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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications, which will be referred to by the corresponding Roman numerals in the text:

I **Õunap K**, Käsper L, Kurg A, Kurg R (2013) The human WBSCR22 protein is involved in the biogenesis of the 40S ribosomal subunits in mammalian cells. PLoS ONE 8(9): e75686. doi:10.1371/journal.pone.0075686

II **Õunap K**, Leetsi L, Matsoo M, Kurg R (2015) The stability of ribosome biogenesis factor WBSCR22 is regulated by interaction with TRMT112 via ubiquitin-proteasome pathway. PLoS ONE 10(7): e0133841. doi:10.1371/journal.pone.0133841

III Kurg R, Reinsalu O, Jagur S, **Õunap K**, Võsa L, Kasvandik S, Padari K, Gildemann K, Ustav M (2016). Biochemical and proteomic characterization of retrovirus Gag based microparticles carrying melanoma antigens. Sci. Rep. 6, 29425; doi: 10.1038/srep29425

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My contributions to the publications are as follows:

I Participated in designing the experiments, carried out most of the experiments, analysed the data, prepared the figures and wrote some parts of the manuscript.

II Participated in experimental design, carried out most of the experiments, analysed the data, prepared the figures and wrote some parts of the manuscript.

III Performed some of the subcellular localization studies of melanoma antigens, carried out the validation of mass spectrometry results by immunoblotting, contributed in preparing the figures 3 and 5.

LIST OF ABBREVIATIONS

ac^4C	N^4-acetylcytidine
C1QBP	complement C1q binding protein
CTD	C-terminal domain
EGFP	enhanced green fluorescent protein
ETS	external transcribed spacer
EV	extracellular vesicle
FBL	fibrillarin
FC	fibrillar centre
FISH	fluorescence *in situ* hybridization
ITS	internal transcribed spacer
LCR	low-copy repeats
LSU	large ribosomal subunit
m^6_2A	N^6, N^6-dimethyladenosine
m^1A	N^1-methyladenosine
$m^1acp^3\Psi$	N^1-methyl-N^3-(amino-3-carboxypropyl) pseudouridine
m^3U	N^3-methyuridine
m^5C	N^5-methylcytosine
m^7G	N^7-methylguanosine
MLV	murine leukaemia virus
MTD	methyltransferase domain
NAHR	non-allelic homologous recombination
NLS	nuclear localization signal
NOR	nucleolar organizing regions
PTC	peptidyl transferase centre
SAM	S-adenosyl-L-methionine
SILAC	stable isotope labelling by amino acids in the cell culture
snoRNA	small nucleolar RNA
snoRNP	small ribonucleoprotein particle
SSU	small ribosomal subunit
SV40	simian virus 40
SVAS	supravalvular aortic stenosis
TRMT112	tRNA methyltransferase 11–2 homolog (*S. cerevisiae*)
tRNA	transfer RNA
UTR	untranslated region
VLP	virus-like particle
WBS	Williams-Beuren syndrome
WBSCR22	Williams-Beuren syndrome chromosome region 22
YBX1	Y-box binding protein 1
Ψ	pseudouridine

1. INTRODUCTION

In human cells, almost 300 trans-acting factors are required for the production of ribosomes. Investigating the functions of individual proteins involved in the biogenesis of ribosomes is relevant because one third of these factors are related to genetic diseases and cancer.

Williams-Beuren syndrome is a multisystem developmental disorder caused by the contiguous deletion of 26–28 genes from chromosome region 7q11.23. This genomic disorder is characterized by cardiovascular abnormalities, connective tissue anomalies, a characteristic neurocognitive and behavioural profile, growth delay, and a subtle but distinctive facial dysmorphology. The 7q11.23 chromosome locus has a complex genomic architecture, consisting of a single-copy region surrounded by segmental duplications that predispose the region to chromosomal rearrangements, including deletions, duplications, and inversions. Although the mutational mechanisms that lead to the development of Williams-Beuren syndrome and a reciprocal duplication syndrome have been thoroughly investigated, it is not known how alterations to the copy number of individual genes contribute to the disease phenotypes. It is likely that several genes contribute to the phenotype and therefore it is important to study the cellular functions of all proteins expressed from the region 7q11.23. The gene that encodes the WBSCR22 protein is deleted in WBS, and studying the function of WBSCR22 is also relevant for cancer biology. Several works have demonstrated that WBSCR22 is upregulated in various types of cancers and regulates the survival and metastatic potential of cancer cells.

The WBSCR22 protein contains an S-adenosyl-L-methionine binding motif that is present in many methyltransferases, and, prior this work, *in silico* predictions indicated that WBSCR22 is a DNA methyltransferase. Later, WBSCR22 was suggested to be involved in histone methylation; only recently, it was confirmed to act as an 18S rRNA base methyltransferase. The first part of this dissertation is focused on studying the function of the WBSCR22 protein in mammalian cells, and, revealed that WBSCR22 is important for cell growth, ribosome biogenesis and pre-rRNA processing. A cell line that stably expresses epitope-tagged WBSCR22 was generated with the aim to study the interaction partners of WBSCR22, revealing that WBSCR22 interacts with several proteins, including the TRMT112. Subsequently, the importance of the interaction between WBSCR22 and TRMT112 proteins was analysed in detail.

The second part of this dissertation is focused on analysing the protein content of virus-like particles (VLPs) induced by the expression of murine leukaemia virus (MLV) Gag protein. The results revealed that these VLPs consist of different groups of cellular proteins, including ribosomal proteins from both subunits.

2. LITERATURE REVIEW

2.1. Williams-Beuren syndrome

Williams-Beuren syndrome (WBS, also referred to as Williams syndrome; OMIM 194050) is a genomic disorder caused by hemizygous deletion of 26–28 genes on chromosome region 7q11.23 (Pober, 2010). The contiguous gene deletion typically ranges from 1.55 to 1.84 Mb in size, but smaller and larger atypical deletions have also been described (Bayés et al., 2003; Ferrero et al., 2010; Schubert, 2009). The prevalence of WBS is relatively rare and has been estimated to occur sporadically in 1/7,500 to 1/20,000 live births (Strømme et al., 2002).

WBS is a multisystem disorder: patients usually have a subtle but distinctive facial dysmorphology, cardiovascular abnormalities, connective tissue anomalies, a characteristic neurocognitive and behavioural profile, and growth delay (Merla et al., 2010). The syndrome is named after two doctors, Dr. Williams and Dr. Beuren, who independently characterized a syndrome with specific facial features, supravalvular aortic stenosis (SVAS) and mental retardation in the 1960s (Beuren et al., 1962; Williams et al., 1961). The main facial features of WBS are a broad forehead, periorbital fullness, a low nasal root, a long philtrum, a wide mouth and full lips (Merla et al., 2010; Popowski et al., 2011).

The prevalence of cardiovascular diseases is high (~80%) in people diagnosed with WBS (Eronen et al., 2002; Pober et al., 2008). The most common cardiovascular symptoms include vascular stenosis, such as SVAS or pulmonary artery stenosis, and hypertension, however, patients often have multiple problems and a lifelong assessment of the cardiovascular system is recommended (Lin et al., 2008). The cardiovascular phenotype of WBS is similar to that of familial SVAS, and both are caused by haploinsufficiency of the elastin gene (*ELN*), which leads to reduced elastin synthesis (Ewart et al., 1993). Individuals with WBS often have endocrine abnormalities related to glucose metabolism, hypothyroidism, infantile hypercalcemia, hypercalciuria and decreased growth spurt (Pober, 2010).

WBS causes a special neurocognitive and behavioural profile that is characterized by developmental delay and mild to moderate intellectual disability with IQ scores typically in the range of 50 to 60 (Merla et al., 2010; Pober, 2010). The language development of people with WBS is below age expectations, however, compared to other groups that display intellectual disabilities, children with WBS have a higher expressive vocabulary level, albeit similar receptive vocabulary levels (Van Den Heuvel et al., 2016). The characteristic pattern of cognitive strengths and weaknesses of WBS include hypersociability, friendliness, and empathy towards others, but also the presence of anxieties, phobias, and a visuospatial construction deficit (Merla et al., 2010; Pober, 2010). Individuals with WBS often have strong attraction to sounds and music but on the other hand, they can be hypersensitive to certain noises like fireworks or thunderstorms (Gothelf et al., 2006; Pober, 2010). It has been shown that high

frequency sensorineural hearing loss has developed in ~75% of WBS adults over the age of thirty (Cherniske et al., 2004).

In addition to clinical observation, a diagnosis of WBS is confirmed either by fluorescence in situ hybridisation analysis (FISH), using the elastin gene as a marker, or by other molecular biology methods, e.g., restriction fragment length polymorphism, microsatellite analysis or array comparative genomic hybridization (Merla et al., 2010). WBS has also been diagnosed prenatally, following an initial diagnosis of intra-uterine growth retardation and nasal bone hypoplasia (Popowski et al., 2011).

2.2. The 7q11.23 duplication syndrome

In addition to deletions of the WBS chromosomal region, a 1.5 Mb duplication in the corresponding region has also been characterized, and causes 7q11.23 duplication syndrome (also referred to as Willams-Beuren region duplication syndrome; OMIM 609757) (Somerville et al., 2005). Compared to WBS, the phenotype of 7q11.23 duplication syndrome is less severe and the syndrome may be underdiagnosed (Merla et al., 2010). The milder phenotype is probably the reason why the first case of 7q11.23 duplication was documented much later (Somerville et al., 2005). Some features of the duplication syndrome are opposite to WBS – patients with 7q11.23 duplication do not have impaired visuospatial cognitive skills but have deficits in social interactions and very poor expressive language (Merla et al., 2010). Although it is not yet fully defined, the phenotype of the WBS region duplication syndrome is also characterized by subtle but recognizable facial features, developmental delay and an increase in the prevalence of autistic-like features (Pober, 2010; Velleman and Mervis, 2011). Abbas et al. reported that ~91% of individuals diagnosed with WBS locus duplication have speech delay and ~75% have at least one anxiety disorder (Abbas et al., 2016). Among the psychiatric features, social anxiety, attention deficit/hyperactivity disorder, selective mutism, autism, and special phobias are the most prominent (Abbas et al., 2016). The diagnosis of WBS duplication syndrome often occurs as the result of an untargeted genomic analysis method such as chromosomal microarray analysis on patients with autistic-like features.

2.3. The structure of the Williams-Beuren syndrome chromosomal region

The structure of 7q11.23, the WBS chromosomal region, is complex (**Fig. 1A**), and consists of a single copy region (~1.2 Mb in size) that is flanked by segmental duplications or low copy repeats (LCRs) (Bayés et al., 2003). Three large LCR sequences in 7q11.23, the centromeric, middle, and telomeric LCRs (ordered by their location in the chromosomal band), are composed of DNA

blocks termed A, B, and C (Bayés et al., 2003; Schubert, 2009). The DNA blocks have a length of ~10–400 bp and they display ~97% sequence identity within each block class, which predisposes to strand misalignment and unequal crossing-over in meiosis, causing deletions, duplications, and inversions (Stankiewicz and Lupski, 2002).

The centromeric and middle LCR blocks in the WBS chromosomal share the same orientation; however, the telomeric LCRs occur in the opposite direction compared with the centromeric and middle LCRs, which leads to the possibility of deletion, duplication, or inversion events in the WBS chromosomal region (**Fig. 1C and D)** (Bayés et al., 2003). The B-cen and B-mid LCRs share the highest degree of sequence identity (99.6%) and therefore form the most frequent site of meiotic non-allelic homologous recombination (NAHR), thereby generating the 1.55 Mb deletion seen in ~95% of WBS patients (Bayés et al., 2003). NAHR within A-cen and A-mid, generates a 1.84 Mb deletion, which occurs in ~3–5% of WBS patients, and the frequency of atypical deletions are even lower (~2–3% of WBS cases) (Bayés et al., 2003; Merla et al., 2010; Schubert, 2009). In addition to unequal meiotic crossing-over between homologous chromosomes (Baumer et al., 1998; Cuscó et al., 2008; Urbán et al., 1996), WBS deletions can also occur as a result of intrachromatid recombination (**Fig. 1D**) (Bayés et al., 2003; Cuscó et al., 2008).

