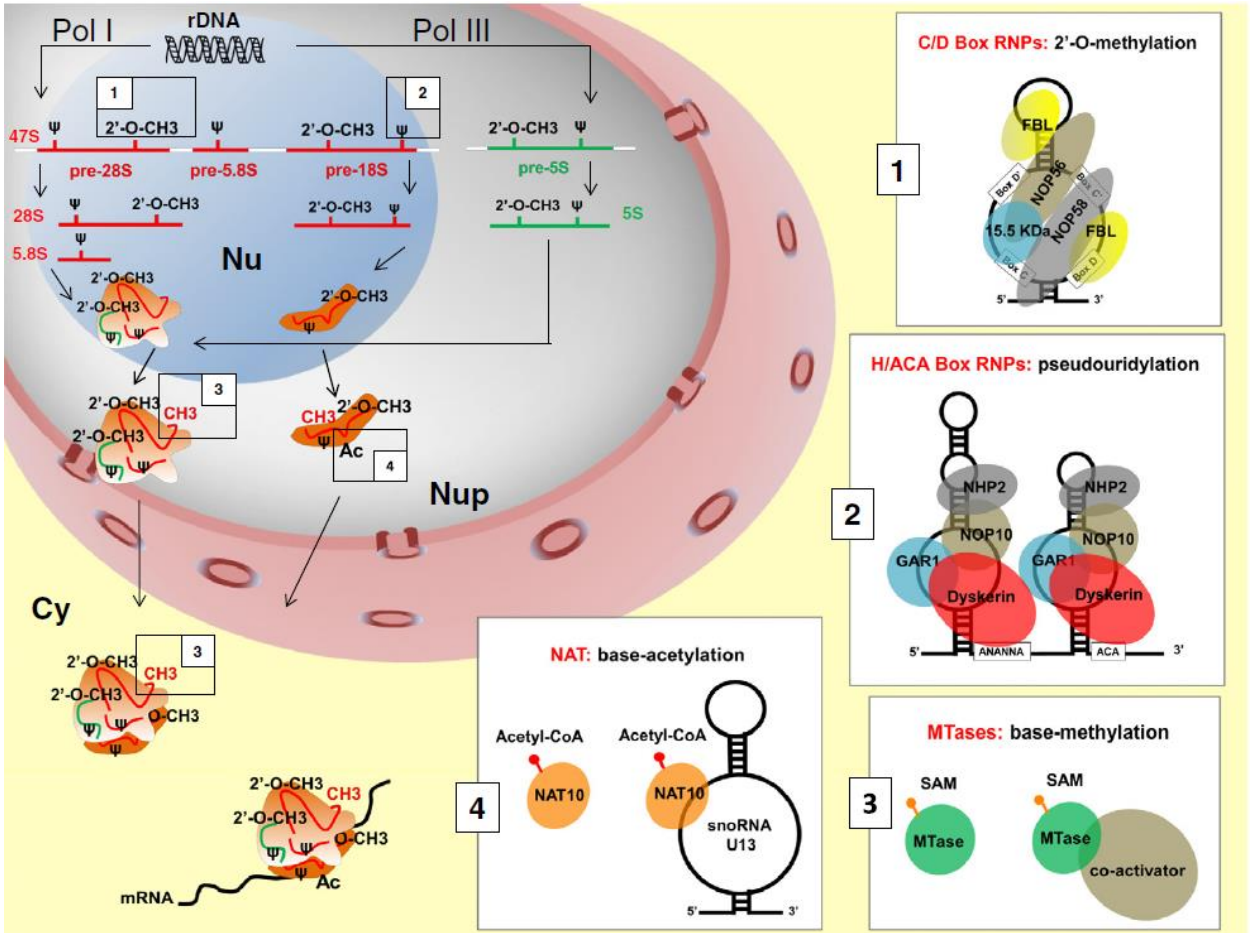

Figure 1 Schematic representation of ribosomal biogenesis and rRNA modifications [10].

Ribosome biogenesis and cell cycle

Ribosomes biogenesis is strictly coordinated with cell cycle progression. Generally, cells enhance the production of ribosomes during the G1 phase, since they need to synthesize enough cellular components to give birth to two daughter cells in the M phase. In addition, the amount of ribosomes produced during the G1 phase is a crucial factor for cell cycle progression[11]. Once cells division occurred, cyclin B phosphorylates TIF1B/SL1 that results inactivated, therefore blocking the constitution of the PIC and the transcription of 45S rRNA. This fine balance, that involve also UBF and PolI activity, guarantees the correct production of RNA precursor and ribosomes[1,6,12]. Interestingly, several studies demonstrated that cell cycle and ribosome biogenesis are regulated by the same oncogenes and tumor suppressor genes[5], respectively upregulating or down-regulating the production of ribosomes. For example, c-MYC, an oncogene overexpressed in several human cancers, can interfere with rDNA transcription at several levels: firstly favoring the binding of SL1 to rDNA promoter therefore activating PolI transcription, secondly stimulating PolII transcription of RPs genes and finally activating the transcription factor TFIIIB which enhance PolIII activity[5]. Another important pathway is the one that involves PI3K/AKT/mTOR and leads to the activation of the protein kinase S6, which phosphorylates the short subunit ribosomal protein 6 (RPS6). RPS6 influences the transcription of a subclass of mRNAs, called Terminal OligoPyrimidine tract in the 5' UTR (TOP mRNAs), that transcribe fundamental proteins for ribosomal biogenesis, such as elongation factors eEF1A and eEF2, known as proto-oncogenes[6,13]. In addition, mTOR activates UBF and PolI transcription, together with PolIII transcription allowing the association between transcription factors TFIIIB and TFIIIC to the 5S rDNA promoter and activating TFIIIA. For these reasons mTOR is target of a therapy based on rapamycin, used also as anti-cancer agent[5,14].

Regarding the tumor suppressor control, p53 has a central role. Firstly, p53 is a sensor of ribosomal stress and it stabilizes when an unbalance in ribosomes production occurs, causing cell cycle arrest. Moreover, some RPs unused for ribosomes production sequester MDM2, that is no longer able to bind and send p53 to proteasomal degradation, leading to cell cycle arrest[11]. Another example of p53 direct influence on ribosomal biogenesis, is its ability to inhibit SL1 recruitment on rDNA promoter blocking PolI and TFIIIB/PolIII activity[5,15].

Finally, the Retinoblastoma Protein (pRb), in its unphosphorylated form, negatively regulates PolI transcription binding both UBF and TFIB, and through its binding with EF2A prevents the G1/S passage. The hyperphosphorylated form of pRb causes an up-regulation of ribosomes production, with an accumulation of 45S precursor and nucleolus enlargement[11,16,17] (Figure 2).

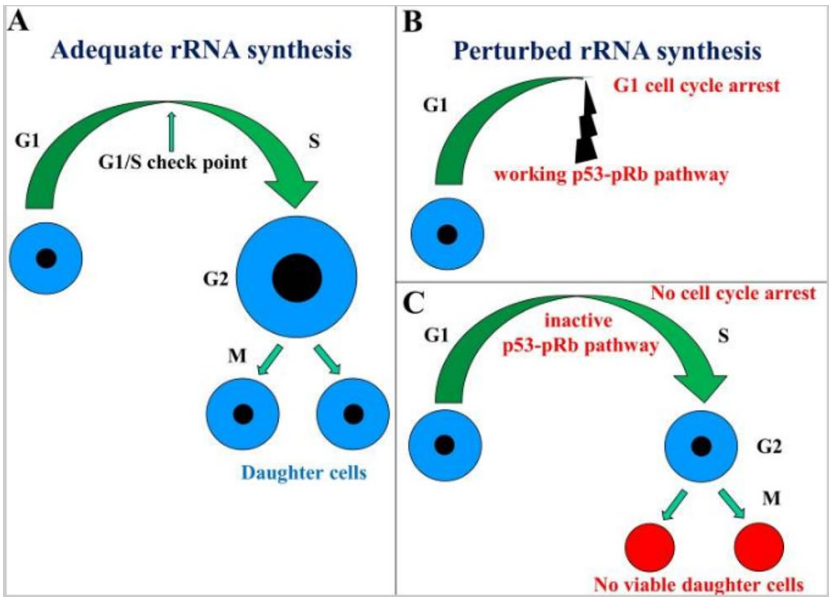

Figure 2 Relationship between ribosome biogenesis and cell cycle progression. Adapted from Brighenti et al[18].

rRNA modifications

As stated before, 45S rRNA processing to mature rRNAs provides for different cuts and co-/post-transcriptional modifications due to specific enzymes. These modifications may occur both on nucleotides (almost 3% of all rRNA is modified) and bases at specific sites possibly altering ribosomal structure, translational capability and ligand interaction[9,10,19,20]. The best characterized modifications in humans are: 1) ribose 2'-O-methylation mediated by the C/D box ribonucleoproteins (RNPs) complexes; 2) base methylation carried out by methyltransferases (MTases, stand-alone or enzymatic complexes); 3) pseudouridylation of specific uridine residues mediated by H/ACA box RNPs complexes; and 4) cytidine acetylation catalyzed by N-acetyltransferases (NAT)[9,10] (Figure 1). Generally, all these modifications confer major rigidity to RNA structure, protect RNA from nuclease action, favor a C3'-*endo* sugar conformation and base/nucleotide stacking capabilities[9].

Pseudouridylation and dyskerin

Among more than 100 different nucleotides modifications, pseudouridine (Ψ) is the first one that has been discovered in 1951 by Cohn and Volking, thereby named the "fifth nucleotide"[21]. At the beginning, the presence of Ψ residues was described in rRNAs, transfer RNAs (tRNAs) and small nuclear RNAs (snRNAs), but in recent years thanks to Next Generation Sequencing (NGS) techniques as PseudoSeq[22], PSI-Seq[23], Ψ-Seq[24] and CeU-Seq[25], pseudouridines have been detected in almost all RNA species in eukaryotes [e.g. in small Cajal Body-specific RNAs (scaRNAs), small nucleolar RNAs (snoRNAs), microRNAs (miRNAs) and messenger RNAs (mRNAs)][26]. The reaction of isomerization of specific uridines in pseudouridines is articulated in

different passages that may be synthetized in the breaking of the linkage between the N1 of the uracil base and the C1' of the ribose, followed by the formation of a new bond between the ribose C1' and the C5 of the uracil. This reaction of internal transglycosylation causes a 180° rotation of the nitrogenous base and the formation of a NH group that allows Ψ to form additional hydrogen bonds[26,27] (Figure 3).

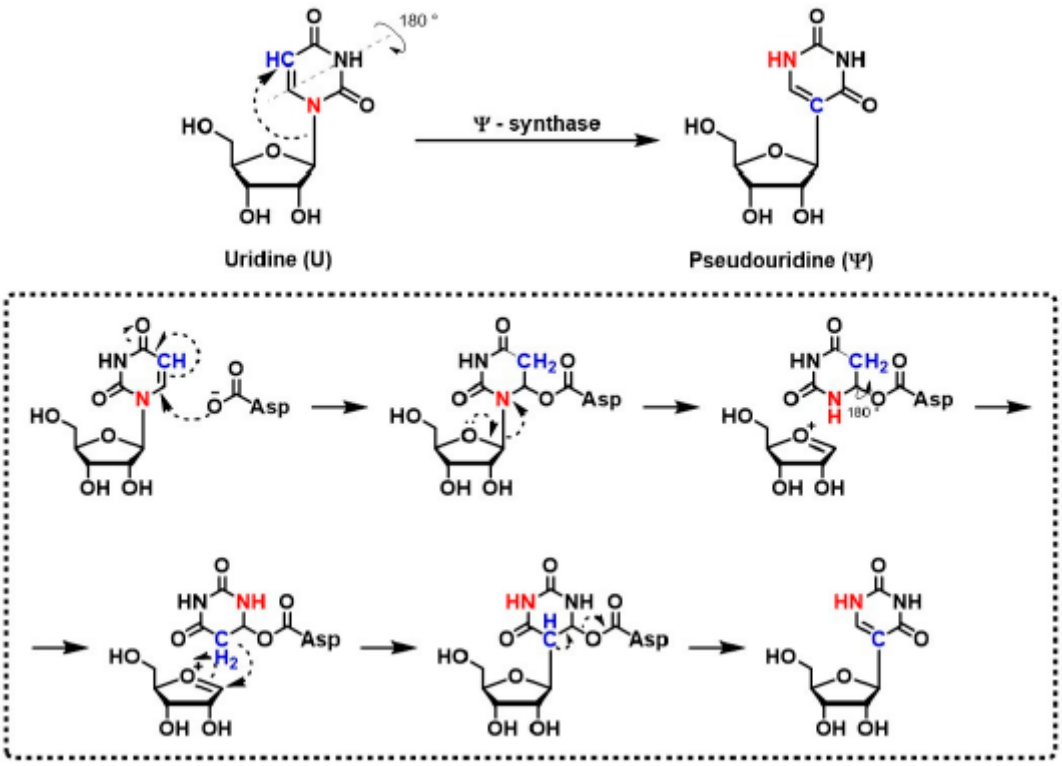

Figure 3 Example of reaction mechanism of the pseudouridylation reaction catalyzed by PUS1[26].

Due to the different molecular configuration, pseudouridines are characterized by distinct physical properties from uridines, such as increased rigidity of the phosphodiester RNA skeleton[28], increased strength of Ψ-A bonds and increased thermal stability up to 2°C [9,26,29].

The pseudouridylation reaction can be catalyzed by both stand-alone pseudouridine synthases (PUSes) and by H/ACA RNPs complexes, which will be further described in this context.

The H/ACA box RNPs consist of an antisense guide snoRNA and four core proteins: dyskerin, which has the catalytic activity, NHP2, NOP10 and GAR1 that have RNA binding and structural functions[30]. The RNPs complex is guided to the specific uridine to be modified by a class of snoRNAs, localized in the nucleolus or in Cajal bodies (scaRNAs[31]) and provided with unique sequence features[32]. These snoRNAs have a complex structure composed by two hairpins linked by a hinge (H) region. The first hairpin is indeed followed at the 5' end by the H box ANANNA, where N is intended for any nucleotide and which seems to be important for the assembly of the RNP complex. At the 5' of the second hairpin instead, there is an ACA box formed by three nucleotides before the 3' terminus, which has the function to stabilize the entire complex. Guide RNAs displaying these sequence features are therefore named H/ACA box snoRNAs. The two antisense motifs located near the hairpins give rise to the "pseudouridylation pocket", which allows the site-specific pairing with uridines that needs to be isomerized to pseudouridines[10,33] (Figure 4).

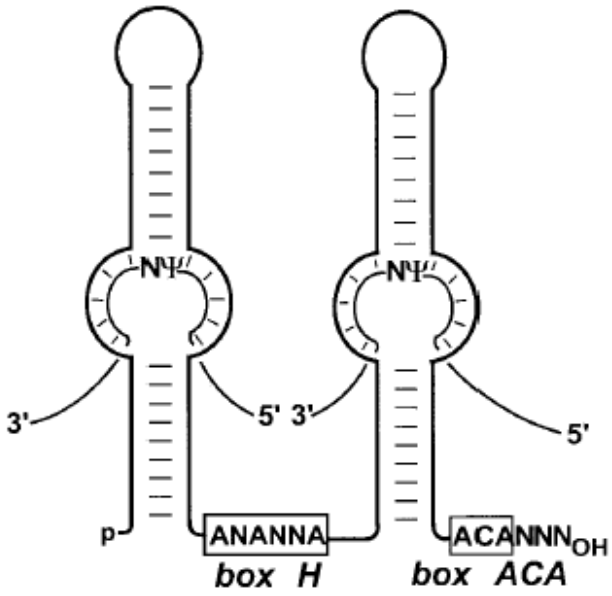

Figure 4 Structure of H/ACA box snoRNAs. Adapted from Kiss et al[33].

The catalytic subunit of the RNPs complex is dyskerin, a 58 KDa nucleolar protein encoded by the DKC1 gene which sequence, mapping on Xq28 chromosome, is ubiquitous and highly conserved in nature[34]. In fact, DKC1 homologues have been identified in Saccharomyces cerevisiae named *Cbf5*[35], in rats as *Nop57*[36] and in Drosophila melanogaster as *Nop60B*[37]. Dyskerin structure is constituted by three different domains: the Dyskerin-like domain (DKCLD) present in eukaryotes and archaea with unknown function; the catalytic TruB_N domain and the PUA (Pseudouridine synthase and Archaeosine transglycosylase) domain which has a common RNA recognition surface allowing the snoRNAs binding[38–40]. Dyskerin has also nuclear localization signals (NLS, lysine rich) and nucleolar localization signals (NoLS) important for intracellular localization[38,41] (Figure 5).

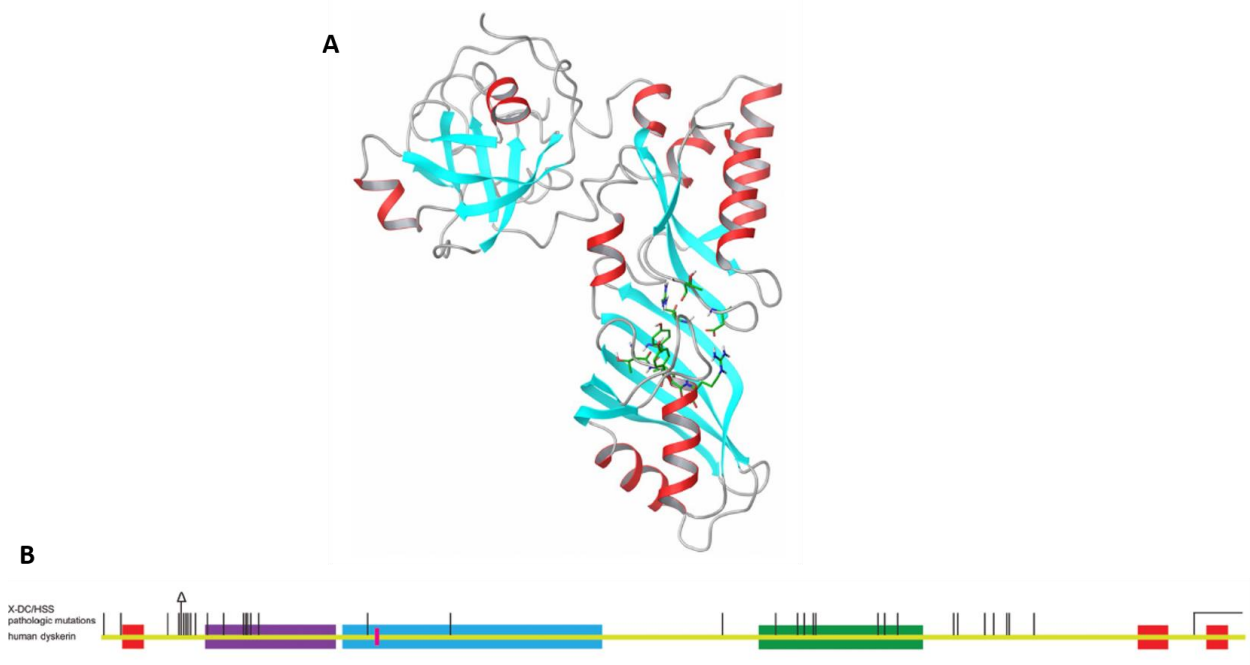

Figure 5 A) Structure of human dyskerin. Adapted from Rocchi et al[42]. B) Organization of human dyskerin domains. N- and C- terminal red boxes represent lysine-arginine rich NLSs; the DKCLD domain is purple, the TruB_N domain in blue, and the PUA domain in green; the pink rod within the TruB_N domain marks the catalytic aspartic acid residue. Adapted from Angrisani et al[43].

Dyskerin is a pleiotropic protein involved in several cellular processes, the main two being RNA modification/processing and telomerase complex stabilization. As described before, dyskerin is part of the pseudouridylation complex (Figure 6A) and so participating in the correct maturation of rRNAs. On the other hand, dyskerin is part of the telomerase complex, in which it stabilizes the human Telomerase RNA (hTR or TERC), characterized by the same H/ACA box structure described above (Figure 6B).

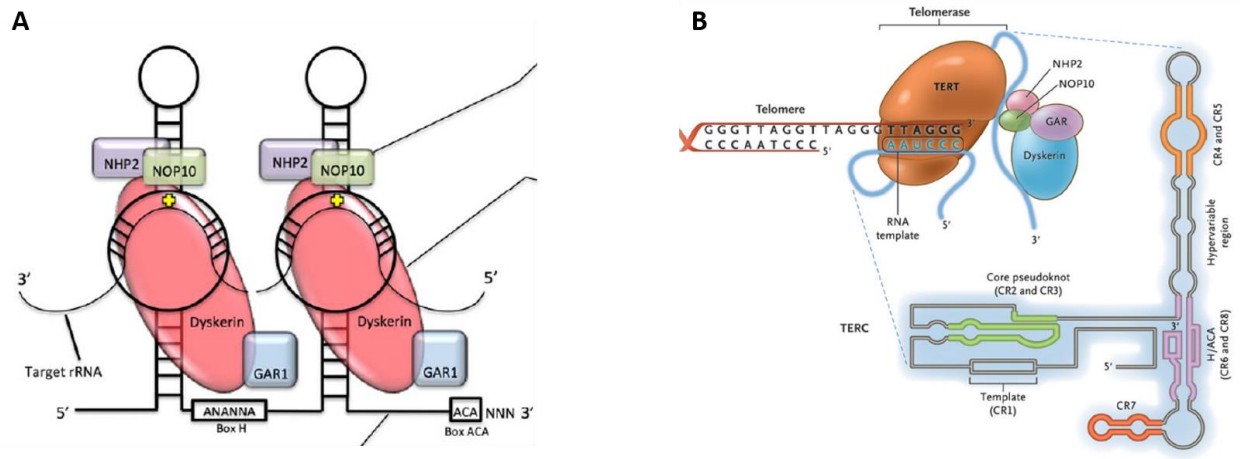

Figure 6 Graphic representations of human dyskerin complexes. A) Pseudouridylation complex. Adapted from Penzo and Montanaro[44]. B) Telomerase complex. Adapted from Calado and Young[45].

Ribosome biogenesis, dyskerin and cancer

The relationship between ribosome biogenesis and cancer is well documented in literature. The first observation was made by Pianese in 1896, who found that more aggressive tumors are characterized by larger nucleoli[46]. During the years, others studies confirmed that highly proliferating tumor cells have an increased need of protein synthesis, therefore leading to increased ribosome biogenesis and nucleoli dimension[12].

Focusing on breast cancer, it has been demonstrated that more aggressive forms with mutated p53 and pRb pathways are characterized by increased ribosomal biogenesis, larger nucleoli and worst prognosis[12] (Figure 7).

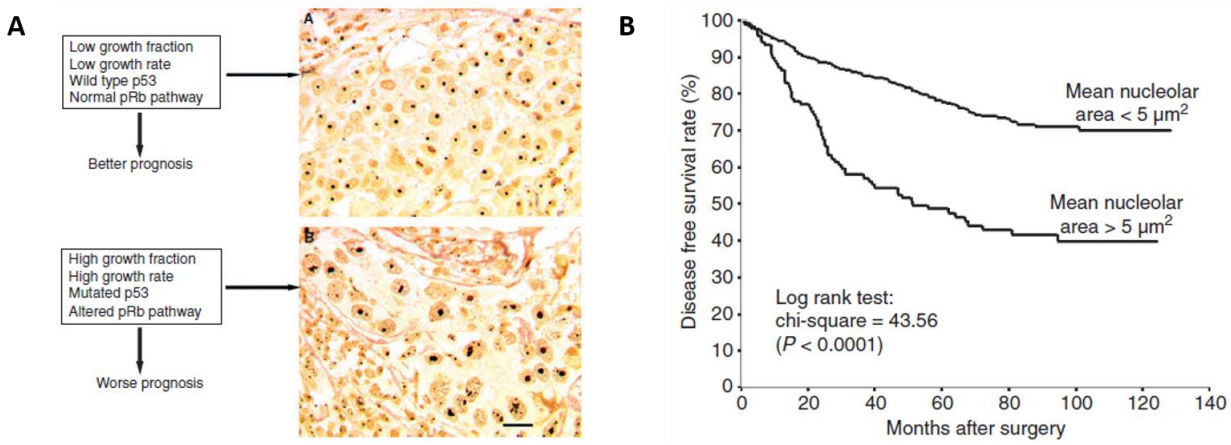

Figure 7 Correlation between tumor suppressor alterations, nucleolar dimension (A) and disease-free survival (B) in breast cancer. Adapted from Derenzini et al[12].

Some enzymes involved in rRNA processing can be altered in cancer, leading to accumulation of 45S rRNA precursor and increased nucleoli dimension[47]. Furthermore, p53 or pRb are frequently mutated in tumors with higher ribosome biogenesis rate and can interfere with rDNA transcription. Moreover, rRNA modifications are altered in different types of cancers also leading to defect in translation[9,10,26]. Finally, RPs themselves are linked to cancer development[48]: for example RPL5 and RPL10 are mutated in multiple cancer types[49,50]. In addition, RPs are characterized by extra-ribosomal functions that are linked with cell proliferation, invasion and migration capabilities, apoptosis and cell cycle arrest[51].

Dyskerin, a key protein for ribosomes biogenesis, has been linked to cancer in different tissues. The first correlation between dyskerin and cancer was made since germline DKC1 mutations are at the basis of the inherited syndrome termed X-linked Dyskeratosis Congenita (X-DC) characterized by failure of proliferating tissues and increased susceptibility to cancer[52,53]. Ruggero et al in 2003 reproduced X-DC patients' phenotype generating hypomorphic mice with 30% of normal dyskerin levels, indicating a tumor suppressor role for dyskerin as its partial lack and/or reduced function lead to cancer onset[6,54,55]. In X-DC patients as in hypomorphic mice, tumor susceptibility has been linked to pseudouridylation defects[24,54,56], snoRNAs levels perturbation[57] without involving the telomerase complex disruption[54].

Although mutations in DKC1 sequence are not so frequent in sporadic cancers[58], *in vitro* models of dyskerin reduced levels shed light on impaired cellular translation capabilities, specially of IRES-containing mRNAs. Internal Ribosomes Entry Sites (IRES) were discovered in viral mRNAs first and successively in some human mRNAs, and are nucleotide sequences where ribosomes can start translation independently from the presence of canonical CAP-dependent translation initiation factors[59,60]. Different studies demonstrated that dyskerin-depleted cells have defective translation of anti-apoptotic factors as Bcl-xL and XIAP[61], tumor suppressor genes as

p53[62] and p27[63], together with increased translation of oncogenic mRNAs such as VEGF and Hsp70[64]. Furthermore, in 2015 Penzo et al. demonstrated that ribosomes isolated from dyskerin-depleted cells have intrinsic defects that alter their translation capabilities, making them more unfaithful during translation (impaired translational fidelity), mis-incorporating amino acids or not recognizing stop codons[60]. In the same work, Penzo et al confirmed also the elongation defects of these ribosomes, previously mentioned by Jack et al[19,60]. Taken together, these findings show an intricate scenario that links Dyskerin down-regulation to cancer onset, underlying its role as tumor suppressor. On the other hand, up to date different studies show that DKC1 is significantly amplified or overexpressed in several human cancers and often correlated to more aggressive forms and worse prognosis. This is true for example in prostate[65], liver[66], ovary[67], lung[68], biliary tract[69], nervous system[70] and breast[71] cancers. Montanaro et al demonstrated that tumors' biopsies from patients with higher dyskerin expression have an increased ribosomal pseudouridylation level together with higher hTR expression levels compared to tumors' biopsies with lower dyskerin expression. Furthermore, patients bearing dyskerin overexpressing tumors display a worse prognosis compared to patients with tumors expressing lower dyskerin levels[71] (Figure 8).

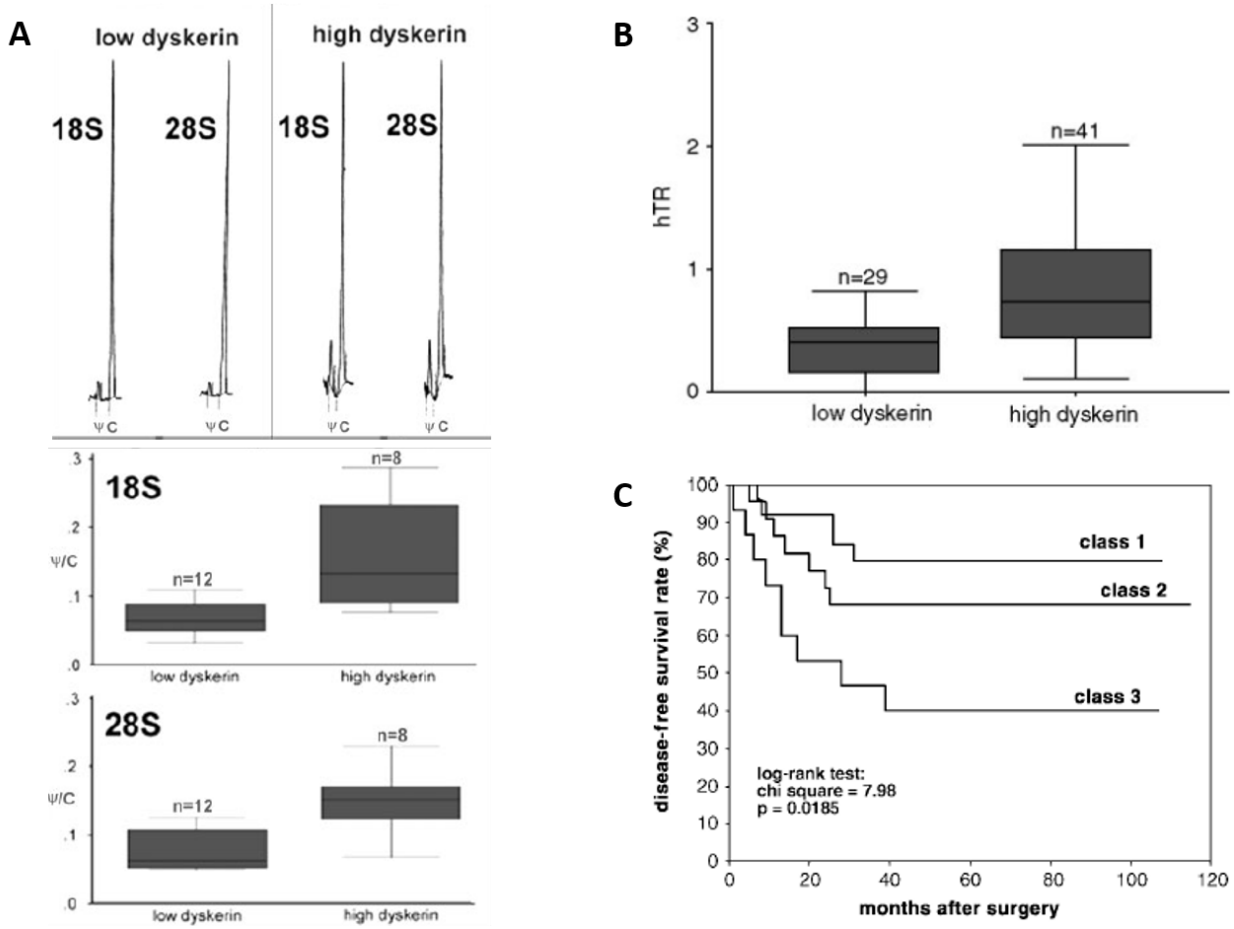

Figure 8 Dyskerin overexpression correlates with increased levels of 18S and 28S rRNAs pseudouridylation (A), stabilization of hTR (B) and lower disease free survival (C) in breast cancer. Adapted from Montanaro et al[71].

In the same study, Montanaro et al found no correlation between dyskerin expression and nucleolar dimension, defining dyskerin expression as an independent prognostic parameter as proxy of tumor malignancy[71].

Literature shows evidences that, not only dyskerin down-regulation, but also dyskerin overexpression may play a key role in neoplastic transformation, but the molecular insights are still unexplored. In 2011 Alawi et al mentioned that transient overexpression of dyskerin did not have remarkable effects in terms of proliferation and telomerase activity (data not shown) in both telomerase-positive and negative cells lines[72]. Interestingly, they demonstrated that

dyskerin is required to sustain cellular growth independently from its role in telomerase complex and only partially for its involvement in rRNA processing[72,73]. Finally, the same group previously demonstrated the direct correlation between c-Myc and dyskerin expression, namely that an increased expression of c-Myc corresponds to higher dyskerin levels, thus explaining the increased ribosomes biogenesis and transcription for these types of cancer[74]. It is plausible that in tumors other factors, apart from c-Myc, can be involved in dyskerin expression regulation and its role in the development of human cancer needs to be further investigated.