Duplications of the WBS chromosomal region arise by interchromosomal NAHR, but not by intrachromosomal rearrangements (**Fig. 1C**) (Schubert, 2009). In addition, NAHR can cause inversions (**Fig. 1E)** that range between 1.79 to 2.56 Mb, but these genomic rearrangements do not appear to affect the expression of genes from the WBS region (Bayés et al., 2003; Tam et al., 2008). Inversions can occur in meiosis or mitosis due to intrachromatid misalignment (Schubert, 2009).

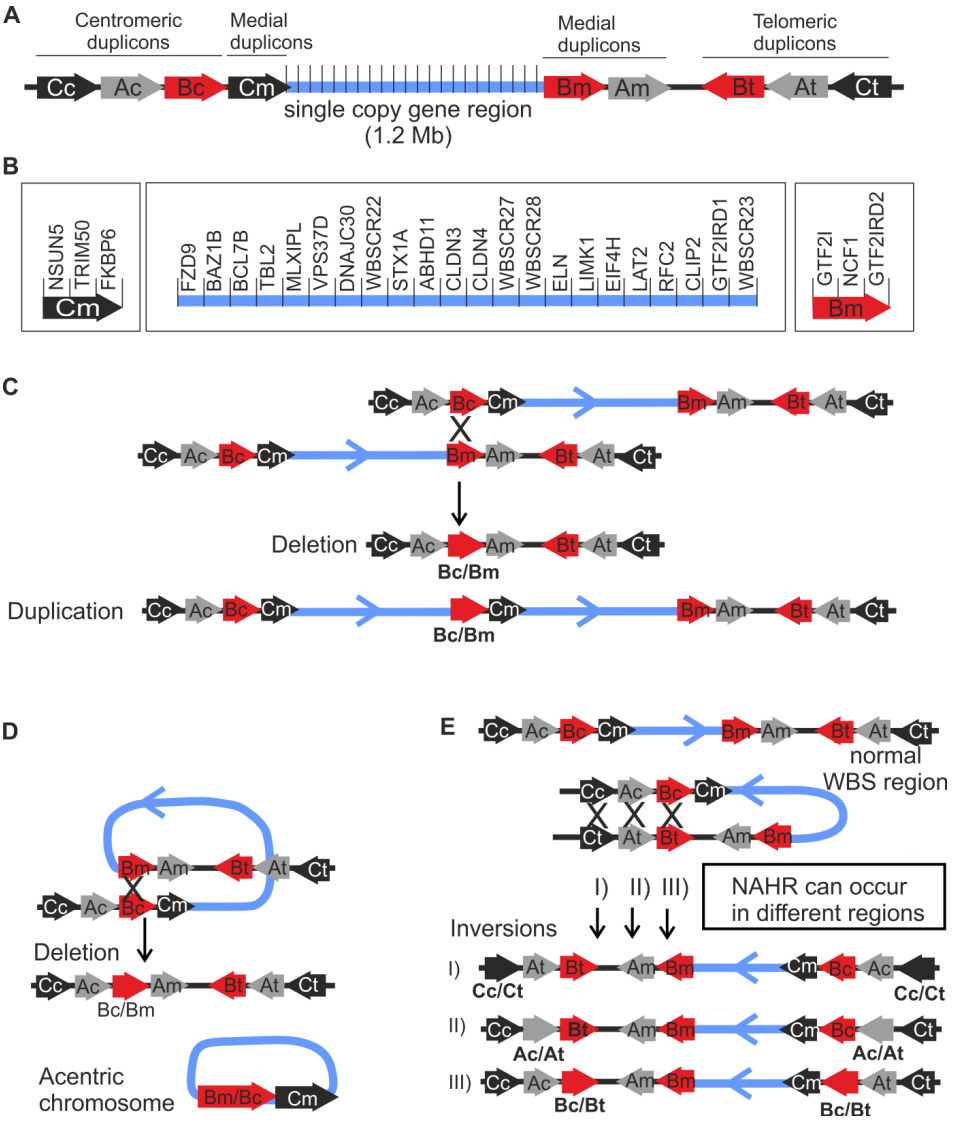

Figure 1. A schematic representation of the Williams-Beuren syndrome chromosome region (7q11.23) and chromosomal rearrangements. A) The Williams-Beuren chromosome region consists of a 1.2 Mb single copy gene region (blue) and highly similar low-copy repeat (LCR) blocks A (grey), B (red), and C (black). Lower case letters (c-centromeric, m-medial, t-telomeric) mark the positions of LCRs within the chromosomal band. B) Protein-coding genes in the single copy gene region, in medial block C (Cm) and B (Bm) duplicons. These genes are commonly deleted in WBS patients. C) Development of deletion and duplication due to interchromosomal or interchromatid NAHR between directly oriented LCRs. Here we present the most frequent deletion and duplications with breakpoints in the centromeric and medial B-blocks. D) Intrachromatid NAHR leads to the deletion and generation of an acentric chromosome, the latter of which can be lost in segragation. E) Development of inversions in the WBS region caused by NAHR of inverted LCR blocks. Misalignment can occur between centromeric and telomeric blocks of type C, A, and B and therefore the size of the inverted region is variable.

2.4. The contributions of individual genes of the WBS locus to the clinical outcome

Although the molecular mechanisms that lead to the development of genomic rearrangements in 7q11.23 have been known for many years, the role of individual genes to the WBS phenotype has not yet been solved. Genomic rearrangements in the WBS region affect the copy number of up to 28 genes (Merla et al., 2010; Pober, 2010). Quantitative PCR analysis from skin fibroblasts and lymphoblastoid cell lines of WBS patients demonstrate that the hemizygous deletion of 7q11.23 leads to a reduced expression of genes located within the deleted interval (Merla et al., 2006). Furthermore, the mRNA level of some genes that flank the deleted region is also decreased in these patients, showing that the number of genes that can influence the development of WBS phenotype is more numerous than previously expected (Merla et al., 2006). Deletion of elastin gene (ELN) is known to cause cardiovascular problems such as SVAS (Ewart et al., 1993), however, determining the role of many other genes requires further study.

2.5. The WBSCR22 protein

2.5.1. The structure and function of WBSCR22 protein

One of the protein coding genes that is mapped within single copy gene regions of the WBS locus is *WBSCR22* (Doll and Grzeschik, 2001; Merla et al., 2002). WBSCR22 mRNA encodes a protein composed of 281 amino acid residues with a predicted molecular weight of 31.8 kDa and an isoelectric point of 8.95 (Doll and Grzeschik, 2001). The protein is conserved in eukaryotes (White et al., 2008). *In silico* analysis has shown that the WBSCR22 protein contains a carboxy-terminal nuclear localization signal (NLS) and an amino-terminal S-adenosyl-L-methionine (SAM) binding motif that is common for methyltransferases (Merla et al., 2002). The human genome encodes 208 methyltransferases that methylate nucleic acids, proteins, lipids, or small molecules, and the WBSCR22 protein belongs to the largest class of methyltransferases – seven-β-strand superfamily or "class I" methyltransferases (Petrossian and Clarke, 2011). The core of these enzymes is composed of seven-stranded β-sheets, where the first six β-sheets are parallel and the seventh occurs in the opposite direction (Falnes et al., 2016; Petrossian and Clarke, 2011). The β-sheets (ordered 3214576) are flanked by α-helices (αZ and αA-αE) – three helices (Z, A, B) are on one side and three helices (C, D and E) on the other (Martin and McMillan, 2002). The yeast orthologue of human WBSCR22 is the Bud23 protein (Ebersberger et al., 2014). A crystal structure analysis of the methyltransferase domain of budding yeast Bud23 protein revealed that it contains a typical class I methyltransferase fold (Létoquart et al., 2014). The 50% sequence similarity of WBSCR22 with yeast Bud23 leads us to believe that the structure of WBSCR22 might be similar to the Bud23 protein.

Despite the presence of the methyltransferase fold, the enzymatic activity of WBSCR22 was unknown for a long time. Based on sequence alignment, it was first suggested to be a DNA methyltransferase, but so far, no DNA methyltransferase activity has been verified experimentally (Doll and Grzeschik, 2001). Several studies have analysed whether WBSCR22 might be a histone methyltransferase (Jangani et al., 2014; Nakazawa et al., 2011). The results of a comparative transcriptome analysis demonstrated a negative correlation between WBSCR22 and tumor-supressor Zac1 expression, which suggested that WBSCR22 might be a protein methyltransferase that renders transcriptionally repressive chromatin environment (Nakazawa et al., 2011). Chromatin-immunoprecipitation results indeed indicated the loss of H3-Lys^{9} methylation in the Zac1 promoter after the depletion of WBSCR22; however, an *in vitro* methylation assay could not confirm the histone methyltransferase activity of WBSCR22 (Nakazawa et al., 2011). In addition to regulating Zac1 expression, downregulation of WBSCR22 expression influences the glucocorticoid receptor target gene GILZ1 by decreasing its response to glucocorticoid receptor induction (Jangani et al., 2014). WBSCR22 affects glucocorticoid receptor binding to its response elements and furthermore, the intact methyltransferase domain and S-adenosyl-methionine binding sequence of WBSCR22 is necessary to support glucocorticoid receptor transactivation (Jangani et al., 2014). Although the downregulation of WBSCR22 causes changes in the status of histone methylation in the GILZ1 promoter, the authors of the latter article could not prove that WBSCR22 possess histone methyltransferase activity (Jangani et al., 2014).

We and others have shown that WBSCR22 is involved in ribosome biogenesis and pre-rRNA processing (Paper I; Tafforeau et al., 2013). Recently, the methyltransferase activity of WBSCR22 and its molecular target were confirmed. As with yeast Bud23, WBSCR22 carries out ribosomal RNA methylation, namely the methylation of G1639 of 18S rRNA and is one of the few rRNA base methyltransferases in humans (Haag et al., 2015; Zorbas et al., 2015).

2.5.2. The expression pattern of WBSCR22

This gene is composed of twelve exons and spans ~45 kbp on genomic DNA (Doll and Grzeschik, 2001; Merla et al., 2002). Using a panel consisting of twenty human cDNA pools from various tissues and developmental stages, Merla et al detected high expression of WBSCR22 mRNA in kidney, spleen, liver, lung, testis, and fetal heart (Merla et al., 2002). Another study, performed by Doll and Grzeschik, analyzed eight human tissues and showed that WBSCR22 mRNA was detected in all samples tested with high expression in skeletal muscle, heart, and kidney (Doll and Grzeschik, 2001). An analysis of human gene expression array data demonstrated that WBSCR22 is widely or ubiquitously expressed with increased expression in bronchial epithelium and activated immune cells – e.g., in B lymphocytes and CD8+ T lymphocytes (Jangani et al.,

2014). Due to the observation that WBSCR22 expression is upregulated in primary plasma cells, Tiedemann et al. suggested that it might have a role in plasma cell biology (Tiedemann et al., 2012).

The expression of WBSCR22 is upregulated in different types of cancer – in invasive breast cancer (invasive ductal carcinoma), hepatocellular carcinoma and multiple myeloma (Nakazawa et al., 2011; Stefanska et al., 2014; Tiedemann et al., 2012). Using a mouse experimental metastasis model, it has been shown that both mouse Wbscr22 and human WBSCR22 proteins are important for metastasis formation by regulating tumour cell survival in the mouse vasculature (Nakazawa et al., 2011). The metastatic potential of Wbscr22 depends on its expression level – overexpression of Wbscr22 enhances, and down-regulation by shRNAs reduces the metastatic ability of cancer cells (Nakazawa et al., 2011). Because the expression level of WBSCR22 seems to affect the oncogenic potential of the cell, its expression level might be tightly controlled. The importance of WBSCR22 in cancer biology was further confirmed by the results of a high throughput siRNA screen that targeted about one third of the human genome. This study showed that WBSCR22 is among 57 multiple myeloma survival genes (Tiedemann et al., 2012). Although the downregulation of WBSCR22 expression affects the survival of non-myeloma cells, as in the 293 and A549 cell-lines, the absence of WBSCR22 seems to be more detrimental to multiple myeloma cells (Tiedemann et al., 2012). The relevance of WBSCR22 for cell survival is supported by recent screens that employed CRISPR/Cas9 genome editing and gene trap methods to show that WBSCR22 is among the genes that are essential in human cells (Blomen et al., 2015; Wang et al., 2015a).