Translational control and cancer

As described before, ribosomes are complexes made by RNAs and proteins, structured in two different subunits, 60S and 40S, exported in the cytoplasm to form the mature 80S ribosome. The 40S subunit has the mRNA binding site that permits its recruitment on the m^7G-CAP 5' structure of the mRNA, which is responsible for the translation initiation. The 60S subunit has three RNA binding sites for tRNAs. tRNAs are highly structured small RNAs that bind both amino acids and mRNAs to pair them through codon-anticodon sequence recognition and transfer specific amino acids from the cytoplasm to the growing peptide. The three tRNAs binding sites are named P, A and E sites. The P site is the Peptidyl site and contains the tRNA bound to the nascent peptide; the A site, or the Acceptor site, houses the aminoacyl-tRNA with the new amino acid to be added to the polypeptide chain; the E site, is the Exit of the completely translated protein that is released into the cytoplasm[75].

Translation is structured in four main phases: initiation, elongation, termination and ribosomes recycling. The vast majority of cellular mRNAs are translated through the canonical m^7G-CAP-dependent mechanism[76]. As for transcription, also for translation initiation is required a Pre-

Initiation Complex (PIC) formed by the 40S ribosomal subunit, the Eukaryotic translation Initiation Factors (eIFs family - eIF1, eIF1A, eIF3, and eIF5), a trimer of eIF2 called Ternary Complex (TC), the starting methionyl-tRNA and GTP. The whole complex is called 43S PIC[75] (Figure 9).

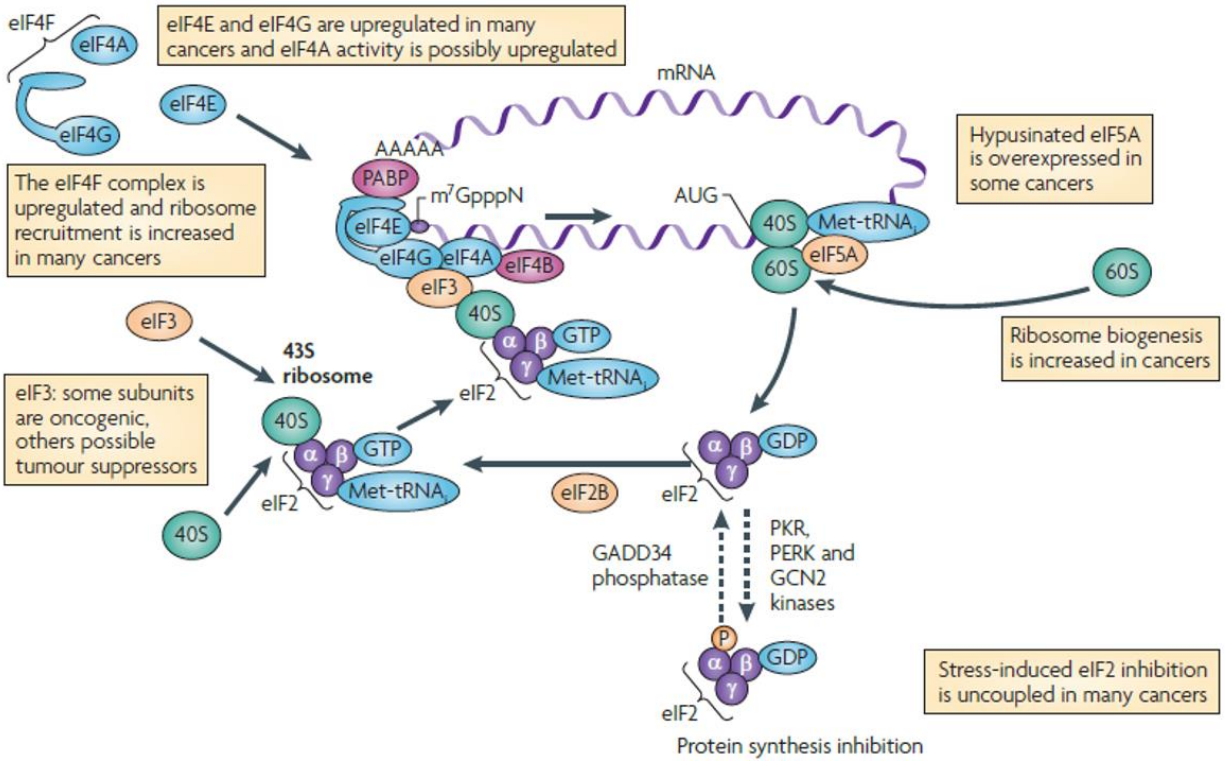

Figure 9 Graphical representation of m^{7}G-CAP-dependent translation initiation in eukaryots[77].

Other members of the eIFs, as eIF4E, eIF4F and eIF4A participating in the recruitment of the mRNA forming the 48S PIC. In particular, eIF4A, as a DEAD- box RNA helicase, helps the 40S ribosomal subunit in the scanning through the structured 5'-UnTraslated Regions (5'-UTR). At this point, the releasing of all the eIFs, determine the beginning of the elongation phase[78–80]. Nearly the 10% of cellular mRNAs utilize a CAP-independent translation initiation mode, the so-called IRES-dependent translation initiation, in which ribosomes bind directly to the mRNA without the need of all the above-mentioned accessory factors. This initiation modality is often preferred in stress conditions, e.g. hypoxia, and in many cancer cells[76,81].

During the elongation phase, tRNAs enter the A site, where the decoding takes place at the 40S side (mRNA-codon/tRNA-anticodon), and translocate to the P site where the nascent peptide chain extends its amino acids sequence[75]. To do so, eukaryotic Elongation factors (eEFs) are required. These enzymes work in a GTP-dependent manner, hydrolyzing GTP to unlock the ribosome and modify its structure to permit tRNA translocation. After that, the tRNA is deacylated at the E-site and the new protein is released. Recent findings did not still clarified if the exit of the polypeptide and the entrance of a new aminoacyl-tRNA in the A site are simultaneous or not[82,83]. Lastly, translation termination is determined by the presence of a stop codon, which cannot be paired to any anticodon and is recognized by the eukaryotic Release Factor 1 (eRF1). eRF1 together with eRF3 mediate the releasing of the newly synthetized protein[75,84].

The entire process of translation is tightly regulated and connected with lots of cellular pathways; deregulation of each phase paves the way to neoplastic transformation[76]. Noteworthy, each factor involved in translation initiation/elongation/termination can be altered and involved in neoplastic transformation. For example, several studies demonstrates that eIFs are overexpressed in different types of malignancies and often correlate with patients worse prognosis[76–78]. The other components of the translation machinery can be also altered and usually overexpressed in human cancer cells that take advantage of these alterations to satisfy their need of proteins and cellular components. Several translation inhibitors are currently in trials for cancer treatment[78].

Beyond the canonical key regulators of translation (Figure 10), other elements regulate this process: e.g. miRNAs, non-coding RNAs (ncRNAs), IRES-Trans Acting Factors (ITAFs) and RNA Binding Proteins (RBPs) frequently interact with the translation machinery and participate to neoplastic transformation[75].

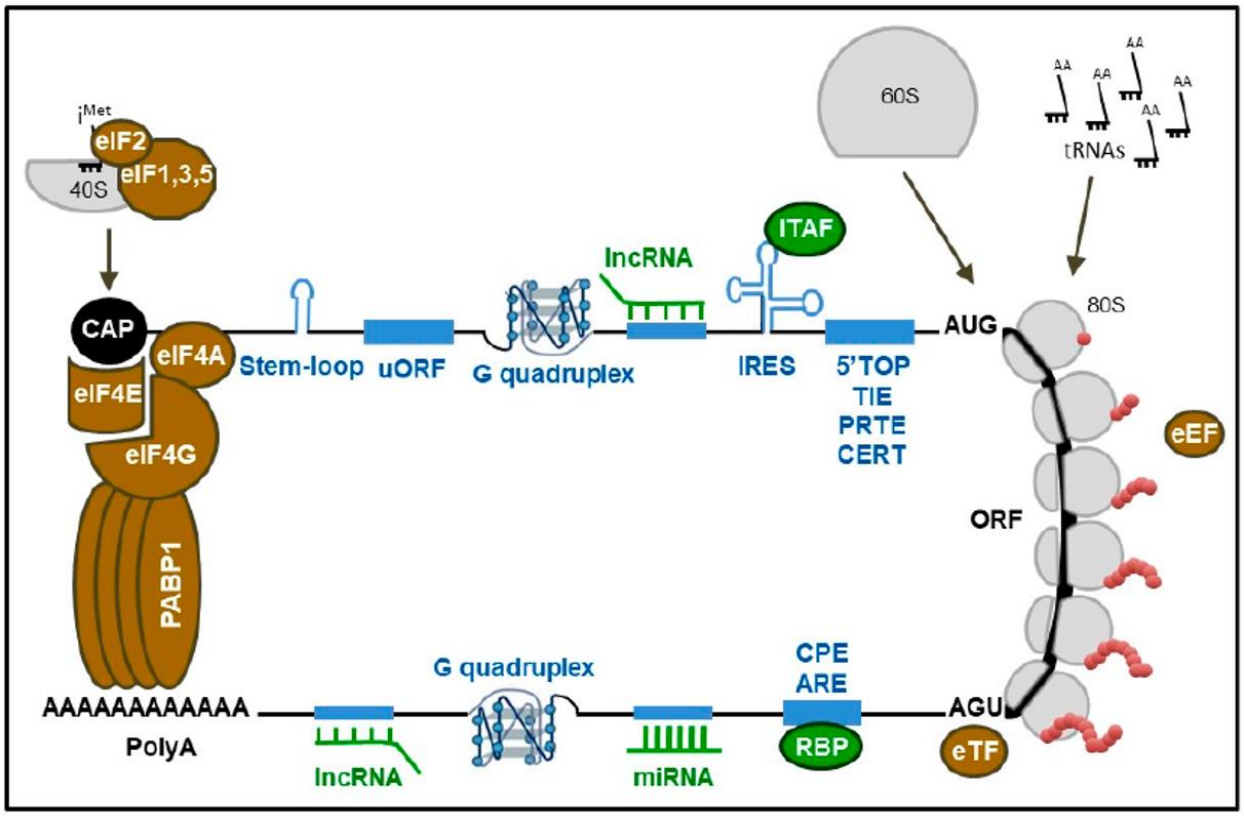

Figure 10 Canonical and *cis*-/*trans*- regulators of mRNA translation in eukaryots[75].

A quite recent review of Truitt and Ruggero describes in detail another class of elements that strongly impact on translation, which are called *cis*-regulators and comprehends structural/sequence-related elements of mRNAs and tRNAs or even ribosomes intrinsic diversities related to cancer development[82]. Regarding structural features of mRNAs, 5'-UTRs tends to be usually unstructured to be easily translated, but some mRNAs display complex secondary structures that inhibit translation; this problem is overcome in cancer by the overexpression of the helicase eIF4A that easily unwinds these structures. It is not by chance that several oncogenes involved in cellular proliferation (e.g. c-MYC) or angiogenesis (e.g. VEGF) have highly structured 5'-UTR[77,82]. Importantly, also the IRES sequences described above are classified as 5'-UTR structural complexities and, as said before, preferred by cancer cells. Between sequence-specific 5'-UTR modifications, some mRNAs have Alternative Translation

Starts (ATS) that can move the initiation starting point from the canonical Open Reading Frame (ORF) to an Upstream ORF (uORF) leading to the production of a different protein[82,85]. Some studies demonstrate that oncogenes and genes involved in fundamental cellular pathways, as proliferation, have uORFs and that this initiation modality is frequent in human disease[82,86,87]. In addition, cancer cells developed mechanisms to overcome the inhibition of the use of uORFs as translation initiation starts[82].

The oncogenic gene expression is also regulated by the presence of specific sequences at the 3'-UTR of mRNAs; these regions often contain miRNAs-binding sites or RBP motifs that participate in the inhibition of mRNAs translation. Some mRNAs isoforms with shortened 3'UTR lack of these binding sites, leading to the loss of miRNAs/RBPs translation inhibition and so to increased gene expression: for these reasons, this shorter sequence is preferred in cancer cells[82,88–90].

Among the class of RNA involved in cancer development, also tRNAs are singled out. The cellular pool of tRNAs is constituted by the so called "isoacceptor" tRNAs, that are tRNAs charged with the same amino acids, thought to be in excess in normal conditions[82]. Recently, NGS studies demonstrate that some species of tRNAs are overexpressed in tumor cells, implicating that also the "choice" or a modification of tRNAs may have a role in neoplastic transformation[82,91,92]. As explained by Truitt and Ruggero, the overexpression of tRNAs is not a mere consequence of the increased protein synthesis request, because only a subset of tRNAs is altered and can stimulate translation elongation factors for specific mRNAs[82]. In addition, the tRNAs usage has an intrinsic bias due to the tissue specific differential expression of tRNAs[93]. Moreover, it has been proven that there is a direct correlation between tRNA-codons pool, tRNAs expression levels and gene expression, especially for pathways that mediate proliferation and cell differentiation[82,94]. Finally, tRNAs are subjected to modifications that influence their

expression: e.g. as cited before, pseudouridylation in tRNAs was among the first to be identified in human RNAs and it confers stability to the tertiary structure of the molecule[26,95]. The presence of Ψs in tRNAs codon sequence has been found in several human diseases and, in some cases, is causative of non-canonical pairing to mRNAs and amino acid mis-incorporation[26,96].

Finally yet importantly, the main protagonist of translation process, the ribosome can be "specialized" to be an onco-ribosome. For example, the presence of mutated RPs in mature 80S ribosomes often confers a functional cellular advantage and it has been well demonstrated that ribosomes can "prefer" to translate a subset of mRNAs, especially IRES-containing mRNAs, involved in neoplastic transformation[48,97].

The process of translation is heavily complicated; taken together these findings let imagine a collaboration between specialized onco-ribosomes (possibly disease/tissue specific?[97]) that can translate preferably specific mRNAs provided with peculiar sequence/structure characteristics therefore promoting and/or contributing to cancer development.

AIM OF THE WORK

Dyskerin is a nucleolar protein encoded by the DKC1 gene which sequence is highly conserved in nature[34]. Dyskerin is involved in several cellular processes, the main two being RNA modification/processing and telomerase complex stabilization. As to the function of RNA modification, it is part of the pseudouridylation complex, in which it has the catalytic activity necessary to isomerize specific uridines residues to pseudouridines mainly on ribosomal RNA (rRNA) and small nuclear RNA (snRNA). In this step, dyskerin is guided by a class of small nucleolar RNAs (snoRNAs), localized in the nucleolus or in Cajal bodies (scaRNAs) and provided with unique sequence features[32]. On the other hand, dyskerin is part of the telomerase complex in which it stabilizes the human Telomerase RNA component (hTR or TERC).

Germline DKC1 mutations are at the basis of the inherited syndrome termed X-linked Dyskeratosis Congenita (X-DC) characterized by failure of proliferating tissues and increased susceptibility to cancer[52,53]. X-DC associated DKC1 mutations alter dyskerin function leading to decreased levels of rRNAs pseudouridylation at specific sites[19,98] and reduced hTR stability with consequently dysregulated telomerase complex activity[99].

In parallel, several tumors types, like for example breast[71], prostate[65], liver[66], and lung[68] cancers, are characterized by an increased dyskerin expression and worse prognosis. In particular, focusing on breast cancer, in a previous study Montanaro et al demonstrated that tumors from patients with higher dyskerin expression have an increased ribosomal pseudouridylation level together with higher hTR expression levels compared to tumors with lower dyskerin expression[71]. Furthermore, patients bearing dyskerin overexpressing tumors display a worse prognosis compared to patients with lower dyskerin levels[71].

Although several studies demonstrated the role of dyskerin as a tumor suppressor[42,53,54,63,100], literature lacks of works experimentally testing the effect of dyskerin overexpression. The aim of this PhD project is to study both the cellular and the molecular *in vitro* effects correlated to dyskerin overexpression generating DKC1 overexpressing cell lines through a stable retroviral transduction approach.