2.5.3. The functions of non-mammalian WBSCR22 homologs

In the beginning of this work, the cellular function of the WBSCR22 protein was unknown. However, as already discussed, WBSCR22 protein is highly conserved in eukaryotes and the functions of some of its orthologs are better characterized. The budding yeast protein Bud23 is not essential, however, the deletion of *Bud23* gene causes a slow growth phenotype (Niewmierzycka and Clarke, 1999). *Saccharomyces cerevisiae* has a bipolar budding pattern which means that it can divide either from its distal or proximal polar end. However, a *bud23* deletion mutant has a random budding pattern, meaning that Bud23 is important for bud-site selection (Ni and Snyder, 2001). In addition, Bud23 is known to be important for ribosome biogenesis, 18S pre-rRNA processing and for the formation of m7G1575 of 18S rRNA (White et al., 2008). Similarly, the Rid2 protein in *Arabidopsis thaliana*'s is important for cell proliferation (Ohbayashi et al., 2011). Rid2 was first characterized from a genetic screen that analysed temperature-sensitive mutants defective in root formation (Konishi and Sugiyama, 2003). The Rid2 protein is involved in rRNA processing while its mutation leads to the enlargement of nucleoli and the accumulation of various

pre-rRNA processing intermediates (Ohbayashi et al., 2011). Although RID2 and Bud23 are both involved in rRNA processing, expression of RID2 protein does not correct the slow growth phenotype of *bud23* mutant (Ohbayashi et al., 2011).

2.6. Eukaryotic ribosome biogenesis

2.6.1. The structure and function of ribosomes

Ribosomes are large ribonucleoprotein complexes essential for translating the genetic information encoded within mRNAs into proteins. These particles are composed of ribosomal RNA (rRNA) and proteins (r-proteins): the RNA component has catalytic activity and therefore the ribosomes are actually ribozymes. The eukaryotic and prokaryotic ribosomes differ in size – eukaryotes have more complex 80S ribosomes, whereas prokaryotes have smaller 70S ribosomes. Each ribosome is composed of two unequal subunits, the large subunit (LSU) and the small subunit (SSU) – e.g., 60S and 40S subunits in eukaryotes and 50S and 30S subunits in prokaryotes. The small subunit contains a decoding centre that monitors the complementarity between the anticodons in tRNA and the codons in mRNA. The large subunit contains a peptidyl-transferase centre that catalyses the formation of peptide bonds. Each ribosome contains aminoacyl-tRNA, peptidyl-tRNA, and deacylated tRNA binding regions termed ribosome A-, P- and E-sites, respectively. In contrast with prokaryotic ribosomes, eukaryotic ribosomes contain additional r-proteins and additional residues in both protein and rRNA content, termed ribosomal protein extensions and rRNA expansion segments, respectively (Wilson and Doudna Cate, 2012). The structural core of ribosomes is highly conserved and eukaryote specific rRNA expansion segments (5 in SSU and 16 in LSU) and ribosomal protein extensions are mostly localized to the surface of ribosomal subunits (Wilson and Doudna Cate, 2012). The deletion experiments conducted in budding yeast indicate that eukaryotic expansion segments affect ribosome biogenesis and are important for growth (Ramesh and Woolford, 2016).

The human 40S subunit has one rRNA (18S rRNA) and 33 proteins, whereas the 60S subunit has three rRNAs (28S, 5.8S and 5S rRNA) and 47 proteins (Anger et al., 2013). The yeast 60S subunit has 25S rRNA and 46 ribosomal proteins, meaning that the total number of proteins in the yeast ribosomes is 79 (Yusupova and Yusupov, 2014).

2.6.2. Factors required for the production of eukaryotic ribosomes

Eukaryotic ribosome biogenesis is a complex and energy demanding process that begins in the nucleolus, proceeds in the nucleoplasm, and is completed in the cytoplasm (Woolford and Baserga, 2013). Eukaryotic ribosomes are subject to more complex regulation and biogenesis pathways compared with prokaryotes and moreover, major differences occur in ribosome biogenesis of higher

and lower eukaryotes (Henras et al., 2015; Woolford and Baserga, 2013). Ribosome biogenesis involves the synthesis of pre-rRNAs and ribosomal proteins, pre-rRNA processing, generation of covalent rRNA modifications – e.g., pseudouridylations and methylations, the assembly and transport of pre-ribosomal particles, as well as quality control and surveillance mechanisms (Sharma and Lafontaine, 2015). The production of yeast ribosomes requires more than 200 synthesis factors and ~80 small nucleolar RNAs (snoRNAs) that transiently associate with pre-ribosomes at certain stages during the maturation process (Henras et al., 2015; Mullineux and Lafontaine, 2012; Woolford and Baserga, 2013). In human cells, more than 200 snoRNAs guide rRNA modifications and pre-rRNA processing (Lestrade and Weber, 2006) and it has been shown by a siRNA screen that at least 286 trans-acting factors are important for pre-rRNA processing (Tafforeau et al., 2013). Understanding the role of individual ribosome biogenesis transacting factors is relevant because about one third of the factors are related to genetic diseases and/or cancer (Tafforeau et al., 2013).

2.6.3. The processing of pre-rRNA in eukaryotes

2.6.3.1. The organization of human 47S pre-rRNA and its processing into 45S pre-rRNA

In human cells, the rDNA that encodes 47S pre-rRNA is tandemly repeated within nucleolar organizing regions (NOR) on five acrocentric chromosomes (chromosomes 13–15, 21 and 22) and the rDNA repeats that encode 5S rRNA are localized on chromosome 1 (Henderson et al., 1973; Sørensen and Frederiksen, 1991). The 47S pre-rRNA is a single polycistronic transcript composed of three rRNAs (18S, 5.8S and 28S) and it is synthesised by RNA polymerase I (Pol I) in the nucleolus. In *Saccharomyces cerevisiae*, the RNA Pol I synthesizes the 35S pre-rRNA with a general architecture similar to mammalian 47S pre-rRNA (Henras et al., 2015; Mullineux and Lafontaine, 2012).

The nucleolus is a non-membranous nuclear compartment that forms around the NOR and it is the major site for ribosome biogenesis (Raska, 2003). The cells of most eukaryotes, including plants, invertebrates, and anamniote vertebrates contain bipartite nucleoli; whereas the nucleoli of amniotic reptiles, birds, and mammals consist of three parts (Thiry and Lafontaine, 2005; Thiry et al., 2011). In the inner part of tripartite nucleolus is the electron-microscope transparent area, fibrillar centre (FC), that contains transcriptionally active rDNA, transcription factors and RNA Pol I (Derenzini et al., 2006). rRNA transcription occurs in the interface between the FC and the dense fibrillar centre, which consists of an electron-dense area that contains nascent rRNA transcripts and ribonucleoprotein complexes that participate in pre-rRNA processing and rRNA modification (Denissov et al., 2011). Maturing ribosomes, which consist of rRNA, ribosomal proteins and transacting factors, move towards the nuclear periphery to the granular component of the nucleolus (Boisvert et al., 2007).

5S rRNA, is transcribed separately by RNA polymerase III in the nucleoplasm; however, it forms a 5S RNP complex with ribosomal proteins RPL5 and RPL11, followed by binding to the pre-60S particles in the nucleolus (Ciganda and Williams, 2011; Pelava et al., 2016). In eukaryotes, both rRNAs are processed by nucleases, and, in *S. cerevisiae,* the exonucleases Rex1p, Rex2p, and Rex3p participate in the processing of the 3′ end of 5S rRNA (van Hoof et al., 2000).

The overall architecture of the precursor rRNA processing pathway is conserved through evolution (Henras et al., 2015; Mullineux and Lafontaine, 2012). In a series of exo- and endonucleolytic cleavages, the external (5′ETS and 3′ETS) and internal transcribed spacers (ITS1 an ITS2) are removed from the primary transcript and the mature 18S, 5.8S and 28S/25S rRNAs are released (Eichler and Craig, 1994; Henras et al., 2015; Mullineux and Lafontaine, 2012). The schemes for pre-rRNA processing in human and budding yeast are provided in Figures 2 and 3, respectively. Pre-rRNA processing in yeast can occur co-transcriptionally and usually starts with cleavages within 5′ETS prior to ITS1 processing (Kos and Tollervey, 2010; Mougey et al., 1993). In higher eukaryotes, pre-RNA processing can follow alternative pathways and there is no evidence of co-transcriptional release of pre-40S particles (Henras et al., 2015).

The processing of human 47S rRNA transcripts begins within 5′ETS at site 01, mapped to the conserved region in close proximity to the box C/D snoRNA U3 binding region (Kass et al., 1987; Mullineux and Lafontaine, 2012). The U3 snoRNA is required for the ribosome small subunit biogenesis and 5′ETS processing at sites 01 and A0 in humans (Langhendries et al., 2016) but also for the initial processing events in mouse (Kass et al., 1990) and yeast (Hughes and Ares, 1991). The human 01 cleavage can occur within two alternative sites, either within C414-C416 or G420-U422 (nucleotide numbering corresponds to the primary transcript) (Kass et al., 1987). The other processing event that occurs shortly after transcription, occurs within 3′ETS at site 02, thereby producing 45S pre-rRNA (Mullineux and Lafontaine, 2012). The human 02 site is located in close proximity to the 3′end of 28S rRNA, and 02 processing is thought to happen either concomitantly with 01 cleavage or soon after 01 cleavage (Mullineux and Lafontaine, 2012).