MATERIALS AND METHODS

Cell Culture and generation of DKC1 overexpression models

MCF10A were cultured in DMEM 1 g/L glucose supplemented with 250 U/L of insulin, 0,5 µg/ml of hydrocortisone, 10 ng/ml of epidermal growth factor, 20% Fetal Bovine Serum (FBS), 2 mM L-Glutamine, 100 U/ml Penicillin and 1 mg/ml Streptomycin.

MCF7 were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-Glutamine, 100 U/ml Penicillin and 1 mg/ml Streptomycin.

MDA-MB-231 were cultured in DMEM 4.5 g/L glucose supplemented with 10% FBS, 2 mM L-Glutamine, 2,5X MEM non-essential amino acids, 100 U/ml Penicillin and 1 mg/ml Streptomycin.

To generate stable DKC1 overexpression cell models, all cell lines were infected with Moloney Murine Leukemia Virus (MoMLV) containing control plasmid {pMMLV[EXP]-Bsd(IRES:Bsd) then named CTRL)} or hDKC1 sequence {pMMLV[EXP]hDKC1[NM_001363.3]:IRES:Bsd then named DKC1 OE}. After the infection cells were selected for at least 10 days with blasticidin (8 µg/ml MCF10A; 5 µg/ml MCF7; 14 µg/ml MDA-MB-231), dyskerin overexpression was verified both at mRNA (Real-Time PCR) and protein levels (Western Blot). Halved blasticidin concentration was added in cell culture media for maintenance.

All cells were cultured in a monolayer at 37°C in a humidified atmosphere containing 5% CO_2.

RNA extraction and real-time RT-PCR

RNAs were extracted using PureZOL™ RNA Isolation Reagent (Bio Rad), following the manufacturer's specifications. cDNA synthesis was performed starting from 500 ng of RNA (10 ng for hTR levels evaluation) using iScript™ cDNA Synthesis Kit (Bio Rad) following protocol instructions.

Real-time PCR analyses were conducted with CFX96™ Real-Time detection System (Bio Rad). A semi-quantitative Taqman approach (SsoAdvanced Universal Probes Supermix Bio Rad) was used to evaluate the expression of DKC1 and b-glucuronidase as endogenous control (Applied Biosystems Hs 00154737_m1 and 4326320E, respectively). Regarding hTR levels evaluation a Taqman approach was used as described by Yajima et al[101]. A SYBR green approach (SsoAdvanced™ Universal SYBR® Green Supermix Bio Rad) was used to measure relative levels of snoRNAs (See Table 1).

Table 1 SnoRNAs primers sequences used for Real-Time PCR validation with a SYBR green approach.

SNORNAs NAME	FORWARD 5'-3'	REVERSE 5'-3'
SNORA64	GTGTGACTTTCGTAACGGGGA	TTGCACCCCTCAAGGAAAGAG
SNORA63	AGCAGGATTCAGACTACAATATAGC	GCTACAGGAGAATAGCAGACAG
SNORA81	AATTGCAGACACTAGGACCAT	GGACATTGGACATTAAGAAAGAGG
SNORA43	GGGCAAAGAGAAAGTGGCGA	GGCCATAAACCATTCTCAGTGC
SNORA46	TCTTGGTTACGCTGTAGTGC	ACTCTATACAGCAACAGCAGAAT
SNORA5A	AGCCGTGTCAAATTCAGTACC	GCCCATGAGTCACAGTGTTT
SNORA44	CATGCAAGAGCAACCTGGAA	TATAGGAAAGCTGAGTGGCAG
SNORA5C	AGTGCCCGTTTCTGTCATAGC	CAAACTTATCCCCAGGTCCCA

SNORA70	CCGACTGAGTTCCTTTCCACA	AGGCTGCGTACACTACCAAG
SNORA5B	AGCCATGTCAAATTCAGTGCCT	ACTGTTTCTGTGGCAGTCTTCT
SNORA12	CAAATGGGCCTAACTCTGCC	TCTCTGATGCAGGAAAGGCT
SNORA38	GTGTCTGTGGTTCCCTGTCTT	GGCCTCAAAGTTTCCCAAATCC
SNORA16B	GCTCCAGGTGCTTCCATGTAG	TCACCATCAAGGAAAACTGTCACT
SNORA59	GTATGTTCACGGGGCGATGC	TCTACGGGTAACTGAGGCAC
SNORA29	CATTTGACTACCACATTTTCTCCTA	TCCCTCTTCAGATCATGGCAAG
SNORA62	GGAGTTGAGGCTACTGACTGG	AGCGAAAACTTGCCCCTCAT
SNORA3	AGTCACGCTTGGGTATCGG	AGCCAGTGAATAAGGTCAGCA
SNORA67	TCAGGAAAGTAGCAGCTTGGA	CTAAGGAAGGCAGAGGAAAT
SNORA14B	CCCTCTTGGTAGCTTCGTCCTA	GACTGAGCCACGGGAGAA

For snoRNAs 64L2, 63L9, 70BL6, 67L1, 43L2 and 12L2, since they have no known target RNA, we decided to validate the levels the snoRNAs with homology in the pseudouridylation pocket sequence or whole sequence, hypothesizing a correspondence in the modified uridines.

Whole cell protein extraction and western blot analysis

Whole cell protein extraction was performed in lysis buffer [KH2PO4 0.1M pH 7.5, NP-40 1%, added with complete protease inhibitors cocktail (Sigma Aldrich) and 0.1 mM b-glycerolphosphate] for 20 minutes on ice and cleared by centrifugation at 14000 RCF for 20 minutes at 4 degrees. Protein extract was quantified spectrophotometrically with the Bio-Rad Protein Assay (Bio Rad). The same amount of proteins was separated in Laemmli loading Dye (2% SDS; 8% glycerol; 62,5 mM TRIS HCL PH 6,8; 0,005% bromophenol blue and 2% b-mercaptoethanol) by SDS PAGE in a polyacrylamide gel (TGX Stain-Free™ FastCast™ Acrylamide

Solutions Bio Rad) in Running Buffer (2,5 mM Tris, 19,2 mM Glycine and 0,1% SDS) at constant 200V for around 30 minutes. Proteins were then transferred on a PVDF membrane (Amersham Hybond P 0.45 PVDF GE) with Transfer Buffer (2,5 mM Tris, 19,2 mM Glycine, 20% MetOH) for 2 hours using a wet transfer device (Biorad). Dyskerin antibody was purchased from Santa Cruz Biotechnology (H-300 sc-48794); β-actin antibody was purchased from Sigma Aldrich (clone AC-74 No. A2228).

Cell invasion assay

Invasion assays were performed in blind well chambers (Neuroprobe Inc.) according to the manufacturer's instructions, using 13 mm-diameter polycarbonate filters (Neuroprobe Inc.) with pore size 8 µm. 5×10^4 cells, DKC1 overexpressing and controls, were seeded in the upper compartment in low FBS cell culture medium, [2% for MCF10A and 1% for MCF7 and MDA-MB-231 CTRL and DKC1 OE], while 20% and 10% FBS (respectively) in cell culture medium were placed in the lower compartment. After a 24 hours incubation at 37°C, 5% CO_2, filters were collected and washed with water, while cells were fixed in absolute ethanol for 1 min. Lastly, cells were stained with Giemsa stain (1:10 in water) at RT for 10 min and filters were washed again twice with water. The non-invading cells were scraped off with a cotton swab. Cells were visualized with a Leitz Diaplan light microscope (Wetzlar Germany) equipped with a video camera (JVC, 3CCD, KY-F55B, Jokohama, Japan) at 10× of magnification; five random fields for each filter were photographed and counted.

Clonogenic assay

In a single 6-well plate, 100 cells for MCF10A CTRL/DKC1 OE and for MDA-MB-231 CTRL/DKC1 OE or 250 cells for MCF7 CTRL/DKC1 OE respectively, were seeded. The colony number was evaluated 10–12 days later, after overnight fixation in 4% formalin at 4°C and staining with a 0.5% crystal violet solution in 25% methanol for 30 min. Cells were then washed 3 times in PBS and counted.

Generation of mammospheres

1.2×10^4 cells were seeded in ultra-low attachment 6-well plates and cultured in Mammary Epithelial Cell Growth Medium (MEGM, Bullet Kit, Lonza). Spheres started forming after 4–6 days and MS were counted between days 7 and 8 under an inverted microscope at 10× magnification.

Telomerase activity assay

Telomerase activity assay was performed following manufacturer's instruction (S7710 - TRAPEZE RT, Sigma Aldrich). In brief, 10^5 cells were lysed with 200 µl of CHAPS lysis buffer and 2 µl of each lysate were loaded for the Real-Time PCR analysis. Positive/negative controls and standard curve were loaded following protocol's instruction. Platinum™ Taq DNA Polymerase (Invitrogen) was used as antibody mediated hot start Taq polymerase for PCR reaction. Results were analyzed following protocol's instruction.

Plasmids preparation and in vitro translation assays

For whole cells system in vitro translation assays, 2 x 10^5 cells (MCF10A DKC1 OE and CTRL) were seeded in 6-well plates. The day after, cells were transfected with 400 ng of the report monocistronic transcript pR-LUC-F-LUC (a kind gift from Kim De Keersmacker, Department of Oncology, Laboratory for Disease Mechanisms in Cancer, KU Leuven) using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fischer), following protocol's instruction. After 5 hours, cells were lysed with 500 µl of Passive Lysis Buffer 5X and luminescence were measured following the instructions of Dual-Luciferase® Reporter Assay System (Promega).

For in vitro IRES-mediated translation assays with highly purified ribosomes we used the pR-CrPV-IRES-F plasmid (a kind gift from Dr. Davide Ruggero, Department of Urology, University of California, San Francisco, CA, USA)[61].

To make these plasmids suitable for in vitro transcription, the T7 promoter sequence was cloned upstream the luciferase gene after enzymatic digestion of the plasmids with HindIII. Capped mRNAs were transcribed from linearized plasmids using AmpliCap-Max T7 High Yield Message Maker kit (CellScript, Madison, WI, USA), following supplier's instructions.

mRNA translation efficiency assay was performed as described in Penzo et al[102].

Ribosomes Purification

Human ribosomes from MCF10A, MCF7 and MDA-MB-231 CTRL/DKC1 OE were purified as described by Penzo et al[102]. In brief, cells were lysed with a lysis buffer that allows the isolation of the cytoplasmatic fraction from the nuclei and mitochondria. After a short incubation of 10 minutes at 37° C, which permits ribosomes to finish translation and detach from the mRNAs

they were translating, up to 500 µl of the cytoplasmic lysate is loaded on discontinuous sucrose gradient and ultracentrifuged for 15 hours. The resulting pellet is resuspended in a suitable amount of a storage solution (10 mM Tris HCl pH 7.5, 2 mM magnesium acetate and 100 mM ammonium acetate in RNAse free water) and ribosomes were quantified following protocol's indications described in Penzo et al[102].

Global pseudouridylation quantification

Evaluation of ribosomal RNA global pseudouridylation was performed through HPLC analysis as described by Montanaro et al[71]. rRNA was extracted by highly purified ribosomes as previously described. At least 5 µg of rRNA were digested with 10U of Nuclease P1, ammonium acetate 50 mM pH 5.5 and $ZnCl_2$ 1mM for 1 hour at 37°C. The so obtained nucleotides were dephosphorylated by incubating with 1 Unit of alkaline phosphatase (Fluka, Sigma-Aldrich, St Louis, MO, USA) for 1 hour at 37°C after the addition of 0.4 vol of 50 mM Tris-base and 0.1 vol of 10 mM $MgCl_2$. The final volume of this reaction was 100 µl. The resulting nucleosides were then subjected to high performance liquid chromatographic (HPLC) separation in a Beckman System Gold Programmable Solvent Module 126 equipped with a detector Module 166 set at 254 nm (Beckman-Coulter, Fullerton, CA, USA). The column (0.39 × 30 cm) was a reversed-phase µBondapak C18 (particle size 10 µm) purchased from Waters Associates (Milford, MA, USA). Mobile phase conditions were 0.1 M phosphate buffer (pH 6)/methanol, 99:1 (v/v) for 12 min, 96:4 (v/v) for 13 min, and 85:15 (v/v) for 25 min. Pseudo-uridine and major nucleosides used as standards were purchased from Berry and Associates, Inc (Dexter, MI, USA).

SnoRNAs expression array

SnoRNAs expression analysis was performed by Arraystar company using the nrStar™ Human snoRNA PCR Array which contains 359 snoRNAs, 7 snoRNA target snRNAs and 4 snoRNP complex members. 2-5 µg of total RNA from MCF10A DKC1 OE/CTRL were shipped to Arraystar Inc. Experiment and data analyses were performed by Arraystar Inc (Rockville, MD).

SILNAS LC/MS based quantitation of Ψs

The SILNAS-based quantitation of the stoichiometry of Ψs was performed in collaboration with Toshiaki Isobe's group who developed the method described in Yamauchi et al[103] (Figure 11).

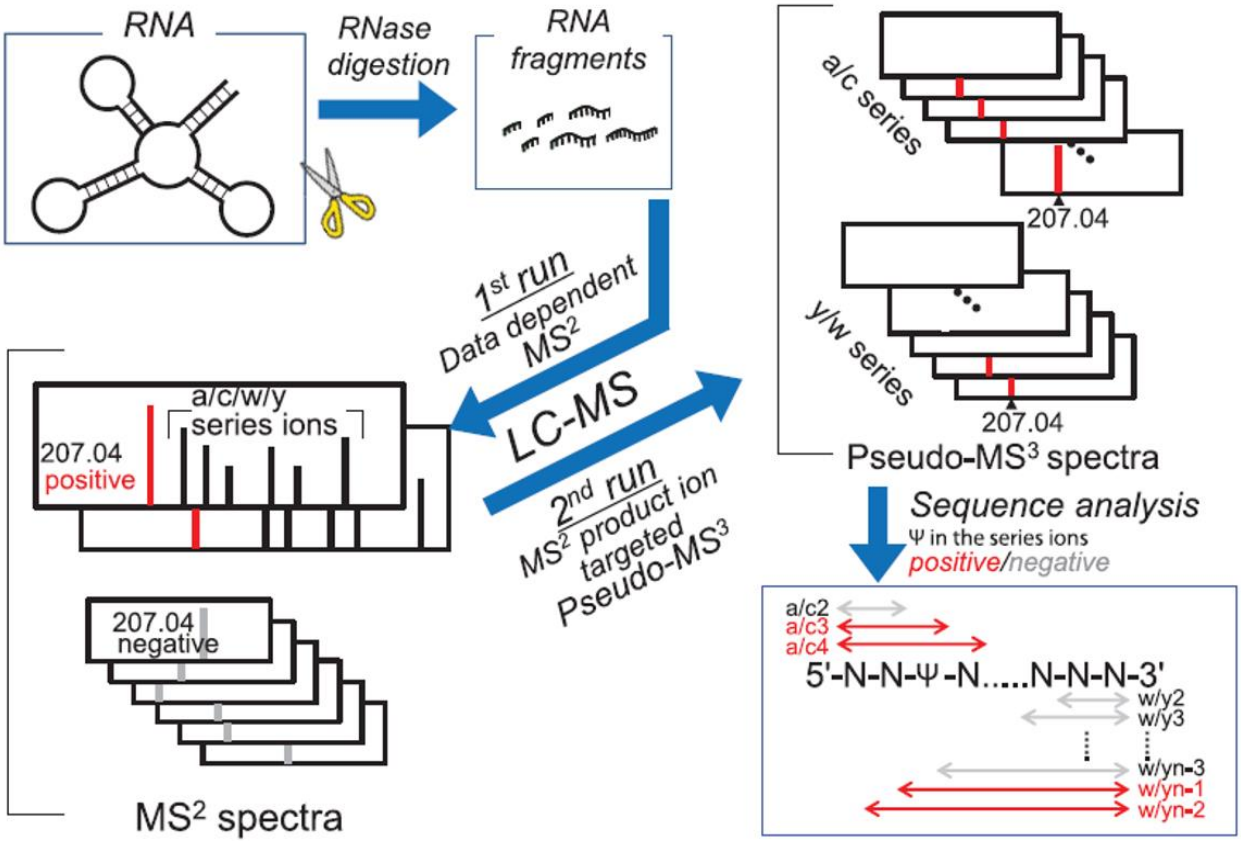

Figure 11 Scheme of the LC/MS method used to identify site-specific Ψs in RNA. Adapted from Yamauchi et al[103].

Patients' material

One hundred and seventy breast carcinomas were selected from a series of consecutive patients who underwent surgical resection for primary breast carcinoma at the Surgical Department of the University of Bologna on the sole basis of frozen tissue availability for DKC1 mRNA expression determination. Part of the cases were obtained from a previous study[71] while additional samples were collected after 2011. Data on tumor histological classification, grading, size and TNM classification were obtained as described[71]. Surrogate bioprofile classification of the cases on the basis of histological results was performed according to St. Gallen 2017 consensus[104]. Informed consent was obtained from all individual participants included in the study.

RESULTS

DKC1 overexpression confers biological features of the neoplastic phenotype in untransformed mammary epithelium cells.

To investigate the effect of dyskerin overexpression in tumors we generated breast cancer cellular models inducing stable DKC1 overexpression through a retroviral transduction in three cell lines with different basal dyskerin expression representing distinct levels of transformation: from lower to higher, MCF10A, MCF7 and MDA-MB-231. Cell lines were infected with the MoMLV containing or control plasmid {pMMLV[EXP]-Bsd(IRES:Bsd) then named CTRL: control} or hDKC1 sequence {pMMLV[EXP]hDKC1[NM_001363.3]:IRES:Bsd then named DKC1 OE: DKC1 overexpressing cells} (Figure 12).

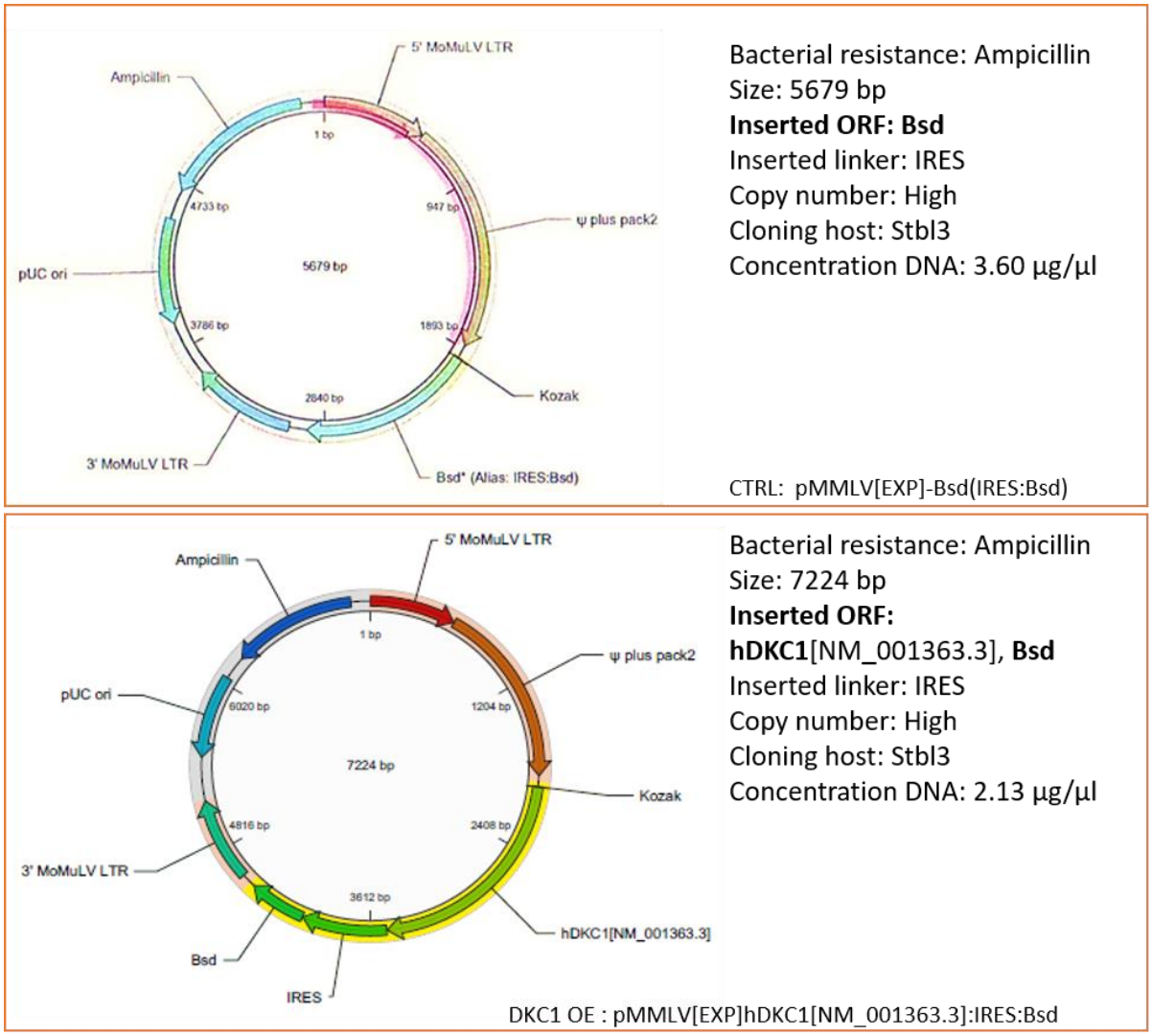

Figure 12 Schemes of control (up) and human DKC1 expression plasmids (down).

After selection, we verified that DKC1 had been successfully overexpressed both at mRNA and protein levels for all the three cell lines (Figure 13). In general, the greatest increase in dyskerin overexpression had been achieved in MCF10 cells, which initially displayed the lowest dyskerin expression.

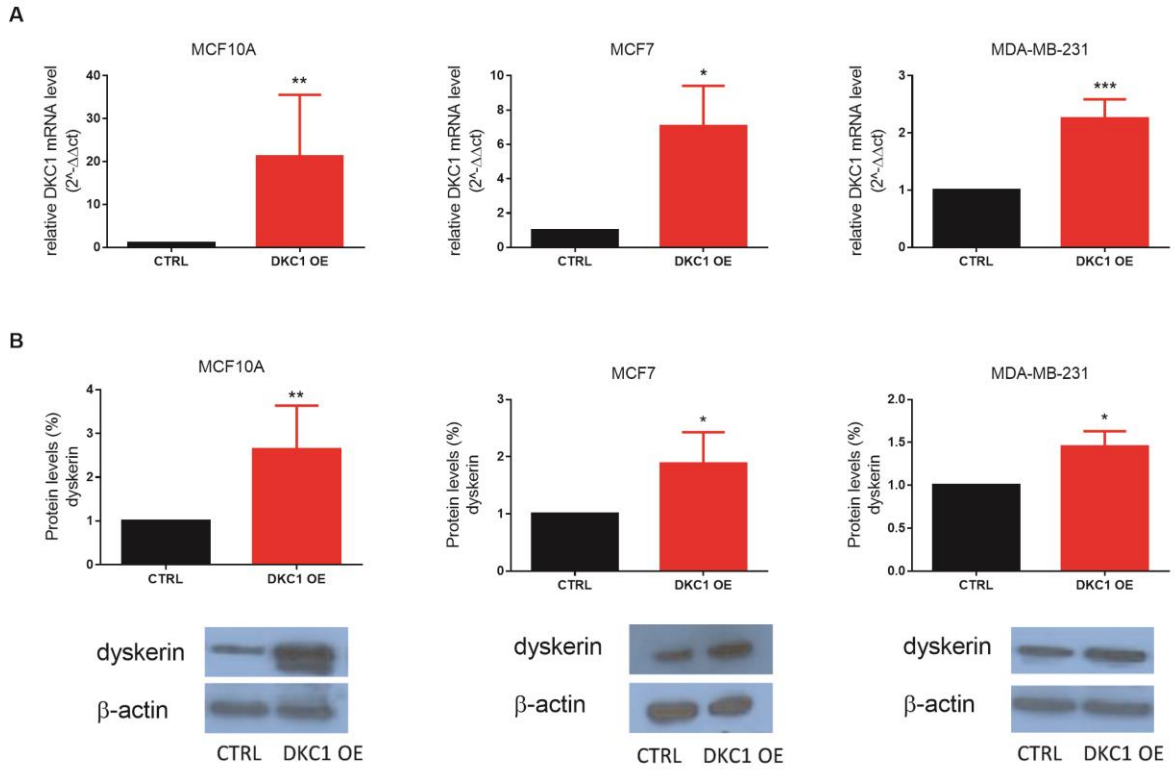

Figure 13 A) DKC1 mRNA expression evaluated in Real-Time PCR. B) Quantification of dyskerin expression after Western Blot analyses. Data were analyzed by unpaired Student's T-test: *P < 0.05; **P < 0.01; ***P < 0.001.

We then performed *in vitro* assays to evaluate the effect of DKC1 overexpression on invasive, stemness and clonogenic potentials. Our results show that increasing DKC1 expression confers a more aggressive phenotype, in terms of increased number of invasive cells, number of colonies and mammospheres, only in untransformed mammary epithelium cells MCF10A in respect of control cells (Figure 14).

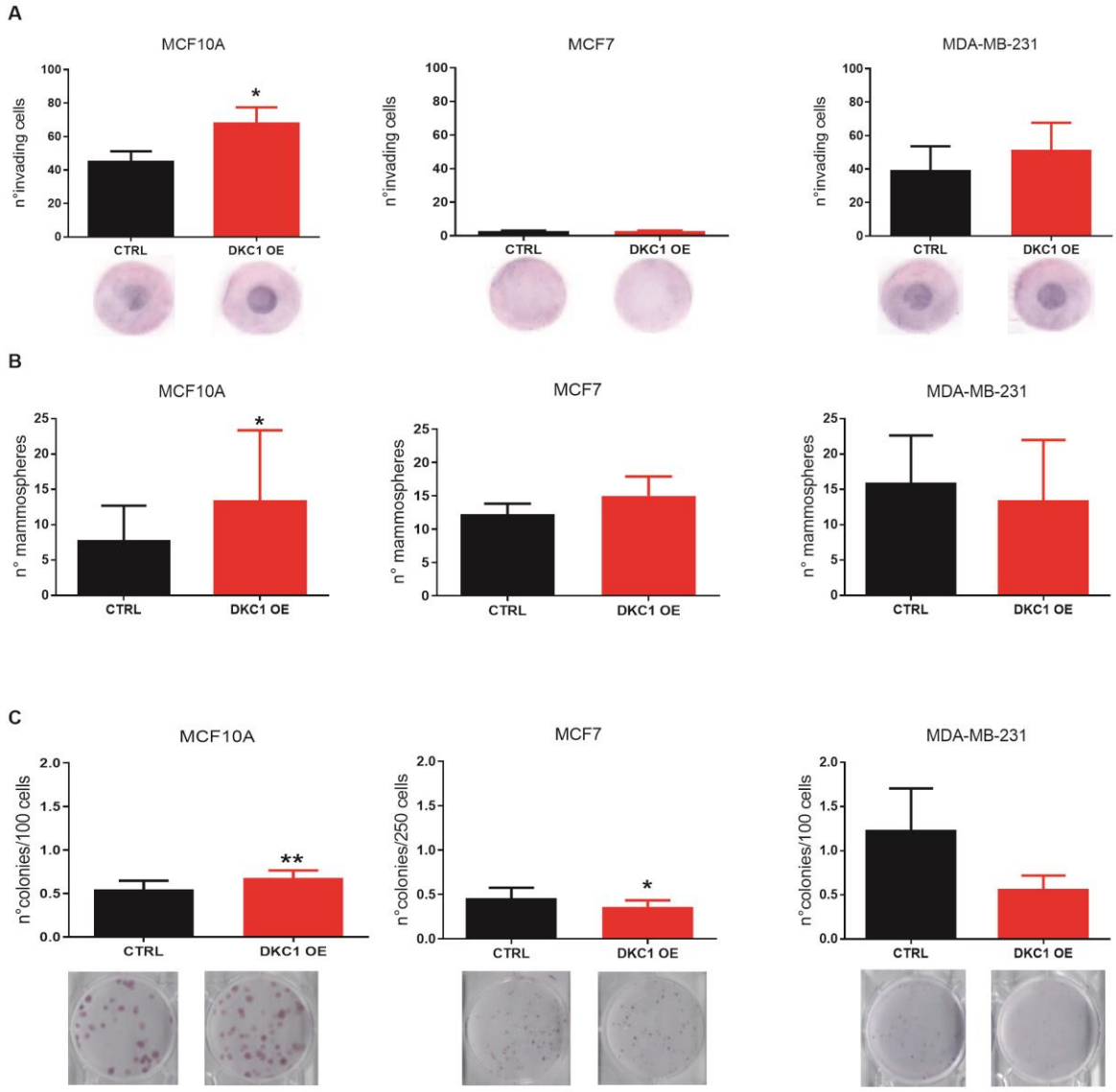

Figure 14 A) Invasive potential assay through Boyden chambers. Data were analyzed by unpaired Student's T-test: *P < 0.05; **P < 0.01; ***P < 0.001. **B**) Stemness potential assay through mammospheres formation. Data were analyzed by paired Student's T-test: *P < 0.05; **P < 0.01; ***P < 0.001. **C**) Clonogenic potential assay. Data were analyzed by paired Student's T-test: *P < 0.05; **P < 0.01; ***P < 0.001.

DKC1 overexpression induce significant increase of telomerase RNA component without influencing telomerase activity.

As mentioned in the Introduction, Montanaro et al demonstrated that breast tumors with higher dyskerin expression show an increased stabilization of hTR[71]. For this reason, we verified if dyskerin overexpression influences telomerase complex and activity. We evaluated hTR levels, finding a significant increase in MCF10 and MCF7 cells after DKC1 overexpression (Figure 15A). In addition, to check if there could be effects on telomerase activity, we performed the Real-Time PCR based TRAPeze® commercial kit (Sigma-Aldrich cat. No. S7710) on MCF10A DKC1 overexpressing and controls cells. Our results show no significant changes in telomerase activity (Figure 15B). Previously, Montanaro et al demonstrated that dyskerin mRNA levels are stricktly related to hTR and that DKC1 silencing in MCF7 cells lead to a drop in telomerase activity[71]. As we discussed before, telomerase activity can be impaired when the fundamental component of the complex, hTR, is missing; on the other hand, an increased stabilization of hTR may not be sufficient to increase the activity of the whole complex.

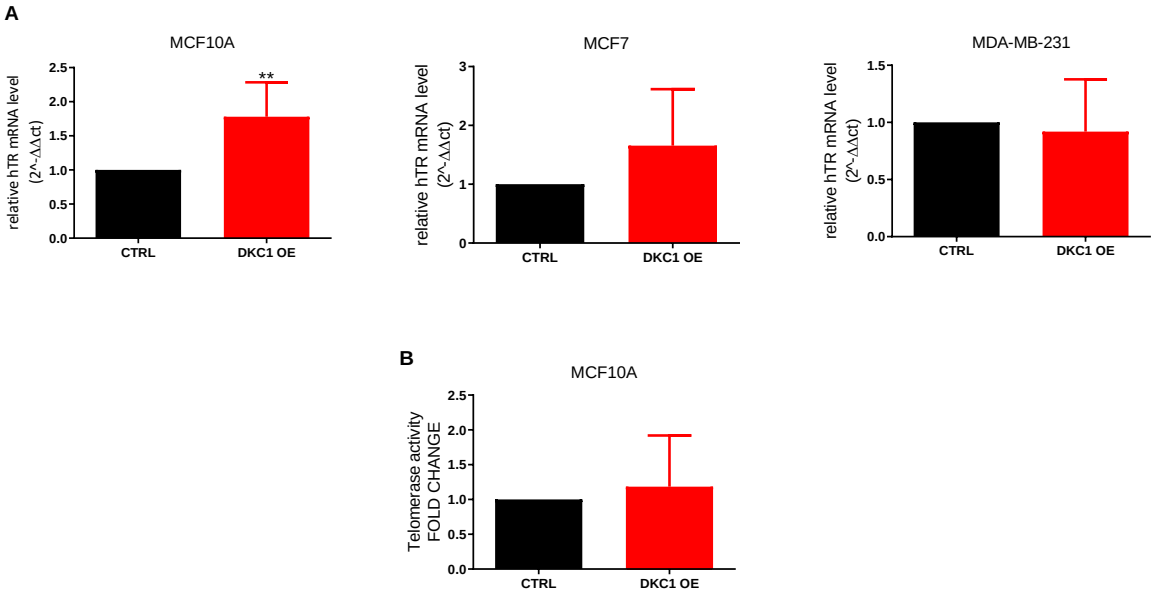

Figure 15 A) Evaluation of hTR levels in Real-Time PCR. Data were analyzed by unpaired Student's T-test: *P < 0.05; **P < 0.01; ***P < 0.001; ****P<0.0001. B) Telomerase activity assay, TRAPeze RT kit Sigma-Aldrich cat No. 7710.