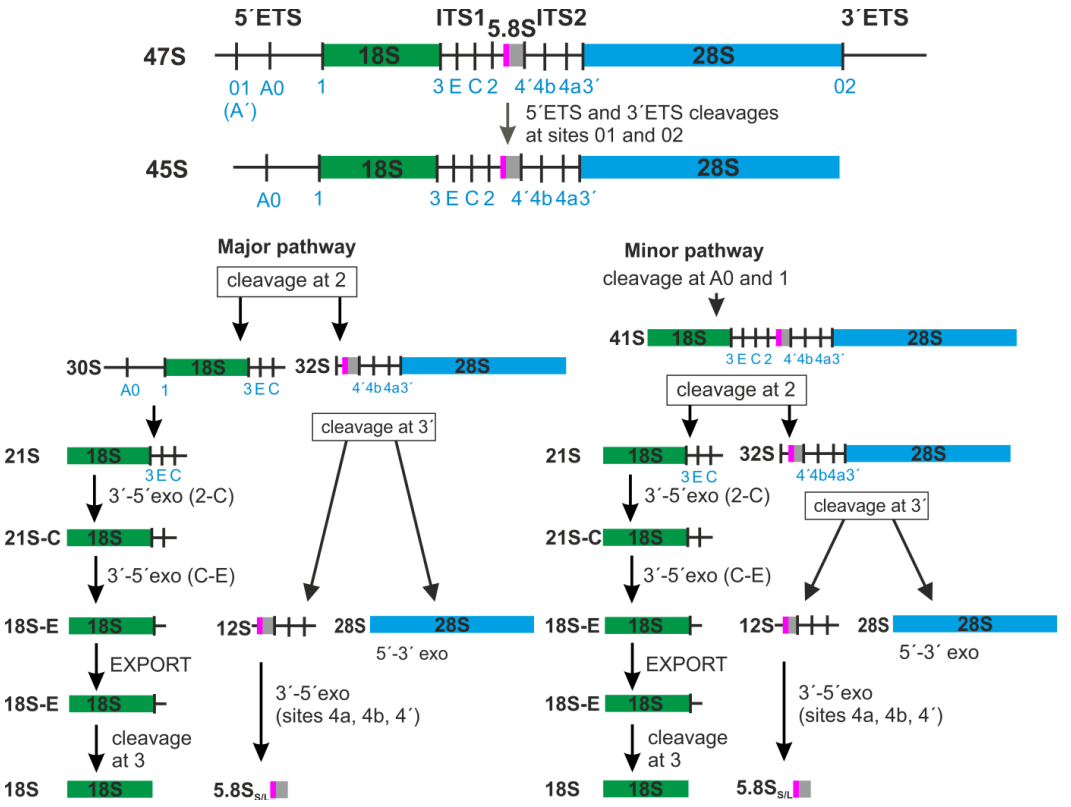

Figure 2. Pre-rRNA processing in human cells. 47S pre-rRNA, transcribed by RNA polymerase I in the nucleolus, is composed of mature 18S (green), 5.8S (grey) and 28S (blue) rRNA. External (5´ETS and 3´ETS) and internal transcribed spacers (ITS1 and ITS2) flank the sequences of mature rRNAs and are removed by exo- and endoribonucleolytic cleavage sites. Individual cleavage sites are marked with blue. The 47S pre-rRNA is cleaved at 5´ETS (site 01) and 3´ETS (site 02), to produce 45S pre-rRNA that can be cleaved by alternative pathways, depending if the next cleavage occurs within ITS1 at site 2 (major pathway) or within 5´ETS at sites A0 and 1 (minor pathway). The cleavage at site 2 produces 30S and 32S pre-rRNA and the further processing of 30S within 5´ETS produces the 21S pre-RNA. In the minor pathway, the cleavage within 5´ETS produces 41S pre-RNA that is further cleaved at site 2 to produce 21S and 32S pre-rRNA. The cleavages at A0 and 1 are tightly coupled; however, their uncoupling can produce 26S and 43S pre-rRNA (not marked on scheme). The 21S pre-rRNA is cleaved at sites C and E to produce 18S-E and at site 3 to produce mature 18S rRNA. The 32S pre-rRNA is cleaved at site 3´ to separate 12S rRNA and 28S rRNA. The 5´-3´and 3´-5´exoribonucleases are involved in the production of 5.8S$_{S/L}$ rRNA and 5´-3´exoribonucleases and are required to produce the 5´end of mature 28S rRNA. There are two types of 5.8S rRNA in human cells, 5.8$_S$ and 5.8$_L$, that differing in size ~7–8 nucleotides. The 5´extension of 5.8S$_L$ is marked with pink.

2.6.3.2. 45S pre-rRNA processing within ITS1 separates the biogenesis pathways of small and large ribosomal subunits

In mammalian cells, the processing of 45S pre-rRNA is flexible and is performed by alternative pathways depending if the processing at site 02 is followed by the cleavage within ITS1 or 5´ETS region (Hadjiolova et al., 1993). In the major pathway of HeLa cells, pre-45S rRNA cleavage occurs within ITS1 at site 2 (C6469-6476), thereby producing 30S and 32S pre-rRNAs (Idol et al., 2007; Mullineux and Lafontaine, 2012). The 30S pre-rRNA that contains mature 18S rRNA is further cleaved at sites A0 (producing 26S rRNA) and at site 1 (producing 21S pre-rRNA) (Mullineux and Lafontaine, 2012). In the minor pathway, 45S rRNA cleavage first occurs within 5´ETS, at site A0 (producing 43S pre-rRNA) and at site 1 (producing 41S pre-rRNA) (Mullineux and Lafontaine, 2012). The 5´ETS processing site A0, which is mapped to position G1643, is located between sites 01 and 1 (Rouquette et al., 2005). It has been shown that processing at sites A0 and 1 is tightly coupled and therefore the 26S and 43S pre-rRNA are present at very low amounts under normal conditions (Rouquette et al., 2005). However, the uncoupling of cleavages at sites A0 and 1 cause the accumulation of 43S pre-rRNA in an aggressive breast cancer cell line (Belin et al., 2009). The cleavage of 41S rRNA within ITS1 at site 2 produces 21S and 32S pre-rRNAs; the same processing intermediates were produced in the major pathway and therefore, the downstream processing of 21S and 32S pre-rRNA are identical in both pathways (Mullineux and Lafontaine, 2012). The primary transcript contains rRNAs of both ribosomal subunits; however, the cleavage in ITS1 leads to the separation of the rRNA processing pathways of the small and large ribosomal subunits.

2.4.3.3. The formation of mature 18S rRNA

The processing of the 21S pre-rRNA continues with 3´-5´ exoribonucleolytic digestion that stops at a conserved region, termed region C (Henras et al., 2015). Removal of ~250 nucleotides that separate site 2 and site C, yields 21S-C rRNA intermediates (Henras et al., 2015). The 21S-C pre-rRNA accumulates after the knock-down of Rps19 (Idol et al., 2007) and ENP1/bystin (Carron et al., 2011), and it is also detected in normal HeLa cells, albeit at a very low level (Carron et al., 2011).

In the nucleolus, further processing of 21S pre-rRNA yields the 18S-E pre-rRNA that is exported to the cytoplasm where the final maturation of 18S rRNA occurs (Rouquette et al., 2005). Processing of 18S-E pre-rRNA is a multistep process in which the first cleavage occurs at site E, located 79 and 82 nucleotides downstream of the 3´ end of 18S rRNA (at G5606 and G5609 of the 47S pre-rRNA) (Preti et al., 2013). Cleavage at site E is followed by rapid 3´-5´exoribonucleolytical trimming (Preti et al., 2013). The exosome complex, namely the EXOSC10/RRP6, exosome cofactors SKIV2L2 and MPP6, exosome core protein RRP46/EXOSC5 and MTR4 helicase, participate in the

production of the 3´end of 18S rRNA (Sloan et al., 2013; Tafforeau et al., 2013). Under normal conditions, ~70-80% of the 18S-E rRNA is found in the cytoplasm (Rouquette et al., 2005). The exonucleolytic processing of 18S-E rRNA occurs during its transport and, as a result, 18S-E pre-rRNA of various lengths can be detected in the nucleus and cytoplasm (Preti et al., 2013). The longest 18S-E rRNA variants contain more than 40 nucleotides of ITS1 and are predominantly localized in the nucleolus, whereas the shorter forms that contain between 14–24 nucleotides of ITS1 are found in the cytoplasm (Preti et al., 2013). The presence of two pools of 18S-E pre-rRNA that differ in size by ~25 nucleotides has also shown by others (Haag et al., 2015).

The final maturation of 18S rRNA occurs in the cytoplasm and requires the cleavage at site 3, which is performed by the conserved endonuclease NOB1 (Preti et al., 2013; Sloan et al., 2013). A recent article provides the three-dimensional structure of human pre-40S particles at 19Å resolution and notes the locations of ribosome biogenesis factors LTV1, ENP1/bystin, TSR1, RIO2, and NOB1 (Larburu et al., 2016). In this model, NOB1 is separated from the 18S rRNA 3´end, and, in order to act at site 3, NOB1 should either change its position or undergo a change in conformation (Larburu et al., 2016). Alternatively, it has been suggested that mature 18S rRNA can be produced by 3´-5´ exoribonucleolytic trimming of 18S-E to site 3 (Henras et al., 2015).

2.4.3.4. The formation of mature 28S and 5.8S rRNA

The 5´end of 32S pre-RNA, which consists of ITS1, 5.8S rRNA, ITS2 and 28S rRNA, is further processed by exonucleolytic digestion (Mullineux and Lafontaine, 2012).). In mouse and human cells, the 5´-3´ exonuclease XRN2, which is an ortholog of the Rat1 protein in budding yeast (Schillewaert et al., 2012), is required for the production of the 5´end of 5.8S pre-rRNA (Wang and Pestov, 2011). As in yeast, the 5´end of human 5.8S rRNA is heterogeneous as seen by the presence of shorter ($5.8S_S$ rRNA) and longer forms ($5.8S_L$ rRNA) that differ in size by ~7–8 nucleotides (Heindl and Martinez, 2010; Morello et al., 2011). The ratio of $5.8S_S$ and $5.8S_L$ is between 60:40 to 70:30 in HeLa cells (Heindl and Martinez, 2010; Schillewaert et al., 2012) and does not change after XRN2 depletion (Schillewaert et al., 2012).

Endonucleolytic cleavage of ITS2 at site 3´ (C7849 on the 47S rRNA) separates the 12S and 28S rRNA (Hadjiolova et al., 1993) and requires the Las1L protein in humans (Schillewaert et al., 2012). 12S pre-rRNA is further cleaved within ITS2 at site 4a (C6947 on the 47S rRNA), thereby producing 7S pre-rRNA (Farrar et al., 2008). The processing of the 3´end of human 5.8S rRNA requires the exosome complex and is performed via 3´-5´ exoribonucleolytic trimming (Schillewaert et al., 2012; Tafforeau et al., 2013). The accumulation of 7S pre-rRNA (~340 nucleotides in size) appear after the depletion of exosome components, including EXOSC2, EXOSC5, EXOSC10, SKIV2L2, and MPHOSPH6 (Tafforeau et al., 2013). However, depletion of EXOSC10 also causes the accumulation of another 5.8S rRNA intermediate, the 5.8S+40

(~190–200 nucleotides in size) (Schillewaert et al., 2012; Tafforeau et al., 2013). The final precursor of 5.8S rRNA is transported to the cytoplasm, where ERI1 is required to remove the last few nucleotides of ITS2 by 3′-5′ exoribonucleolytical digestion (Ansel et al., 2008; Schillewaert et al., 2012).

The 3′end of 28S rRNA is generated in an early processing event, namely the 02 cleavage of 47S rRNA (Mullineux and Lafontaine, 2012). The vertebrate specific snoRNA U8 is important for ribosome 60S subunit biogenesis and its depletion leads to the accumulation of 3′ extended processing products (45S-L, 43S-L, 41S-L, 36S-L and 32S-L) (Langhendries et al., 2016). As discussed above, the production of the 5′end of 28S rRNA requires the processing of ITS2 by the 5′-3′exonuclease XRN2 (Wang and Pestov, 2011). In addition to producing mature rRNA ends, XRN2 is involved in the degradation of 5′ETS, ITS1, and ITS2 spacer fragments (Preti et al., 2013; Schillewaert et al., 2012). As with other rRNAs in mammals, the last intermediate of 28S rRNA, which is extended by 4–6 nucleotides at its 5′end, is transported to the cytoplasm, where its final maturation occurs (Mullineux and Lafontaine, 2012).

2.4.3.5. pre-rRNA processing in the eukaryotic model organism budding yeast

For decades, *Saccharomyces cerevisiae* has been the model organism in the field of eukaryotic ribosome biogenesis. This makes it important to introduce the main points gained from studies of budding yeast and compare these with human cells. The 35S pre-rRNA of budding yeast consists of 18S, 5.8S, and 25S rRNAs that are interspersed with external and internal transcribed spacers; however, compared to the human 47S pre-rRNA, the spacers of 35S pre-rRNA, are smaller and the processing of 35S pre-rRNA generates the precursors that are similar, but not identical, to those of higher eukaryotes (**Fig. 3**) (Henras et al., 2015; Woolford and Baserga, 2013). In budding yeast, ~70% of nascent transcripts are cleaved co-transcriptionally at the early processing sites A_0, A_1, and A_2, thereby generating 20S and $27SA_2$ pre-rRNAs while remaining pre-rRNA transcripts, which are processed post-transcriptionally within the 90S pre-ribosome complex (Kos and Tollervey, 2010). Another study demonstrates that the frequency of co-transcriptional processing ranges from 41% to 79% and is dependent on the growth phase of budding yeast (Osheim et al., 2004). Co-transcriptional processing requires the formation of the SSU processome, which consists of several processing factors including the core U3 snoRNP complex that base pairs via U3 snoRNA with sequences of 5′ETS and 18S rRNA (Dragon et al., 2002). These complexes are visible as terminal knobs at the 5′ ends of nascent pre-rRNA transcripts using cryo-electron microscopy (Miller and Beatty, 1969; Mougey et al., 1993; Osheim et al., 2004). The 5′ terminal knobs on nascent transcripts appear in most eukaryotes, however, co-transcriptional separation of the precursors of the small and large subunits of the ribosome is rare (Osheim et al., 2004).