DKC1 overexpression lead to an increased translation efficiency in MCF10A cells.

Due to the fundamental role of dyskerin in rRNA processing and maturation and the impairing of cellular translating capabilities after DKC1 silencing discussed above (See Introduction)[60], we tested translational efficiency in our model through an *in vitro* assay based on the transfection of a monocistronic reporter vector containing both *Firefly* Luciferase (F-LUC) and *Renilla* Luciferase (R-LUC) mRNAs. The measured luminescence is directly proportional to cellular translational activity. Interestingly, we found that overexpressing dyskerin induces significant increase in translational efficiency (Figure 16).

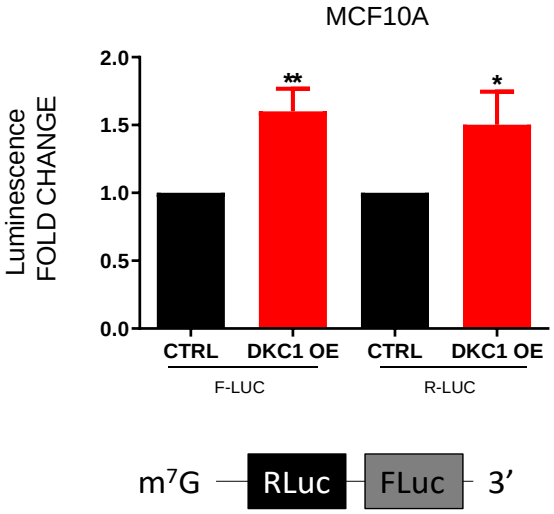

Figure 16 mRNA translation efficiency assay on MCF10A DKC1 overexpressing and control cells. Data were analyzed by unpaired Student's T-test: *P < 0.05; **P < 0.01; ***P < 0.001.

Ribosomes from MCF10A DKC1 overexpressing show significantly increased translational efficiency independently from translation initiation modality.

To further investigate the impact of increased dyskerin levels in translation, we tested highly purified ribosomes from MCF10A DKC1 overexpressing and control cells for their translation efficiency. We challenged the ribosomes with the bicistronic R-LUC/F-LUC reporter vector to test both CAP dependent and independent (IRES mediated) translation[102]. Interestingly, we found that ribosomes from dyskerin overexpressing cells are significantly more efficient in translation, independently from the modality of translation initiation (CAP or IRES translation) (Figure 17).

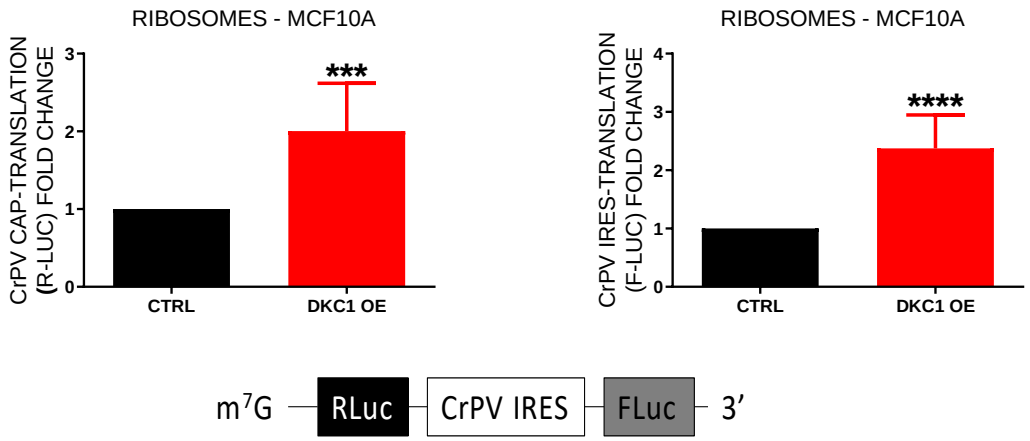

Figure 17 mRNA translation efficiency assay conducted on highly purified ribosomes extracted from MCF10A DKC1 overexpressing and controls cells. Data were analyzed by unpaired Student's T-test: *P < 0.05; **P < 0.01; ***P < 0.001; ****P<0.0001.

DKC1 overexpression does not affect global pseudouridylation on rRNA but induce a remodulation in snoRNAs expression levels.

In order to investigate the effects of dyskerin overexpression on global pseudouridylation, we performed HPLC analysis on rRNA (from highly purified ribosomes). Results showed no significant changes in global pseudouridylation in all samples (Figure 18A). However, this result does not exclude a possible site-specific modification effect. For this reason, we tested snoRNAs expression through a comprehensive expression array, which analyze 359 of both H/ACA and C/D boxes snoRNAs, focusing only on the first ones, which guide dyskerin and the pseudouridylation complex during its activity (See Introduction). The array showed that in MCF10A, DKC1 overexpression lead to a remodulation in snoRNAs expression with a majority of up-regulated and a few down-regulated snoRNAs. We focused on the top-20 up-regulated snoRNAs (Fold Change from 1,5 to 2,5) and, after Real-Time PCR validation, we found four snoRNAs that resulted significantly up-regulated: SNORA64, SNORA70, SNORA67 and SNORA38 (Figure 18B). We decided to focus only on snoRNAs with known target uridines on rRNAs: SNORA64 U1492 on 18S rRNA, SNORA70 U4975 and SNORA67 U1445 on 28S rRNA respectively. We then performed LC-MS (SILNAS-MS based quantitation) analyses to quantify site-specific pseudouridylation on the corresponding uridine sites on 18S and 28S rRNAs of the selected snoRNAs[103]. Results showed no significant changes in all samples (p value NS), but we measured a slight increase on Ψ1692 and Ψ1445 on 18S rRNA (corresponding to less uridines, shown in Figure 18C). These findings prompted us to measure a more relevant biological sample, thus we analyzed highly purified cytoplasmic ribosomes of MCF10A DKC1 overexpressing cells and relative controls. Data shows significant reduction of the percentage of U1445 on 18S rRNA of

cytoplasmic ribosomes in MCF10 dyskerin overexpressing cells in respect of control cells (Fig. 18D).

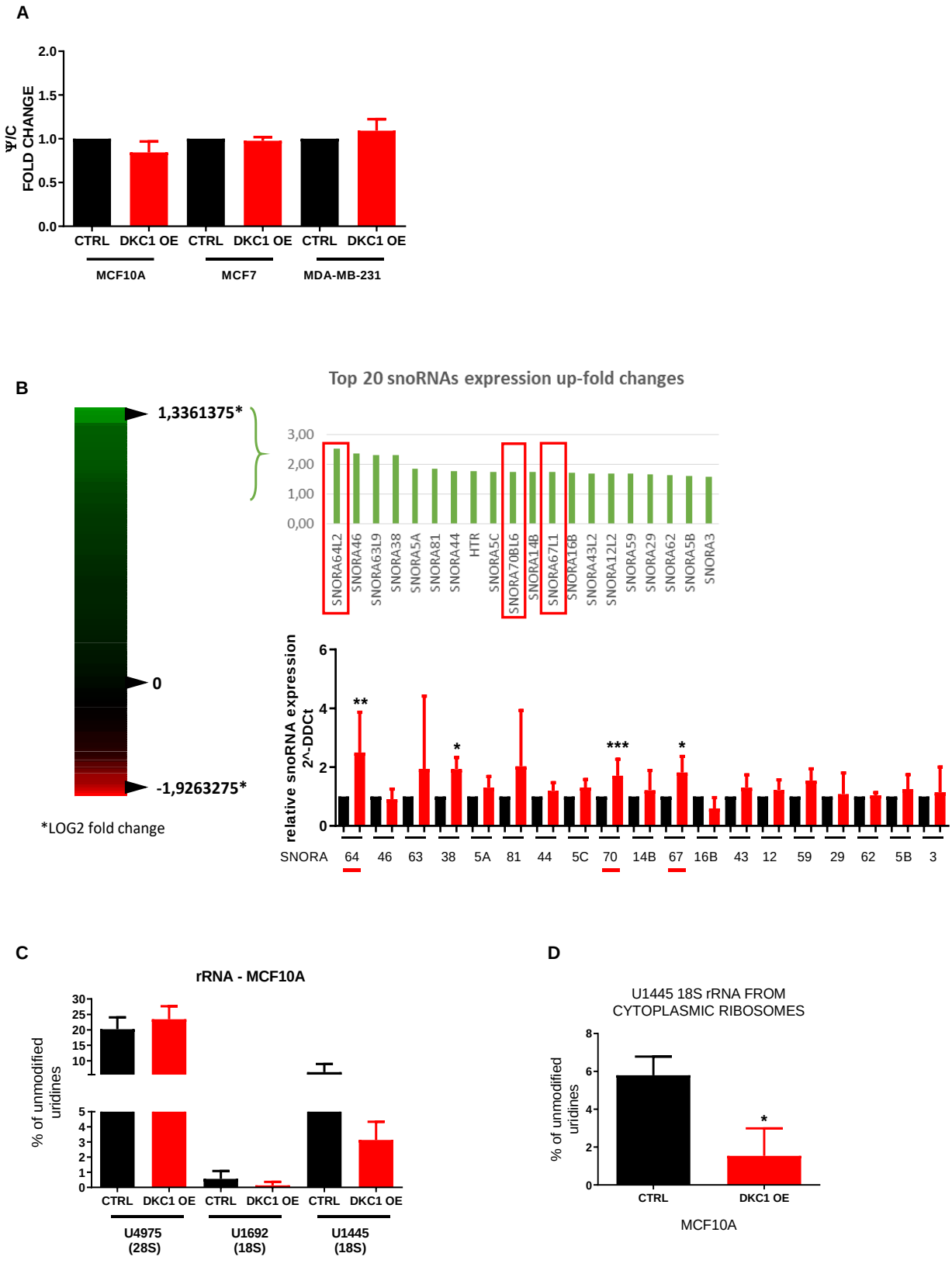

Figure 18 A) HPLC analysis of rRNA extracted from highly purified ribosomes. Results are shown as fold change of DKC1 OE/CTRL cells. **B Left)** Heat map of all H/ACA box snoRNAs ordered for fold change (DKC1 OE/CTRL) LOG2. Green values are for more expressed snoRNAs, red values for less expressed. **B Right)** Focus on top 20 up-regulated snoRNAs in MCF10A dyskerin overexpressing cells from Arraystar expression array analyses (up). SnoRNAs expression validated in Real-Time PCR (down). Data were analyzed by unpaired Student's T-test: *P < 0.05; **P < 0.01; ***P < 0.001; ****P<0.0001. **C)** LC-MS analyses of sites U4975 on 28S rRNA, U1692 and U1445 on 18S rRNA of total RNA samples. Results are shown as percentage of unmodified uridines and were calculated from peak intensity (U4975, U1692) or peak area (U1445). **D)** LC-MS analyses of U1445 on 18S rRNA of highly purified cytoplasmic ribosomes from MCF10A DKC1 overexpressing cells and relative controls.

Patients with tumors with higher dyskerin expression have worse prognosis, lower disease-free survival and advanced lymph node status respect of patients expressing low dyskerin levels.

To check if dyskerin overexpression correlates with more malignant neoplastic phenotype also *in vivo*, we updated data from a breast cancer cases series of 170 patients with primary breast carcinoma previously published in Montanaro et al[71]. We confirmed that patients with higher dyskerin expression have a lower Disease-Free Survival (DFS) in respect of patients with intermediate/low dyskerin levels (Figure 19A). In addition, we found a significant correlation between DKC1 mRNA levels and lymph node status. Consistently with our *in vitro* results in which DKC1 overexpressing untransformed cells acquire greater invasive potential, tumors with higher dyskerin expression levels show a lymph node status of N1 or higher (grouped N+) (Figure 19B). Furthermore, tumors with higher dyskerin expression present also higher levels of the snoRNAs found up regulated in MCF10A DKC1 overexpressing cells (Figure 19C).

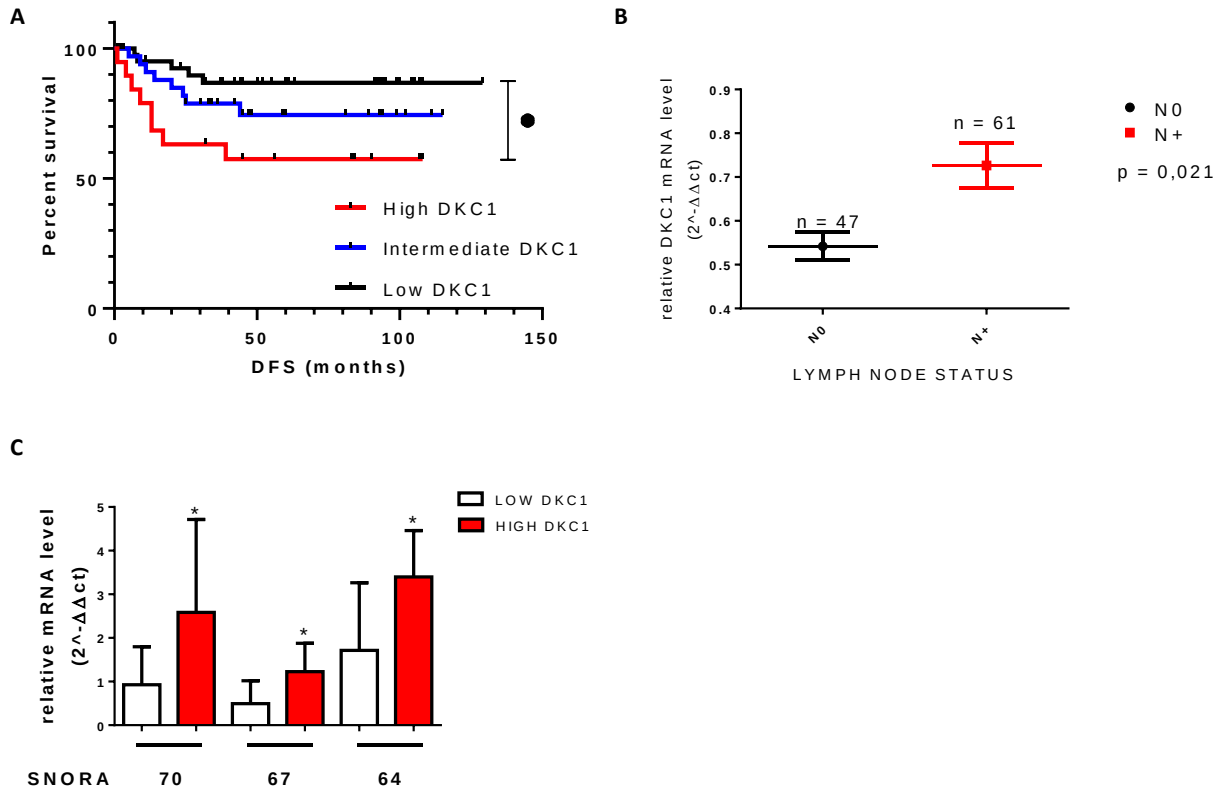

Figure 19 A) Updated DFS of 170 patients who undergone surgery for primary breast carcinoma at the Sant'Orsola Hospital. Previous data were published by Montanaro et al in 2006[71]. B) Correlation with DKC1 mRNA expression and lymph node status in the same breast cancer cases group. C) Evaluation of snoRNAs levels in Real-time PCR in RNA extracted from tumors derived from the same breast cancer cases group. At this purpose, five samples with high dyskerin and five with low dyskerin levels have been selected.

DISCUSSION

The aim of this PhD project is to deeply characterize both the molecular and phenotypic changes that occur in cells with high dyskerin expression focusing on human mammary epithelium.

Firstly, it is interesting to note that we achieved the best DKC1 overexpression levels in untransformed epithelium cells (MCF10A), which harbor in principle the lowest dyskerin amount (see Cancer Cells Line Encyclopedia, Broad Institute), underlying that at the basal condition our models require different dyskerin expression because of their diverse transformation status and their intrinsic differences. It is probably for this reason that increasing dyskerin expression confers a more aggressive phenotype only in untransformed mammary epithelium cells MCF10A and that in parallel, we observed not significant changes (MCF7) or even a "toxic" effect in neoplastic cell models (MDA-MB-231) (See Figure 14). Cells that are already transformed and represent an advanced tumor stage may not have benefits of even higher dyskerin levels that can disturb their balance. Basing on that, we can state that dyskerin overexpression may be an early event occurring in neoplastic transformation of breast epithelium, although would be fundamental to confirm these data in alternative models as Human Mammary Epithelium Cells (hMEC) systems. hMECs are different cell populations at progressive levels of neoplastic transformation stages deriving from the same healthy cells obtained from breast reduction intervention. This model would eliminate biases derived from various genetic backgrounds. Some experiments in this sense have been conducted and are still on going (data not shown).

Successively, we found that MCF10A DKC1 overexpressing cells are significantly more efficient in translation (See Figure 16) and more specifically that DKC1 overexpressing cells make

ribosomes more efficient in mRNA translation independently on the initiation modality (Figure 17). It has been previously reported that dyskerin depletion induce a defect in translation elongation[19,60]; in this sense, our results suggest that DKC1 overexpression may, in contrast, enhance the synthetic activity of ribosomes during the elongation phase. Preliminary results obtained inhibiting the elongation phase with selective inhibitors, as cycloheximide and puromycin, seem to confirm this hypothesis, since the treatment drops the differences observed in untreated cells (data not shown). In addition, translation elongation factors are altered in many cancer types[105,106] and, regarding breast tumors, overexpression of some of these factors is predictive of worse prognosis and associated with subtypes stratification[105].

Since results indicate that DKC1 overexpression provide ribosomes with intrinsic different characteristics that improve translation efficiency, we investigated what make these ribosomes more efficient. Even if there are no changes in global pseudouridylation levels (Figure 18A), we found that after DKC1 overexpression the majority of the snoRNAs are up regulated (Figure 18B). We focused on the target modification sites of the three significantly up-regulated snoRNAs: U1492 on 18S rRNA for SNORA64, U1445 and U4975 on 28S rRNA for SNORA67 and SNORA70 respectively and thanks to a collaboration with Dr. Toshiaki Isobe's group of the Tokyo Metropolitan University, we analyzed site-specific pseudouridylation. Initially we found not significative changes in the levels of pseudouridylation in the analysed sites but it is important to consider that, as demonstrated by Dr. Isobe's group in Taoka et al [98], these sites are almost fully pseudouridylated at basal state conditions. However, the decrease in the percentage of not-modified uridines observed for some sites (e.g. 1445 on 18S ranged from 6% to 3% in DKC1 overexpressing cells) suggested that more important changes may concern specific subset of ribosomes. Therefore, to exclude the possible confounding effect of nuclear maturing pre-ribosomes, we evaluated highly purified cytoplasmic ribosomes of MCF10A DKC1

overexpressing in respect of control cells. In this specific set of ribosomes, we found a significant decrease of U1445 on 18S rRNA (shown in Figure 18D) highlighting a possible role of this specific pseudouridine on translation elongation efficiency. In support to our hypothesis, Abeyrathne et al. recently published Cryo-EM structures of S. Cerevisiae 80S ribosome-eEF2-GTP complex that show the involvement of U1445 in ribosomes structural changes during the elongation phase[107].

In parallel, several works found a role for snoRNAs themselves in human cancers[28,108,109]. Noteworthy, SNORA64 has been found up-regulated in metastatic tumors rather than in primary prostate cancer cases[109]. In addition, data from a group of 994 patients of the Breast Invasive Carcinoma study of The Cancer Genome Atlas (TCGA) database show that SNORA64 is altered in 5% of cases and, specifically, these alterations are genetic amplification or mRNA up regulation. Moreover, patients harbouring these alterations have a significantly shorter disease free survival compared to the other patients with normal SNORA64 (Figure 20).

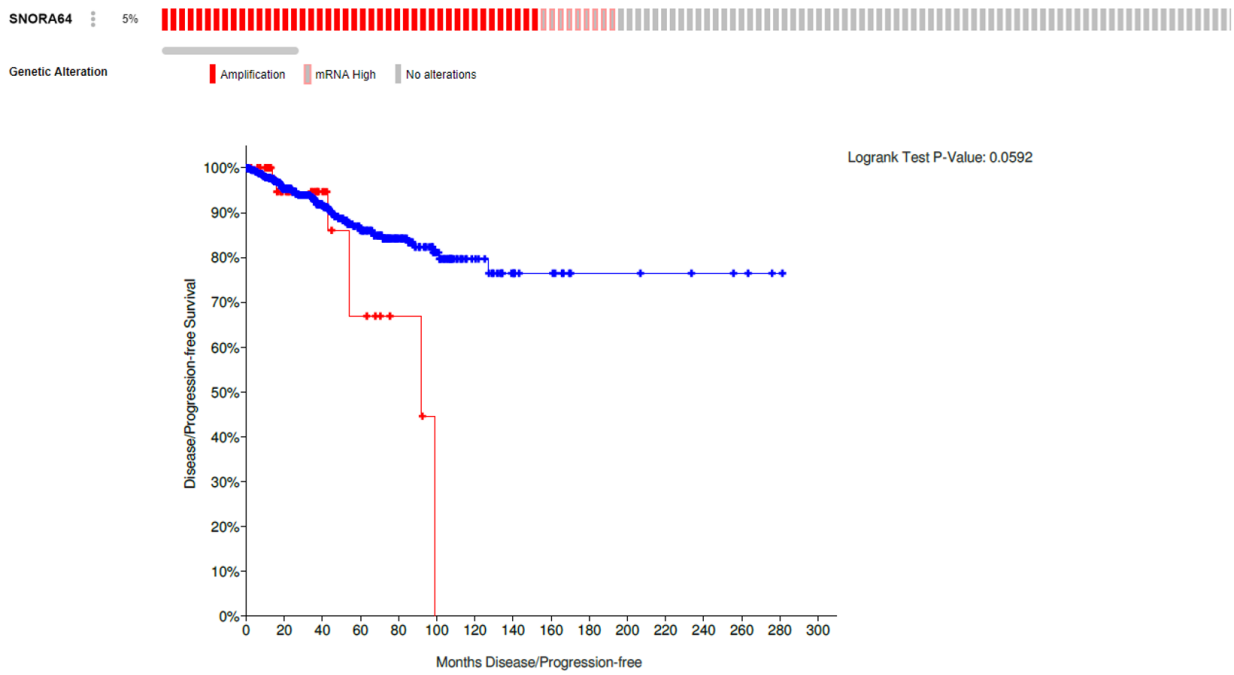

Figure 20 Data from the The Cancer Genome Atlas database for SNORA64 alterations in Breast Invasive Carcinoma. cBioPortal for Cancer Genomics.

Finally, we found that our *in vitro* results have a correspondence *in vivo* on a primary breast cancer cases series available in our laboratory. We updated data from these patients regarding DFS, confirming what Montanaro et al previously published in 2006, namely that patients with higher dyskerin expression have shorter DFS and worse prognosis than patients with lower DKC1 levels. These patients with higher dyskerin expression more frequently display lymph node involvement as compared to patients with lower DKC1 expression, in line with our *in vitro* findings, in which DKC1 overexpressing untransformed cells acquire a greater invasive potential. Basing on this clinical-experimental concordance, dyskerin overexpression can be considered a proxy of tumor aggressiveness. In addition, the fact that tumors with higher dyskerin expression have increased levels of SNORA64, SNORA67 and SNORA70 is an important result that indicate that the molecular mechanism we characterized *in vitro* may be also true for *in vivo* neoplastic transformation of mammary epithelium.

CONCLUSIONS

Our *in vitro* study propose for the first time that dyskerin overexpression may be an early event in the neoplastic transformation of mammary epithelium, suggesting a new role for dyskerin as an oncogene in breast cancer. Additional experiments are needed to further confirm the obtained results by extending the cellular models panel with human Mammary Epithelium Cells (hMECs), which nowadays can be considered as the best model to analyze tumor onset/progression of human breast epithelium. To our knowledge, this is the first study that investigate the effects of dyskerin overexpression in breast cancer.

Our work also contributes to expand the knowledge on the involvement of dyskerin in protein synthesis and ribosomes biogenesis, and lets to the hypothesis that ribosomes from tumor with higher dyskerin expression are more efficient in translation of selected mRNAs. In this sense, we are currently performing the Clariom S human expression array (Thermo Fisher) comparing total RNA and mRNAs that are actively translated (polysomes-bound RNAs) in MCF10A DKC1 overexpressing and control cells in order to understand how and how much dyskerin influences the cellular “translatome”. Finally, *in vitro* translation experiments indicate that ribosomes from dyskerin overexpressing cells are different in translation, probably being characterized by a more efficient elongation phase. This result may open to innovative therapeutic strategies based on the usage of antibiotics that interfere specifically with the elongation phase of translation. In this sense, further studies are needed.

BIBLIOGRAPHY

1 Montanaro L, Treré D, Derenzini M. Nucleolus, ribosomes, and cancer. *Am J Pathol* 2008; **173**: 301–310.

2 Mager WH. Control of ribosomal protein gene expression. *Biochim Biophys Acta* 1988; **949**: 1–15.

3 Wlotzka W, Kudla G, Granneman S, Tollervey D. The nuclear RNA polymerase II surveillance system targets polymerase III transcripts: Targets for nuclear surveillance. *EMBO J* 2011; **30**: 1790–1803.

4 Lafontaine DLJ. Noncoding RNAs in eukaryotic ribosome biogenesis and function. *Nat Struct Mol Biol* 2015; **22**: 11–19.

5 Montanaro L, Treré D, Derenzini M. Changes in ribosome biogenesis may induce cancer by down-regulating the cell tumor suppressor potential. *Biochim Biophys Acta* 2012; **1825**: 101–110.

6 Ruggero D, Pandolfi PP. Does the ribosome translate cancer? *Nat Rev Cancer* 2003; **3**: 179–192.

7 Panse VG, Johnson AW. Maturation of eukaryotic ribosomes: acquisition of functionality. *Trends Biochem Sci* 2010; **35**: 260–266.

8 Karbstein K. Quality control mechanisms during ribosome maturation. *Trends Cell Biol* 2013; **23**: 242–250.

9 Sharma S, Lafontaine DLJ. 'View From A Bridge': A New Perspective on Eukaryotic rRNA Base Modification. *Trends Biochem Sci* 2015; **40**: 560–575.

10 Penzo M, Galbiati A, Treré D, Montanaro L. The importance of being (slightly) modified: The role of rRNA editing on gene expression control and its connections with cancer. *Biochim Biophys Acta* 2016; **1866**: 330–338.

11 Donati G, Montanaro L, Derenzini M. Ribosome Biogenesis and Control of Cell Proliferation: p53 Is Not Alone. *Cancer Res* 2012; **72**: 1602–1607.

12 Derenzini M, Montanaro L, Treré D. What the nucleolus says to a tumour pathologist. *Histopathology* 2009; **54**: 753–762.

13 Stępiński D. The nucleolus, an ally, and an enemy of cancer cells. *Histochem Cell Biol* 2018; **150**: 607–629.

14 Mayer C, Grummt I. Ribosome biogenesis and cell growth: mTOR coordinates transcription by all three classes of nuclear RNA polymerases. *Oncogene* 2006; **25**: 6384–6391.

15 Zhai W, Comai L. Repression of RNA polymerase I transcription by the tumor suppressor p53. *Mol Cell Biol* 2000; **20**: 5930–5938.

16 Treré D, Ceccarelli C, Montanaro L, Tosti E, Derenzini M. Nucleolar size and activity are related to pRb and p53 status in human breast cancer. *J Histochem Cytochem Off J Histochem Soc* 2004; **52**: 1601–1607.

17 Hannan KM, Hannan RD, Smith SD, Jefferson LS, Lun M, Rothblum LI. Rb and p130 regulate RNA polymerase I transcription: Rb disrupts the interaction between UBF and SL-1. *Oncogene* 2000; **19**: 4988–4999.

18 Brighenti E, Treré D, Derenzini M. Targeted cancer therapy with ribosome biogenesis inhibitors: a real possibility? *Oncotarget* 2015; **6**: 38617–38627.

19 Jack K, Bellodi C, Landry DM, Niederer RO, Meskauskas A, Musalgaonkar S *et al.* rRNA pseudouridylation defects affect ribosomal ligand binding and translational fidelity from yeast to human cells. *Mol Cell* 2011; **44**: 660–666.

20 Liang X, Liu Q, Fournier MJ. rRNA modifications in an intersubunit bridge of the ribosome strongly affect both ribosome biogenesis and activity. *Mol Cell* 2007; **28**: 965–977.

21 Cohn WE, Volkin E. Nucleoside-5′-Phosphates from Ribonucleic Acid. *Nature* 1951; **167**: 483–484.

22 Carlile TM, Rojas-Duran MF, Zinshteyn B, Shin H, Bartoli KM, Gilbert WV. Pseudouridine profiling reveals regulated mRNA pseudouridylation in yeast and human cells. *Nature* 2014; **515**: 143–146.

23 Lovejoy AF, Riordan DP, Brown PO. Transcriptome-wide mapping of pseudouridines: pseudouridine synthases modify specific mRNAs in S. cerevisiae. *PloS One* 2014; **9**: e110799.

24 Schwartz S, Bernstein DA, Mumbach MR, Jovanovic M, Herbst RH, León-Ricardo BX *et al.* Transcriptome-wide mapping reveals widespread dynamic-regulated pseudouridylation of ncRNA and mRNA. *Cell* 2014; **159**: 148–162.

25 Li X, Zhu P, Ma S, Song J, Bai J, Sun F *et al.* Chemical pulldown reveals dynamic pseudouridylation of the mammalian transcriptome. *Nat Chem Biol* 2015; **11**: 592–597.

26 Penzo M, Guerrieri AN, Zacchini F, Treré D, Montanaro L. RNA Pseudouridylation in Physiology and Medicine: For Better and for Worse. *Genes* 2017; **8**. doi:10.3390/genes8110301.

27 Zhao Y, Dunker W, Yu Y-T, Karijolich J. The Role of Noncoding RNA Pseudouridylation in Nuclear Gene Expression Events. *Front Bioeng Biotechnol* 2018; **6**: 8.

28 McMahon M, Contreras A, Ruggero D. Small RNAs with big implications: new insights into H/ACA snoRNA function and their role in human disease. *Wiley Interdiscip Rev RNA* 2015; **6**: 173–189.

29 Spenkuch F, Motorin Y, Helm M. Pseudouridine: still mysterious, but never a fake (uridine)! *RNA Biol* 2014; **11**: 1540–1554.

30 Meier UT. The many facets of H/ACA ribonucleoproteins. *Chromosoma* 2005; **114**: 1–14.

31 Richard P, Darzacq X, Bertrand E, Jády BE, Verheggen C, Kiss T. A common sequence motif determines the Cajal body-specific localization of box H/ACA scaRNAs. *EMBO J* 2003; **22**: 4283–4293.

32 Kiss T. Small nucleolar RNAs: an abundant group of noncoding RNAs with diverse cellular functions. *Cell* 2002; **109**: 145–148.

33 Kiss AM, Jády BE, Bertrand E, Kiss T. Human box H/ACA pseudouridylation guide RNA machinery. *Mol Cell Biol* 2004; **24**: 5797–5807.

34 Knight SW, Heiss NS, Vulliamy TJ, Greschner S, Stavrides G, Pai GS *et al.* X-linked dyskeratosis congenita is predominantly caused by missense mutations in the DKC1 gene. *Am J Hum Genet* 1999; **65**: 50–58.

35 Jiang W, Middleton K, Yoon HJ, Fouquet C, Carbon J. An essential yeast protein, CBF5p, binds in vitro to centromeres and microtubules. *Mol Cell Biol* 1993; **13**: 4884–4893.

36 Meier UT, Blobel G. NAP57, a mammalian nucleolar protein with a putative homolog in yeast and bacteria. *J Cell Biol* 1994; **127**: 1505–1514.