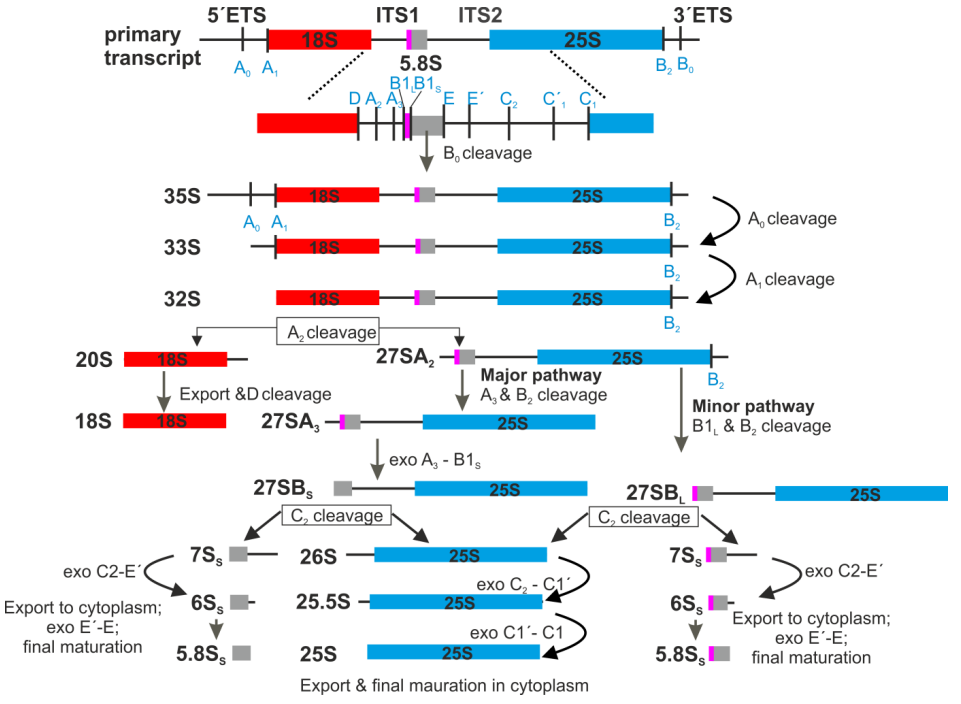

Figure 3. Schematic representation of pre-rRNA processing in *Saccharomyces cerevisiae*. The 35S pre-rRNA consists of 18S (red), 5.8S (grey), and 25S (blue) rRNAs, flanked by external (5´ETS and 3´ETS) and internal transcribed spacers (ITS1 and ITS2). The processing sites are marked. Pre-rRNA processing can occur co-transcriptionally or after transcription. During post-transcriptional processing, cleavage in 3´ETS at site B_0 generates the 35S pre-rRNA that is processed by coupled digestions at A_0, A_1, and A_2, yielding 20S and $27SA_2$ pre-rRNAs. The processing of 20S pre-rRNA continues with nuclear export and cleavage at site D, thereby generating mature 18S rRNA. There are two alternative routes for $27SA_2$ processing, either at site A_3 followed by 5´-3´exonucleolytic digestion to site $B1_S$, or at $B1_L$, yielding $27SB_S$ or $27SB_L$, respectively. $27SB_S$ and $27SB_L$ differ in their 5´ ends; however, their exonucleolytic digestions in ITS2 lead to the production of 25S rRNA and $5.8S_S$ or $5.8S_L$ rRNA, respectively. The 5´extension present in $5.8S_L$ is marked with pink.

During post-transcriptional processing, the cleavage in 3`ETS at site B_0 is performed by Rnt1 and releases the 90S pre-ribosome (Kufel et al., 1999; Venema and Tollervey, 1999). The initial cleavage is followed by cleavages at site A_0 of 5´ETS and at site A_1, generating the 33S and 32S pre-rRNAs, respectively (Venema and Tollervey, 1999). The A_1 cleavage generates the 5´ end of mature 18S rRNA and the cleavage at site A_2 in ITS1 results in the separation of the 32S pre-rRNA into the 20S and $27SA_2$ pre-rRNA molecules, which are the precursors of SSU and LSU (Henras et al., 2015; Mullineux and Lafontaine, 2012; Woolford and Baserga, 2013). Processing at sites A_0, A_1, and A_2 is tightly coupled; however, their uncoupling or inhibition leads to cleavage

at site A_3, thereby yielding aberrant 23S, 22S, and 21S pre-rRNAs that are probably degraded by the nucleolar surveillance pathway (Mullineux and Lafontaine, 2012). However, 23S pre-rRNA is detected in low amounts in wild type cells and can be a normal rRNA precursor (Granneman and Baserga, 2004).

Unlike the processing of 21S pre-rRNA in higher eukaryotes, which require both exo- and endonucleolytic cleavages, the processing of 20S pre-rRNA in budding yeast requires a single cleavage at site D, which is performed by the endonuclease Nob1, releasing ITS1 fragment D-A_2, which is degraded by the 5′-3′ exoribonuclease Xrn1 (Mullineux and Lafontaine, 2012). Site D corresponds to site 3 in humans and in both organisms, the final processing step is performed in the cytoplasm by Nob1/NOB1 (Larburu et al., 2016; Lebaron et al., 2012; Pertschy et al., 2009; Preti et al., 2013; Sloan et al., 2013).

The processing of $27SA_2$ pre-rRNA can occur in two alternative pathways either at site A_3 or $B1_L$ (Woolford and Baserga, 2013). About 80% of $27SA_2$ pre-rRNA is cleaved by MRP RNase at site A_3 in ITS1 (Chu et al., 1994; Lygerou et al., 1996), thereby generating $27SA_3$, which is further trimmed by 5′-3′ exoribonucleases Rat1-Rai1 heterodimer (Henry et al., 1994), Rrp17 (Oeffinger et al., 2009) and Xrn1 (Henry et al., 1994) and requires the presence of A_3 processing factors (Granneman et al., 2011). In the major pathway, 5′-3′ exonucleases stop at $B1_S$, yielding the $27SB_S$ pre-rRNA (Woolford and Baserga, 2013). The remaining 20% of $27SA_2$ pre-rRNA is processed by direct cleavage at site $B1_L$, producing the $27SB_L$ (Faber et al., 2006). The processing of $27SB_S$ and $27SB_L$ is identical and the cleavage in ITS2 at site C_2 separates the processing pathways of 5.8S and 25S rRNAs, thereby generating the $7S_S/7S_L$ and 26S pre-rRNAs (Woolford and Baserga, 2013). The 26S pre-rRNA is processed by a two-step 5′-3′ exonucleolytic digestion that includes the formation of 25.5S pre-rRNA, and is rapidly digested to 25S rRNA by 5′-3′ exonuclease Rat1 or Xrn1 (El Hage et al., 2008; Geerlings et al., 2000). The $7S_S/7S_L$ pre-rRNA is processed to $6S_S/6S_L$ pre-rRNA by the exosome complex and is then transported to the cytoplasm where Ngl2 is required to produce mature 5.8S rRNA (Faber et al., 2006; Thomson and Tollervey, 2010). Due to the different pathways and processing sites, the two types of 5.8S rRNA molecules generated, the major $5.8S_S$ and the minor $5.8S_L$, differ in their 5′ends by either 7 or 8 nucleotides (Henry et al., 1994).

2.7. rRNA modifications

Ribosome biogenesis involves the formation of modified nucleotides in pre-rRNA, which allows changing the biochemical and biophysical properties of certain nucleotides (Motorin and Helm, 2011). ~2–3% of the nucleotides in rRNA are modified (Sloan et al., 2016) and the majority of rRNA modifications are introduced already in the nucleolus (Watkins and Bohnsack, 2012). The most abundant posttranscriptional modifications in eukaryotes are pseudo-

uridylation (Ψ) and ribose methylation – namely, there are 44 Ψs and 55 ribose methylations in the ribosome of budding yeast (Machnicka et al., 2013; Piekna-Przybylska et al., 2008) and 95 Ψs and 106 ribose methylations in the human ribosome (Krogh et al., 2016). In addition, approximately ten base modifications are intoduced in rRNA. The number of modified bases in rRNA is similar in budding yeast and human and most, but not all, modifications are introduced at equivalent positions (Maden and Hughes, 1997; Piekna-Przybylska et al., 2008; Sharma and Lafontaine, 2015). The rRNA modifications are often conserved and clustered at functionally important regions of the ribosome, namely the peptidyl transferase centre in the LSU and the decoding centre in the SSU as well as the A, P, and E sites of tRNA and mRNA binding, the polypeptide exit tunnel, and intersubunit bridges (Decatur and Fournier, 2002). Posttranscriptional modification of rRNA can affect the functions of the ribosome, most probably the efficiency and fidelity of translation (Decatur and Fournier, 2002; Motorin and Helm, 2011).

For decades, it was assumed that each organism contains only one type of ribosome and all positions in the rRNA are always fully methylated; however, measuring the fraction of molecules modified at particular sites demonstrates that in human cells only ~2/3 of methylation sites are fully or close to fully methylated, and the remainder are partially methylated, thereby supporting the concept that ribosomes can be heterogeneous (Krogh et al., 2016). In the rRNA of budding yeast, 94 nucleotides, including the majority of base modifications, are close to fully modified, whereas 18 nucleotides are modified in less than 85% of ribosomes (Taoka et al., 2016). In actively growing yeast cells, approximately 68% of adenosines at position 100 of 18S rRNA in both monosomes and polysomes are methylated, which shows that the ribosomes that lack A100 methylation are engaged in translation (Buchhaupt et al., 2014). The extent of 25S rRNA A100 methylation depends on the expression level of the guide RNA, the box C/D snoRNA snR51, which is required for introducing this modification (Buchhaupt et al., 2014).

2.7.1. rRNA pseudouridylation and ribose 2´-O-methylation are carried out by ribonucleoprotein complexes

In eukaryotes, the pseudouridylation and ribose methylation reactions are performed by small ribonucleoprotein (snoRNP) complexes, and guided by box H/ACA (Ganot et al., 1997; Ni et al., 1997) and box C/D snoRNAs (Kiss-László et al., 1996), respectively. The formation of ribose methylation requires the addition of a methyl group to the 2´-oxygen of ribose, catalysed by a methyltransferase fibrillarin (FBL) in humans and by Nop1 in yeast (Tollervey et al., 1993). The ribose methylation reaction takes place five nucleotides upstream of the conserved D or D´ box of snoRNA and one box C/D snoRNA can guide the methylation at two alternative sites in the rRNA (Kiss-László et al., 1996; Maden and Hughes, 1997). The formation of Ψ occurs via iso-

merization of uridine and is performed by Ψ synthase dyskerin in humans and by Cbf5 in yeast cells (Lafontaine et al., 1998a). The precise role of pseudouridine formation in ribosomes remains unknown, however it offers an additional hydrogen bond donor site and may be required to stabilize the structure and function of rRNA, as shown with the Ψs located in human 28S rRNA helix 69, which are part of the intersubunit bridge B2a (Sumita et al., 2005).

Fibrillarin/Nop1 and dyskerin/Cbf5 belong to the box C/D and box H/ACA snoRNP complexes, respectively (Balakin et al., 1996). In addition to enzymes, the box C/D snoRNP complex is composed of RNA binding protein 15.5K/NHPX, NOP56, and NOP58, whereas the box H/ACA snoRNP complex consists of RNA binding protein NHP2, GAR1, and NOP10 (Watkins and Bohnsack, 2012). The genes that encode the components of the snoRNP complexes are essential for the viability of human cells (Blomen et al., 2015) and their down-regulation causes defects in early steps of pre-rRNA processing (Tafforeau et al., 2013)

Although variations in ribosome structure occur under normal conditions, differential expression of ribosome biogenesis factors, including rRNA modification enzymes, can lead to several diseases (Sloan et al., 2016; Tafforeau et al., 2013). The expression level of both snoRNAs and FBL are upregulated in different types of malignancies (Marcel et al., 2013; Martens-Uzunova et al., 2012; Su et al., 2014) and mutations in the DKC1 are the cause of X-linked dysceratosis congenita, characterized by bone-marrow failure and increased susceptibility to cancer (Heiss et al., 1998). It has been shown using the xenograft model that decreasing the expression of fibrillarin reduces the tumorigenicity of human breast cancer cell line MCF-7 (Su et al., 2014).

2.7.2. Stand-alone enzymes catalyse the formation of rRNA base modifications

In eukaryotes, the formation of base methylation and acetylation are catalysed by stand-alone enzymes. The 25S rRNA of budding yeast contains six methylated bases, located in the peptidyl transferase centre (m^1A645, m^5C2870, and m^3U2634), in the subunit interface (m^1A2142 and m^5C2278) and in the phosphostalk (m^3U2843) (Sharma and Lafontaine, 2015). The 18S rRNA of budding yeast contains six modified bases, located in the decoding site (ac^4C1773, m^6_2A1781, and m^6_2A1782), mRNA channel (ac^4C1280) and near the tRNA binding sites ($m^1acp^3\Psi$1191 and m^7G1575) (Sharma and Lafontaine, 2015). In addition to performing rRNA modifications, several rRNA methyltransferases are also required for ribosome biogenesis and pre-rRNA processing; however, only the presence of these proteins, and not their catalytic activity is important for ribosome biogenesis (Sharma and Lafontaine, 2015).

2.7.2.1. 28S/25S rRNA methyltransferases involved in ribosome biogenesis

Rrp8 of budding yeast catalyses the formation of m^1A645 of 25S rRNA (Peifer et al., 2013), while nucleomethylin (NML) is responsible for the formation of 28S rRNA m^1A1309 in human (Waku et al., 2016). Rrp8 is not essential, however, it affects the biogenesis of both ribosomal subunits (Peifer et al., 2013; Schilling et al., 2012) and pre-rRNA processing within ITS1 at site A_2 (Bousquet-Antonelli et al., 2000). The methyltransferase activity of Rrp8 is not required to process ITS2, which indicates that the roles of Rrp8 in rRNA methylation and ribosome biogenesis are separated (Peifer et al., 2013). Human NML is not required for pre-rRNA processing (Tafforeau et al., 2013), however, it is important for ribosome 60S subunit biogenesis and its depletion supresses cell proliferation, by activating the p53 pathway (Waku et al., 2016). Under nutrient deficiency, NML inhibits rDNA synthesis and protects cells from energy deprivation-dependent apoptosis (Murayama et al., 2008; Yang et al., 2013).

In budding yeast, the essential nucleolar protein Nop2/Nsun1 (de Beus et al., 1994) is responsible for the formation of m^5C2870 of 25S rRNA (Sharma et al., 2013a). A decrease in the expression level of Nop2 (Nucleolar protein gene 2) leads to lengthened doubling time and defects in ribosome biogenesis (Hong et al., 1997). It has been shown that the presence of m^5C2870 methylation is critical because the expression of a catalytically inactive mutant of Nop2 does not rescue either the growth defects, the impairment of ribosome biogenesis, nor the reduced amount of polysomes (Sharma et al., 2013a). However, another study demonstrated that a catalytically dead mutant of Nop2 rescued the growth defect of a Δ*Nop2* yeast strain, showing that the presence of m^5C2870 is not important for ribosome biogenesis (Bourgeois et al., 2015). Based on sequence alignment, the homologous protein NSUN1 (also known as p120 and NOL1) is considered as a putative candidate for human 28S rRNA m^5C4447 modification (Sharma et al., 2013a). NSUN1 is not required for rRNA processing; however, it possesses m^5C methyltransferase activity and the expression of human NSUN1 in a Δ*Nop2* yeast strain completely restores the 25S rRNA C2870 methylation and partially rescues its growth defect (Bourgeois et al., 2015; Tafforeau et al., 2013).

In budding yeast, the nucleolar protein Bmt5 and the nucleocytoplasmic protein Bmt6 catalyse the formation of two m^3U modifications in 25S rRNA (Sharma et al., 2014). Both proteins and methylated residues are conserved among lower eukaryotes, however, these enzymes are not essential and their absence does not affect ribosome biogenesis or cell growth (Sharma et al., 2014).

The budding yeast Bmt2 introduces the m^1A2142 of 25S rRNA (Sharma et al., 2013b) and Rcm1 catalyses the formation m^5C2278 of 25S rRNA (Gigova et al., 2014; Sharma et al., 2013a). Both methylations lie in the subunit interface of budding yeast ribosomes, however, neither the presence of these enzymes nor the methylations are essential for cell growth (Sharma et al., 2013b, 2013a).

Yeast 25S rRNA m^1A2142 is conserved in lower eukaryotes and a *Bmt2* deletion does not cause defects in ribosome biogenesis (Sharma et al., 2013b).

25S rRNA m^5C2278 is part of the eukaryote specific intersubunit bridge eB14 (Sharma and Lafontaine, 2015). In this bridge, ribosomal protein eL41 connects the LSU and modified nucleotides in the decoding site of SSU, namely the ac^4C1773, m_2^6A1781 and m_2^6A1782 of 18S rRNA (Sharma and Lafontaine, 2015). Deletion of *Rcm1* does not interfere with ribosome 60S subunit synthesis (Schosserer et al., 2015; Sharma et al., 2013a). Downregulation of *Rcm1* in yeast and its orthologs in worms and flies lengthens the lifespan of these organisms and increases their resistance to different types of stress, including heat shock and oxidative stress (Schosserer et al., 2015). m^5C2278 corresponds to 28S rRNA m^5C3782 in humans and NSUN5/WBSCR20, a conserved protein in eukaryotes, is considered to be the enzyme that catalyses this reaction in human cells (Schosserer et al., 2015; Sharma et al., 2013a). The methyltransferase activity of human NSUN5 has not yet been elucidated, however, it is known to affect ribosome synthesis in the early steps of pre-rRNA processing (Tafforeau et al., 2013).

2.7.2.2. 18S rRNA methyltransferases in ribosome biogenesis

Dim1/DIMT1 catalyses the formation of dimethyladenosines in 3´ end of 18S rRNA

In the small subunit of the ribosome, the structure of 3´ end of rRNA (either 16S and 18S rRNA) is highly conserved and contains two dimethylated adenosines, located in helix 45 (Piekna-Przybylska et al., 2008). The formation of these modifications is carried out by a family of methyltransferases that are present in all domains of life (O'Farrell et al., 2008). The Dim1 protein modifies 18S rRNA m_2^6A1781 and m_2^6A1782 in budding yeast (Lafontaine et al., 1995) and the homologous human protein DIMT1 (also known as DIMT1L and DIM1) (Stanchi et al., 2001) is involved in the formation of 18S rRNA m_2^6A1850 and m_2^6A1851 (Zorbas et al., 2015). The nucleolar proteins DIMT1 and Dim1 are essential for human and yeast cells, respectively (Blomen et al., 2015; Lafontaine et al., 1994). Downregulation of Dim1 causes severe defects in pre-rRNA processing, at sites A_1 and A_2, and leads to both the upregulation of 22S pre-rRNA and the downregulation of 27SA and 20S pre-rRNAs and this, in turn, is followed by a depletion in the pool of mature 18S rRNA (Lafontaine et al., 1995). In HeLa cells, depletion of DIMT1 by RNA interference causes uncoupling of cleavages within sites A0 and 1 which leads to the accumulation of 26S and 43S pre-rRNAs (Zorbas et al., 2015). DIMT1 depletion in other human cell lines (WI-38, RKO and HCT116) causes the level of 26S pre-rRNA to rise and also leads to the accumulation of 21S and 21S-C pre-rRNAs, which results in a reduction of mature 18S rRNA (Zorbas et al., 2015). The expression of catalytically inactive Dim1/DIMT1 mutants is able to rescue defects in pre-

rRNA processing, which demonstrates that the presence of 18S rRNA dimethylation is not required for ribosome biogenesis (Lafontaine et al., 1998b; Zorbas et al., 2015).

Dim1 is a nucleolar protein that binds to the pre-rRNA processing machinery and affects early pre-rRNA cleavages; however, it remains bound with pre-rRNA and catalyses the modification of two m_2^6A on 20S pre-rRNA in the cytoplasm (Lafontaine et al., 1998b). In human cells, the formation of m_2^6A1850 and m_2^6A1851 within 18S rRNA occurs earlier, already in the nucleus, but after the cleavages that are regulated by the presence of DIMT1 (Zorbas et al., 2015). Delay in rRNA processing and modification events support the hypothesis that the presence of Dim1 and DIMT1 within pre-ribosomes might serve as a quality control mechanism that regulates ribosome biogenesis (Lafontaine et al., 1998b; Zorbas et al., 2015).

Kre33/NAT10 catalyses the formation N4-acetylcytidines

The Kre33 protein within budding yeast and the human protein NAT10 are important for the introduction of two N4-acetylcytidines within 18S rRNA (Sharma et al., 2015). In humans, the vertebrate-specific box C/D snoRNA U13 (Tyc and Steitz, 1989) is also critical for the formation of ac^4C1842 (Sharma et al., 2015). Expression of a catalytically inactive form of Kre33 demonstrates that 18S rRNA acetylation is not essential for the growth of budding yeast cells (Sharma et al., 2015). However, Kre33 interacts with pre-40S ribosomes and its presence is essential for the biogenesis of the ribosome small subunit, pre-rRNA processing, and cell growth (Ito et al., 2014a; Sharma et al., 2015). Kre33 affects the early nucleolar cleavages (at sites A_0-A_2), and, as with Dim1, down-regulation of Kre33 expression leads to the accumulation of 22S pre-rRNA (Lafontaine et al., 1995; Sharma et al., 2015). The depletion of NAT10 causes morphological changes in nucleoli and leads to a slow growth phenotype (Shen et al., 2009), increased apoptosis (Ito et al., 2014b), and reduces the level of 18S rRNA precursors (21S and 18S-E pre-rRNA), which results in a decrease in the production of ribosome 40S subunits (Ito et al., 2014b; Sharma et al., 2015; Tafforeau et al., 2013).

snoRNP complex and stand-alone enzymes participate in the formation of hypermodified residue ($m^1acp^3\Psi$)

The hypermodified residue, $m^1acp^3\Psi1191$ of 18S rRNA is synthesized in three steps and requires the action of the snoRNP complex as well as stand-alone enzymes. The first step occurs in the nucleolus where the H/ACA snoRNP complex (snoRNP35) carries out a pseudouridylation reaction (Samarsky et al., 1995). Thereafter, the methyltransferase Nep1 (Emg1) catalyses N1-methylation of Ψ (Meyer et al., 2011) and in the cytoplasm, Tsr3 catalyses the transfer of aminocarboxypropyl (acp) to $m^1\Psi$ thereby producing $m^1acp^3\Psi$ (Meyer et al.,

2016). 18S rRNA hypermodification and the enzymes that catalyse its formation are conserved in eukaryotes and it has been shown that the H/ACA box snoRNP complex (contains snoRNA ACA13) and TSR3 are required for the formation of 18S rRNA $m^1acp^3\Psi1248$ in human cells (Kiss et al., 2004; Meyer et al., 2016). The results obtained from an *in vitro* methylation assay demonstrate that human NEP1 is able to methylate Ψ1248 (Wurm et al., 2010).