37 Phillips B, Billin AN, Cadwell C, Buchholz R, Erickson C, Merriam JR *et al.* The Nop60B gene of Drosophila encodes an essential nucleolar protein that functions in yeast. *Mol Gen Genet MGG* 1998; **260**: 20–29.

38 Cerrudo CS, Mengual Gómez DL, Gómez DE, Ghiringhelli PD. Novel insights into the evolution and structural characterization of dyskerin using comprehensive bioinformatics analysis. *J Proteome Res* 2015; **14**: 874–887.

39 Pan H, Agarwalla S, Moustakas DT, Finer-Moore J, Stroud RM. Structure of tRNA pseudouridine synthase TruB and its RNA complex: RNA recognition through a combination of rigid docking and induced fit. *Proc Natl Acad Sci U S A* 2003; **100**: 12648–12653.

40 Rashid R, Liang B, Baker DL, Youssef OA, He Y, Phipps K *et al.* Crystal structure of a Cbf5-Nop10-Gar1 complex and implications in RNA-guided pseudouridylation and dyskeratosis congenita. *Mol Cell* 2006; **21**: 249–260.

41 Hassock S, Vetrie D, Giannelli F. Mapping and characterization of the X-linked dyskeratosis congenita (DKC) gene. *Genomics* 1999; **55**: 21–27.

42 Rocchi L, Barbosa AJM, Onofrillo C, Del Rio A, Montanaro L. Inhibition of human dyskerin as a new approach to target ribosome biogenesis. *PloS One* 2014; **9**: e101971.

43 Angrisani A, Vicidomini R, Turano M, Furia M. Human dyskerin: beyond telomeres. *Biol Chem* 2014; **395**: 593–610.

44 Penzo M, Montanaro L. Turning Uridines around: Role of rRNA Pseudouridylation in Ribosome Biogenesis and Ribosomal Function. *Biomolecules* 2018; **8**. doi:10.3390/biom8020038.

45 Calado RT, Young NS. Telomere diseases. *N Engl J Med* 2009; **361**: 2353–2365.

46 Pianese G. *Beitrag zur Histologie und Aetiologie des Carcinoms*. Jena : G. Fischer, 1896http://archive.org/details/beitragzurhistol00pian (accessed 6 Oct2019).

47 Galbiati A, Penzo M, Bacalini MG, Onofrillo C, Guerrieri AN, Garagnani P *et al.* Epigenetic up-regulation of ribosome biogenesis and more aggressive phenotype triggered by the lack of the histone demethylase JHDM1B in mammary epithelial cells. *Oncotarget* 2017; **8**. doi:10.18632/oncotarget.16181.

48 Goudarzi KM, Lindström MS. Role of ribosomal protein mutations in tumor development (Review). *Int J Oncol* 2016; **48**: 1313–1324.

49 Fancello L, Kampen KR, Hofman IJF, Verbeeck J, De Keersmaecker K. The ribosomal protein gene RPL5 is a haploinsufficient tumor suppressor in multiple cancer types. *Oncotarget* 2017; **8**: 14462–14478.

50 Hofman IJF, Patchett S, van Duin M, Geerdens E, Verbeeck J, Michaux L *et al.* Low frequency mutations in ribosomal proteins RPL10 and RPL5 in multiple myeloma. *Haematologica* 2017; **102**: e317–e320.

51 Xu X, Xiong X, Sun Y. The role of ribosomal proteins in the regulation of cell proliferation, tumorigenesis, and genomic integrity. *Sci China Life Sci* 2016; **59**: 656–672.

52 Heiss NS, Knight SW, Vulliamy TJ, Klauck SM, Wiemann S, Mason PJ *et al.* X-linked dyskeratosis congenita is caused by mutations in a highly conserved gene with putative nucleolar functions. *Nat Genet* 1998; **19**: 32–38.

53 Montanaro L. Dyskerin and cancer: more than telomerase. The defect in mRNA translation helps in explaining how a proliferative defect leads to cancer. *J Pathol* 2010; **222**: 345–349.

54 Ruggero D, Grisendi S, Piazza F, Rego E, Mari F, Rao PH *et al.* Dyskeratosis congenita and cancer in mice deficient in ribosomal RNA modification. *Science* 2003; **299**: 259–262.

55 Montanaro L, Calienni M, Ceccarelli C, Santini D, Taffurelli M, Pileri S *et al.* Relationship between dyskerin expression and telomerase activity in human breast cancer. *Cell Oncol Off J Int Soc Cell Oncol* 2008; **30**: 483–490.

56 Karijolich J, Yi C, Yu Y-T. Transcriptome-wide dynamics of RNA pseudouridylation. *Nat Rev Mol Cell Biol* 2015; **16**: 581–585.

57 Bellodi C, McMahon M, Contreras A, Juliano D, Kopmar N, Nakamura T *et al.* H/ACA small RNA dysfunctions in disease reveal key roles for noncoding RNA modifications in hematopoietic stem cell differentiation. *Cell Rep* 2013; **3**: 1493–1502.

58 Penzo M, Casoli L, Ceccarelli C, Treré D, Ludovini V, Crinò L *et al.* DKC1 gene mutations in human sporadic cancer. *Histol Histopathol* 2013; **28**: 365–372.

59 Hellen CU, Sarnow P. Internal ribosome entry sites in eukaryotic mRNA molecules. *Genes Dev* 2001; **15**: 1593–1612.

60 Penzo M, Rocchi L, Brugiere S, Carnicelli D, Onofrillo C, Couté Y *et al.* Human ribosomes from cells with reduced dyskerin levels are intrinsically altered in translation. *FASEB J Off Publ Fed Am Soc Exp Biol* 2015; **29**: 3472–3482.

61 Yoon A, Peng G, Brandenburger Y, Brandenburg Y, Zollo O, Xu W *et al.* Impaired control of IRES-mediated translation in X-linked dyskeratosis congenita. *Science* 2006; **312**: 902–906.

62 Montanaro L, Calienni M, Bertoni S, Rocchi L, Sansone P, Storci G *et al.* Novel dyskerin-mediated mechanism of p53 inactivation through defective mRNA translation. *Cancer Res* 2010; **70**: 4767–4777.

63 Bellodi C, Krasnykh O, Haynes N, Theodoropoulou M, Peng G, Montanaro L *et al.* Loss of function of the tumor suppressor DKC1 perturbs p27 translation control and contributes to pituitary tumorigenesis. *Cancer Res* 2010; **70**: 6026–6035.

64 Rocchi L, Pacilli A, Sethi R, Penzo M, Schneider RJ, Treré D *et al.* Dyskerin depletion increases VEGF mRNA internal ribosome entry site-mediated translation. *Nucleic Acids Res* 2013; **41**: 8308–8318.

65 Sieron P, Hader C, Hatina J, Engers R, Wlazlinski A, Müller M *et al.* DKC1 overexpression associated with prostate cancer progression. *Br J Cancer* 2009; **101**: 1410–1416.

66 Liu B, Zhang J, Huang C, Liu H. Dyskerin overexpression in human hepatocellular carcinoma is associated with advanced clinical stage and poor patient prognosis. *PloS One* 2012; **7**: e43147.

67 Schaner ME, Ross DT, Ciaravino G, Sorlie T, Troyanskaya O, Diehn M *et al.* Gene expression patterns in ovarian carcinomas. *Mol Biol Cell* 2003; **14**: 4376–4386.

68 Fernandez-Garcia I, Marcos T, Muñoz-Barrutia A, Serrano D, Pio R, Montuenga LM *et al.* Multiscale in situ analysis of the role of dyskerin in lung cancer cells. *Integr Biol Quant Biosci Nano Macro* 2013; **5**: 402–413.

69 Vasuri F, Rocchi L, Degiovanni A, Giunchi F, Brandi G, Treré D *et al.* Dyskerin expression in human fetal, adult and neoplastic intrahepatic bile ducts: correlations with cholangiocarcinoma aggressiveness. *Histopathology* 2015; **66**: 244–251.

70 O'Brien R, Tran SL, Maritz MF, Liu B, Kong CF, Purgato S *et al.* MYC-Driven Neuroblastomas Are Addicted to a Telomerase-Independent Function of Dyskerin. *Cancer Res* 2016; **76**: 3604–3617.

71 Montanaro L, Brigotti M, Clohessy J, Barbieri S, Ceccarelli C, Santini D *et al.* Dyskerin expression influences the level of ribosomal RNA pseudo-uridylation and telomerase RNA component in human breast cancer. *J Pathol* 2006; **210**: 10–18.

72 Alawi F, Lin P. Dyskerin is required for tumor cell growth through mechanisms that are independent of its role in telomerase and only partially related to its function in precursor rRNA processing. *Mol Carcinog* 2011; **50**: 334–345.

73 Alawi F, Lin P, Ziober B, Patel R. Correlation of dyskerin expression with active proliferation independent of telomerase. *Head Neck* 2011; **33**: 1041–1051.

74 Alawi F, Lee MN. DKC1 is a direct and conserved transcriptional target of c-MYC. *Biochem Biophys Res Commun* 2007; **362**: 893–898.

75 Dalla Venezia N, Vincent A, Marcel V, Catez F, Diaz J-J. Emerging Role of Eukaryote Ribosomes in Translational Control. *Int J Mol Sci* 2019; **20**. doi:10.3390/ijms20051226.

76 Blagden SP, Willis AE. The biological and therapeutic relevance of mRNA translation in cancer. *Nat Rev Clin Oncol* 2011; **8**: 280–291.

77 Silvera D, Formenti SC, Schneider RJ. Translational control in cancer. *Nat Rev Cancer* 2010; **10**: 254–266.

78 Bhat M, Robichaud N, Hulea L, Sonenberg N, Pelletier J, Topisirovic I. Targeting the translation machinery in cancer. *Nat Rev Drug Discov* 2015; **14**: 261–278.

79 Sriram A, Bohlen J, Teleman AA. Translation acrobatics: how cancer cells exploit alternate modes of translational initiation. *EMBO Rep* 2018; **19**. doi:10.15252/embr.201845947.

80 Merrick WC. Eukaryotic protein synthesis: still a mystery. *J Biol Chem* 2010; **285**: 21197–21201.

81 Spriggs KA, Stoneley M, Bushell M, Willis AE. Re-programming of translation following cell stress allows IRES-mediated translation to predominate. *Biol Cell* 2008; **100**: 27–38.

82 Truitt ML, Ruggero D. New frontiers in translational control of the cancer genome. *Nat Rev Cancer* 2016; **16**: 288–304.

83 Dever TE, Green R. The elongation, termination, and recycling phases of translation in eukaryotes. *Cold Spring Harb Perspect Biol* 2012; **4**: a013706.

84 Jackson RJ, Hellen CUT, Pestova TV. Termination and post-termination events in eukaryotic translation. *Adv Protein Chem Struct Biol* 2012; **86**: 45–93.

85 Somers J, Pöyry T, Willis AE. A perspective on mammalian upstream open reading frame function. *Int J Biochem Cell Biol* 2013; **45**: 1690–1700.

86 Sendoel A, Dunn JG, Rodriguez EH, Naik S, Gomez NC, Hurwitz B *et al.* Translation from unconventional 5' start sites drives tumour initiation. *Nature* 2017; **541**: 494–499.

87 Barbosa C, Peixeiro I, Romão L. Gene expression regulation by upstream open reading frames and human disease. *PLoS Genet* 2013; **9**: e1003529.

88 Akman HB, Oyken M, Tuncer T, Can T, Erson-Bensan AE. 3'UTR shortening and EGF signaling: implications for breast cancer. *Hum Mol Genet* 2015; **24**: 6910–6920.

89 Hoffman Y, Bublik DR, Ugalde AP, Elkon R, Biniashvili T, Agami R *et al.* 3'UTR Shortening Potentiates MicroRNA-Based Repression of Pro-differentiation Genes in Proliferating Human Cells. *PLoS Genet* 2016; **12**: e1005879.

90 Sun M, Ding J, Li D, Yang G, Cheng Z, Zhu Q. NUDT21 regulates 3'-UTR length and microRNA-mediated gene silencing in hepatocellular carcinoma. *Cancer Lett* 2017; **410**: 158–168.

91 Goodarzi H, Nguyen HCB, Zhang S, Dill BD, Molina H, Tavazoie SF. Modulated Expression of Specific tRNAs Drives Gene Expression and Cancer Progression. *Cell* 2016; **165**: 1416–1427.

92 Huang S-Q, Sun B, Xiong Z-P, Shu Y, Zhou H-H, Zhang W *et al.* The dysregulation of tRNAs and tRNA derivatives in cancer. *J Exp Clin Cancer Res CR* 2018; **37**: 101.

93 Dittmar KA, Goodenbour JM, Pan T. Tissue-specific differences in human transfer RNA expression. *PLoS Genet* 2006; **2**: e221.

94 Gingold H, Tehler D, Christoffersen NR, Nielsen MM, Asmar F, Kooistra SM *et al.* A dual program for translation regulation in cellular proliferation and differentiation. *Cell* 2014; **158**: 1281–1292.

95 Davis DR. Stabilization of RNA stacking by pseudouridine. *Nucleic Acids Res* 1995; **23**: 5020–5026.

96 Parisien M, Yi C, Pan T. Rationalization and prediction of selective decoding of pseudouridine-modified nonsense and sense codons. *RNA N Y N* 2012; **18**: 355–367.

97 Kampen KR, Sulima SO, De Keersmaecker K. Rise of the specialized onco-ribosomes. *Oncotarget* 2018; **9**: 35205–35206.

98 Taoka M, Nobe Y, Yamaki Y, Sato K, Ishikawa H, Izumikawa K *et al.* Landscape of the complete RNA chemical modifications in the human 80S ribosome. *Nucleic Acids Res* 2018; **46**: 9289–9298.

99 Mitchell JR, Wood E, Collins K. A telomerase component is defective in the human disease dyskeratosis congenita. *Nature* 1999; **402**: 551–555.

100 Sbarrato T, Horvilleur E, Pöyry T, Hill K, Chaplin LC, Spriggs RV *et al.* A ribosome-related signature in peripheral blood CLL B cells is linked to reduced survival following treatment. *Cell Death Dis* 2016; **7**: e2249.

101 Yajima T, Yagihashi A, Kameshima H, Kobayashi D, Furuya D, Hirata K *et al.* Quantitative reverse transcription-PCR assay of the RNA component of human telomerase using the TaqMan fluorogenic detection system. *Clin Chem* 1998; **44**: 2441–2445.

102 Penzo M, Carnicelli D, Montanaro L, Brigotti M. A reconstituted cell-free assay for the evaluation of the intrinsic activity of purified human ribosomes. *Nat Protoc* 2016; **11**: 1309–1325.

103 Yamauchi Y, Nobe Y, Izumikawa K, Higo D, Yamagishi Y, Takahashi N *et al.* A mass spectrometry-based method for direct determination of pseudouridine in RNA. *Nucleic Acids Res* 2016; **44**: e59.

104 De-escalating and escalating treatments for early-stage breast cancer: the St. Gallen International Expert Consensus Conference on the Primary Ther... - PubMed - NCBI. https://www.ncbi.nlm.nih.gov/pubmed/?term=st.+gallen+ann+oncol+2017+Xu (accessed 15 Jul2019).

105 Hassan MK, Kumar D, Naik M, Dixit M. The expression profile and prognostic significance of eukaryotic translation elongation factors in different cancers. *PloS One* 2018; **13**: e0191377.

106 Mathews MB, Hershey JWB. The translation factor eIF5A and human cancer. *Biochim Biophys Acta* 2015; **1849**: 836–844.

107 Abeyrathne PD, Koh CS, Grant T, Grigorieff N, Korostelev AA. Ensemble cryo-EM uncovers inchworm-like translocation of a viral IRES through the ribosome. *eLife* 2016; **5**. doi:10.7554/eLife.14874.

108 Nallar SC, Kalvakolanu DV. Regulation of snoRNAs in cancer: close encounters with interferon. *J Interferon Cytokine Res Off J Int Soc Interferon Cytokine Res* 2013; **33**: 189–198.

109 Stockert JA, Gupta A, Herzog B, Yadav SS, Tewari AK, Yadav KK. Predictive value of pseudouridine in prostate cancer. *Am J Clin Exp Urol* 2019; **7**: 262–272.