In budding yeast, deletion of either *snR35* or *Tsr3* causes minor growth defects (Meyer et al., 2016), while the presence of *Nep1* is essential (Eschrich et al., 2002). Like other 18S rRNA methyltransferases, yeast Nep1 and human NEP1, are important for ribosome small subunit biogenesis (Eschrich et al., 2002; Tafforeau et al., 2013). Downregulation of NEP1 expression slightly decreases the levels of 47S, 45S, and 41S pre-rRNAs (Tafforeau et al., 2013) and in another study, upregulation of 30S, 21S, and 21S-C pre-rRNAs is also documented (Warda et al., 2016). A missense mutation in the gene encoding NEP1/EMG1 (D86G) is the cause of a severe autosomal recessive disease known as Bowen-Conradi syndrome (BCS; OMIM 211180) (Armistead et al., 2009). The BCS mutation does not abolish the methyltransferase activity of NEP1, however, it does disrupt a salt bridge which leads to protein aggregation (Armistead et al., 2009) and its subsequent degradation in a proteasome-dependent manner (Warda et al., 2016). As a result, a decrease in the level of NEP1 protein impairs ribosome SSU maturation in BCS (Warda et al., 2016). TSR3 affects several steps in ribosome small subunit maturation and its depletion in human cells causes the accumulation of 47S and 18S-E pre-rRNAs (Meyer et al., 2016). As with the human phenotype, deletion of *Tsr3* in budding yeast causes accumulation of 35S pre-rRNA as well as an increase in the level of the final precursor of 18S rRNA, and the 20S pre-rRNA (Meyer et al., 2016). Unlike most rRNA methyltransferases, the expression of catalytically inactive mutants of Tsr3 does not rescue the processing defects (Meyer et al., 2016).

Bud23/WBSCR22 catalyses the formation of N7-methylated guanosine

Eukaryotic 18S rRNA contains another base methylation, N7-methylated guanosine (m^7G), which, in the three-dimensional structure of the ribosome, is located in the ridge between the P-site and E-site tRNAs (Sharma and Lafontaine, 2015). The presence of a guanosine base at this location is highly conserved, and corresponds to G1338 in *Escherichia coli,* however, its m^7G methylation is only present in eukaryotes (m^7G1575 and m^7G1639 in budding yeast and human 18S rRNA, respectively) (Piekna-Przybylska et al., 2008; White et al., 2008). Bud23 in yeast and its human ortholog WBSCR22 catalyse these methyl transfer reactions (Haag et al., 2015; White et al., 2008; Zorbas et al., 2015).

rRNA base methyltransferase Bud23 is not essential in yeast, however its absence leads to loss of 18S rRNA m^7G1575 methylation, pre-rRNA processing defects, impaired ribosome small subunit biogenesis, and decreased cell growth (White et al., 2008). Bud23 interacts with components of the SSU processome, Utp2 and Utp14, but not with U3 snoRNA (Sardana et al., 2013). The loss of

Bud23 expression causes accumulation of the initial pre-rRNA transcript, 35S pre-rRNA, as well as a decrease in 27SA pre-rRNA, which demonstrates that the early pre-rRNA processing step of cleaving A_2 within ITS1 is impaired (Sardana and Johnson, 2012; White et al., 2008). Furthermore, after cleavage within A_2, Bud23 remains bound to 20S pre-rRNA (Sardana et al., 2013). 20S pre-rRNA accumulation upon *Bud23* deletion shows that the protein is also engaged in late steps of 18S rRNA formation (White et al., 2008). Similarly, pre-rRNA processing defects occur in WBSCR22 depleted human cells (Haag et al., 2015; Tafforeau et al., 2013; Zorbas et al., 2015). On the other hand, the pre-rRNA intermediates that accumulate as a result of WBSCR22 depletion are not identical with those that increase after *Bud23* deletion. The details of this process are discussed in section 5.1.3.

2.8. Trm112/TRMT112 is an interaction partner for several methyltransferases

Budding yeast Trm112 is a 15-kDa zinc-finger protein that interacts with several methyltransferases important for translation. This protein is conserved in all domains of life, however, the residues important for binding the zinc atom are not conserved in metazoans (Bourgeois et al., 2017a). In budding yeast, the interactions of Trm112 with four methyltransferases, i.e., Trm9, Trm11, Mtq2, and Bud23, are considered direct interactions because these proteins co-purify with Trm112 when they are co-expressed in bacterial cells (Figaro et al., 2012; Heurgué-Hamard et al., 2006; Mazauric et al., 2010). A recent review compares various crystal structures of Trm112-methyltransferase complexes and found that Trm112 interacts with its methyltransferase partners in a very similar way, and, methyltransferases probably compete with each other to interact with Trm112 (Bourgeois et al., 2017a). During binding, the β3 of the methyltransferase forms a β-zipper interaction with β4 of Trm112 and this complex formation buries a large hydrophobic region on the surface of both partners (Létoquart et al., 2014, 2015; Liger et al., 2011).

Trm9-Trm112 and Trm11-Trm112 complexes are involved in the modification of tRNAs. Trm9-Trm112 modifies the wobble uridine (U34) of some tRNAs and the Trm11-Trm112 complex catalyses the formation of 2-methylguanosine (m^2G) at position 10 of several tRNAs (Mazauric et al., 2010; Purushothaman et al., 2005). The Mtq2-Trm112 complex catalyses the methylation of translation termination factor eRF1 (Heurgué-Hamard et al., 2006), whereas the Bud23-Trm112 complex catalyses the formation of m^7G1575 within 18S rRNA and participates in ribosome 40S subunit biogenesis In addition to direct interactions, co-immunoprecipitation analysis demonstrates that the Trm112 protein forms complexes with 25S rRNA methyltransferases Nop2 and Rcm1 and is bound to 27S pre-rRNA, 7S pre-rRNA, and 25S rRNA, which affects the biogenesis of the large ribosomal subunit (Sardana and Johnson, 2012).

2.9. Extracellular vesicles as carriers of biological information

In order to facilitate intercellular communication in normal and pathophysiological processes, cells release various particles, collectively termed extracellular vesicles (EVs), to the extracellular environment (Abels and Breakefield, 2016). EVs present a heterogeneous family of small membranous particles that originate from either endosomes or from the plasma membrane, and these particles are divided into three groups – e.g., exosomes, microvesicles and apoptotic bodies (Kalra et al., 2012). Exosomes range from 40 to 120 nm in size, are produced inside multivesicular endosomes or multivesicular bodies, and their release occurs when these compartments fuse with the plasma membrane (Colombo et al., 2014). Microvesicles (50–1000 nm) are formed and released via budding from the plasma membrane of the cell (Colombo et al., 2014).

EVs are complex structures that are covered with a lipid bilayer that contains transmembrane proteins and encloses soluble hydrophilic components derived from the cytosol of the donor cell (Colombo et al., 2014). These particles are internalized via endocytosis or membrane fusion, which enables the release of the contents of the particle into the recipient cell (Mulcahy et al., 2014). Cell-derived membrane vesicles are endogenous carriers of cellular proteins, as well as genetic material (DNA, RNA and non-coding RNAs), lipids or sugars and thus, the contents of a particular EV can influence the phenotype of recipient cells (Yáñez-Mó et al., 2015). EVs are deregulated in cancer and the possibility of using EVs to deliver therapeutic cargo is being investigated (van Dommelen et al., 2012). Virus-like particles (VLPs) are produced by ectopic expression of retrovirus Gag protein and are biochemically very similar to EVs and could potentially be used in immunotherapy and for the delivery of therapeutic cargo.

In order to use EVs for medical purpose, it is important to precisely know their contents. Mass-spectrometry based analyses have shown that exosomes contain specific groups of cellular proteins, some of which vary with cell type; whereas others are found in most exosomes regardless of cell type (Colombo et al., 2014).

3. AIMS OF THE STUDY

Ribosome biogenesis is a complex process that, in human cells, requires at least 284 trans-acting factors that participate in pre-rRNA processing and modification, ribosome subunit assembly and transport of pre-ribosomal particles (Tafforeau et al., 2013). One third of these factors are medically important because they are related to genetic diseases and cancer (Tafforeau et al., 2013). The gene encoding the WBSCR22 protein is deleted in Williams-Beuren syndrome and its expression is increased in cancers (Doll and Grzeschik, 2001; Merla et al., 2002; Nakazawa et al., 2011; Tiedemann et al., 2012). Orthologs of WBSCR22 – e.g., the budding yeast's Bud23 and *Arabidopsis thaliana'*s Rid2 have nucleolar functions and participate in ribosome biogenesis (Ohbayashi et al., 2011; White et al., 2008). The cellular functions of WBSCR22 were unknown when this current work was undertaken and the aim of the first part of this dissertation is to investigate the functions of WBSCR22 protein in human cells.

The specific aims of the first part of this dissertation (Papers I and II) are the following:

- to compare the expression level of WBSCR22 protein in cell-lines derived from Williams-Beuren syndrome patients and healthy controls,
- to define the subcellular localization of WBSCR22 protein in order to become closer to clarifying its functions in the cells,
- to elucidate, whether WBSCR22 protein is important for cell growth, pre-rRNA processing and ribosome biogenesis,
- to determine the interaction partners of WBSCR22 protein,
- to elucidate whether the interacting proteins influence the functions of WBSCR22 protein.

The second part of this work includes the formation and characterization of MLV Gag based virus-like particles carrying melanoma antigens. These are chimeric enveloped VLPs, formed by outward budding at the plasma membrane of host cells and therefore, host proteins are always included in VLPs. The aim of Paper III is to study the protein content of MLV-Gag induced virus-like particles using mass-spectrometry.

4. MATERIALS AND METHODS

Detailed descriptions of the materials and methods used in this study are provided in the original publications and briefly reproduced here.

In general, the cellular functions of WBSCR22 protein were studied in human tissue culture cells in which the expression level of WBSCR22 was reduced using RNA interference. For WBSCR22 depletion, WBSCR22 siRNAs were transfected into cells and analysed 72 hours after transfection by various methods discussed below. In order to analyse the role of WBSCR22 downregulation on cell growth, the number of siWBSCR22 treated and control cells was counted up to 5 days. Studying the role of WBSCR22 in ribosome biogenesis and pre-rRNA processing required fractionating the cells into cytoplasmic and nuclear fractions. The cytoplasmic lysate from siWBSCR22 treated cells was used in polyribosome profile analysis. For pre-rRNA processing analysis, RNA from whole cells, cytoplasmic and nuclear fractions was extracted and separated by agarose gel electrophoresis and the abundance of different 18S rRNA processing precursors were evaluated by Northern blot. HeLa cell lysate ultracentrifugation through a sucrose gradient, followed by gradient fractionation, immunoblot, and RNA analysis enabled us to estimate the sedimentation properties of endogenous WBSCR22 protein. Immunoblot analysis was used to study the expression level of WBSCR22 protein in cell-lines derived from the Williams-Beuren syndrome patients.

In order to investigate which proteins interact with WBSCR22, a cell line that stably expresses epitope tagged WBSCR22 protein was generated and then studied using co-immunoprecipitation analysis. For this purpose, these cells, together with control cells, were metabolically labelled in SILAC media and analysed using mass-spectrometry. The most significant interactions found by analysing the SILAC data were then evaluated using immunoblot analysis. Based on these results, the immunofluorescence analysis was used to study the subcellular localization of WBSCR22, TRMT112, and C1QBP. For this, the fluorescent fusion proteins EGFP-WBSCR22 and TRMT112-mCherry were expressed in order to determine the subcellular localisation of the WBSCR22-TRMT112 complex in live cells. Both immunoblot analysis and flow cytometry were used to monitor the expression of ectopically expressed WBSCR22 and EGFP-WBSCR22, respectively. Treatment with a 26S proteasome inhibitor (MG132) followed by immunoblot or flow cytometry enabled us to analyse whether the expression WBSCR22 is regulated by proteasomes.

In order to produce MLV Gag based VLPs, the mouse fibroblast cell line COP5 was transfected with either plasmids that encode the MLV Gag protein to produce MLV Gag VLPs, or with plasmids that encode both the MLV Gag protein and the melanoma antigen (MART1, TRP1, MAGEA4, MAGEA10 or MCAM), to produce VLPs that carry one of the melanoma antigens. The VLPs were purified from cell culture media and characterized using various methods. To study the protein content of MLV Gag VLPs and VLPs that carry melanoma antigens, fractions that contain the VLPs were pooled and studied using mass-spectrometry.

5. RESULTS AND DISCUSSION

5.1. WBSCR22 protein is required for cell growth, ribosome small subunit biogenesis and pre-rRNA processing (Papers I and II)

5.1.1. The level of WBSCR22 protein is reduced in cell lines derived from Williams-Beuren syndrome patients

WBSCR22 is one of the genes expressed from the single-copy gene region of Williams-Beuren syndrome chromosome locus (Doll and Grzeschik, 2001; Merla et al., 2002). Expression of most of the transcripts encoded by the 7q11.23 chromosome region, including *WBSCR22* mRNA, is reduced in WBS patients (Merla et al., 2006). However, for two genes, dosage-compensation mechanisms are known to exist, and their mRNA levels remain unaffected compared to healthy controls (Merla et al., 2006). Crucially, the existence of possible compensatory mechanisms acting at the protein level remains largely unexplored. To analyse the expression level of WBSCR22 protein, the immunoblot analysis from lymphoblastoid cell lines (LCL) derived from Williams-Beuren syndrome patients, was performed (Paper **I, Fig. 6A**). This analysis demonstrates that the expression level of WBSCR22 protein is reduced to a level similar to the level of mRNA found in cell lines derived from WBS patients, which indicates that WBSCR22 is a dosage-sensitive gene and therefore studying its role in human cells is of great interest.

5.1.2. WBSCR22 depletion reduces cell growth

Several works have shown that the mRNA of *WBSCR22* is upregulated in different types of malignancies, including invasive breast cancer, hepatocellular carcinoma, and multiple myeloma (Nakazawa et al., 2011; Stefanska et al., 2014; Tiedemann et al., 2012). In cancer cells, the expression level of WBSCR22 regulates cell survival and metastatic potential (Nakazawa et al., 2011; Tiedemann et al., 2012). In the cell lines derived from WBS patients, the levels of WBSCR22 mRNA (Merla et al., 2006) and protein (Paper **I, Fig. 6**) are reduced. The gene-dosage of multiple genes is altered in microdeletion syndromes (such as WBS) and also in malignancies, and one way to understand the function of individual genes is to observe cells deficient in the gene of interest. To determine the role of WBSCR22 in regulating cell growth, RNA interference (RNAi) was used to silence the expression of the *WBSCR22* gene in HeLa cells, and cell growth was monitored for 5 days (Paper **I, Fig. 1B**). Surprisingly, when compared to control cells, the cells transfected with siRNA targeting *WBSCR22* mRNA displayed significantly reduced growth at 72, 96, and 120 hours after transfection. The doubling time of control cells was 21 hours, whereas in WBSCR22-depleted cells, the doubling time was lengthened to 25 hours. This

clearly demonstrates that WBSCR22 is important for cell growth. This observation is in accordance with the results obtained by gene-trap mutagenesis and CRIPR/Cas9-directed gene editing systems that both demonstrate that WBSCR22 is essential for cell viability under laboratory experimental conditions (Blomen et al., 2015; Wang et al., 2015a). In comparison, the budding yeast protein Bud23, which is an ortholog of WBSCR22, is not essential for cell viability, yet the deletion of *Bud23* causes a slow-growth phenotype (White et al., 2008). Similarly, in *Arabidopsis thaliana,* the presence of the WBSCR22 ortholog RID2 is required for plant growth (Ohbayashi et al., 2011). Therefore, as with its orthologs RID2 and Bud23, WBSCR22 is a factor that helps to regulate cell growth.

5.1.3. WBSCR22 is required for ribosome small subunit biogenesis and pre-rRNA processing

In order to determine whether human WBSCR22 affects ribosome biogenesis, its expression was reduced by siRNAs and a polyribosome profile analysis of cytoplasmic extracts was performed. As shown in Paper I, Fig. 2A, down-regulation of *WBSCR22* causes defects in ribosome small subunit biogenesis that are characterized by a decrease in the amount of free 40S subunits in the cytoplasm, together with an increase in the amount of free 60S subunits. This ribosome subunit imbalance results in a diminished amount of 80S ribosomes in WBSCR22 depleted cells, compared to control cells. This, in turn, can reduce the capability to translate and may be the main cause of increased cell doubling time (Paper **I, Fig. 1A)**. Compared to controls, the amount of mature 18S rRNA decreases in the cytoplasm of WBSCR22 depleted cells, and the decreased ratio of 18S to 28S rRNA (Paper **I, Fig. 2B)** indicates that the observed defects in ribosome small subunit biogenesis may arise due to errors in the production of mature 18S rRNA.

Mature 18S rRNA is produced from 47S pre-rRNA transcript by exo- and endoribonucleolytic cleavages in a complex and hierarchical pre-rRNA processing pathway (Henras et al., 2015; Mullineux and Lafontaine, 2012). In human cells, at least 286 proteins affect pre-rRNA processing (Tafforeau et al., 2013). With the aim to analyse the role of WBSCR22 in pre-rRNA processing, cells transfected with siRNAs against *WBSCR22* were fractionated into cytoplasmic and nuclear parts, and the RNA from each fraction as well as whole cells was analysed by Northern blot (Paper **I, Fig. 3B and C)**. In this experiment, a oligonucleotide probe that hybridizes to the 5′ end of ITS1 and recognizes 18S rRNA processing precursors, including 45S, 41S, 30S, 21S and 18S-E pre-rRNAs, was used. As depicted in Paper I (**Fig. 3B and C**), WBSCR22 does not affect the early pre-rRNA processing events because the relative amount of 45S, 41S and 30S pre-rRNAs remained unaffected in the HeLa and HEK293 cells used in this study. However, when the amount of WBSCR22 was depleted, a slight decrease in 21S pre-rRNA occurs in the cell nucleus, together with a

3-fold accumulation of 18S-E pre-rRNA. This indicates that WBSCR22 is involved in late nuclear steps of pre-rRNA processing. Accumulation of 18S-E pre-rRNA upon WBSCR22 depletion was also uncovered in a siRNA screen that analysed the involvement of nucleolar proteins in pre-rRNA processing (Tafforeau et al., 2013) and later in other articles (Haag et al., 2015; Zorbas et al., 2015).

18S-E pre-rRNA is the final precursor of 18S rRNA that appears in the nucleus, and is then exported to the cytoplasm where the final maturation of pre-40S subunits take place (Henras et al., 2015; Mullineux and Lafontaine, 2012; Rouquette et al., 2005). In whole cell extract, the accumulation of 18S-E pre-rRNA does not indicate, whether the late nuclear processing events, or subsequent cytoplasmic cleavage at site, 3 is impaired. In WBSCR22 depleted cells, the diminished amount of free 40S particles and the nuclear accumulation of 18S-E pre-rRNA indicate that WBSCR22 can be important for ribosome small subunit export. Alternatively, the effect can be indirect, and instead of influencing transport to the cytoplasm, defective pre-40S particles can become trapped in the nucleus and accumulate. In mammalian cells, 18S-E pre-rRNA is comprised of mature 18S rRNA with an additional ~15 to 80 nucleotides of ITS1 sequence at its 3′ end (Haag et al., 2015; Preti et al., 2013; Rouquette et al., 2005). This variation in ITS1 sequence length arises because 18S-E pre-rRNA processing is performed by 3′-5′ exoribonucleolytic trimming that occurs during the transport of pre-40S particles (Preti et al., 2013). As a result of WBSCR22 depletion, the average length of 18S rRNA 3′ end extensions increases, which indicates that WBSCR22 is required for proper 3′-5′ trimming of 18S-E pre-rRNA prior to its export to the cytoplasm (Haag et al., 2015).

The expression of exogenous WBSCR22 is able to rescue the pre-rRNA processing defect present in WBSCR22 siRNA treated cells because the accumulation of 18S-E pre-rRNA disappears at an expression level similar to the endogenous protein (Paper **I, Fig. 3D and E**).

WBSCR22 is a methyltransferase that catalyses the formation of 18S rRNA m^7G1639 methylation; however, as with the wild-type protein, it has been shown that the expression of catalytically inactive mutants, WBSCR22-G63E and WBSCR22-D82K, also rescue the ribosome biogenesis defect which implies that WBSCR22 protein also has a methyltransferase activity-independent role in ribosome biogenesis (Haag et al., 2015; Zorbas et al., 2015). Likewise, the methyltransferase activity of the budding yeast protein Bud23 is not critical for pre-rRNA processing because the exogenous expression of catalytically inactive forms of Bud23 rescues the pre-rRNA processing defects and the slow-growth phenotype of a *Bud23*Δ yeast strain (White et al., 2008). Similarly, the rRNA methyltransferase activities of human DIMT1 as well as yeast Dim1 and Emg1/Nep1 are dispensable for ribosome biogenesis and cell growth (Lafontaine et al., 1995; Leulliot et al., 2008; Zorbas et al., 2015). Thus, rRNA methyltransferases often have two independent roles in ribosome biogenesis, and their participation in pre-rRNA processing seems to be more critical than their enzymatic activity as rRNA methyltransferases. Nevertheless, evolution has

maintained the catalytic activity of enzymes that modify rRNA, and rRNA modifications are clustered at functionally important regions of the ribosome, thereby implying that they are beneficial for the function of ribosomes (Decatur and Fournier, 2002).

5.1.4. WBSCR22 sediments with ribosome 40S subunits

To both perform 18S rRNA G1639 methylation and participate in pre-rRNA processing, WBSCR22 should bind to pre-ribosomes. Therefore, the sedimentation behaviour of WBSCR22 protein in sucrose density gradient ultracentrifugation was analysed (Paper **I, Fig. 5B and C**). The results of this experiment revealed that WBSCR22 sediments at positions that correspond to ribosome 40S/pre-40S subunits. However, it did also sediment in fractions smaller than 40S subunits, which indicates that WBSCR22 is not exclusively associated with ribosomal particles. Compared to WBSCR22, Bud23 exhibits a broader sedimentation profile, namely in peaks corresponding to 40S/pre-40S and 80S/90S sized fractions (Figaro et al., 2012; Létoquart et al., 2014; Sardana and Johnson, 2012). The 90S pre-ribosome or SSU processome represents an early ribosome biogenesis complex that consists of pre-rRNA, U3 snoRNA, early binding transacting factors and ribosomal proteins (Dragon et al., 2002; Grandi et al., 2002). Bud23 sedimentation at 90S pre-ribosomes is in agreement with the genetic and physical interaction between Bud23 and SSU processome components Utp2 and Utp14 (Sardana et al., 2013). It has been proposed that Bud23 acts at the transition between the early and middle stages of 40S subunit synthesis at the time of A_2 cleavage, which separates the biogenesis pathways of LSU and SSU in budding yeast (Sardana et al., 2013). In contrast, WBSCR22 depletion affects processing events that occur after the cleavage within ITS1, which may explain why it does not sediment in large pre-ribosome fractions (Paper **I, Fig. 3B and C**).

5.1.5. WBSCR22 is a nuclear protein that can accumulate within nucleoli

The subcellular localization analysis of WBSCR22 in HeLa cells demonstrates that endogenous WBSCR22 exhibits a diffuse signal throughout the cell nucleus and it is neither accumulated nor excluded from the nucleoli (Paper **I, Fig. 5A)**. A similar pattern of WBSCR22 staining is also reported for the human melanoma cell line A375M (Nakazawa et al., 2011). However, in some works, WBSCR22 displays a nuclear and cytoplasmic staining with enrichment in the nucleus (Jangani et al., 2014; Zorbas et al., 2015). WBSCR22 contains a C-terminal nuclear localization signal in addition to its N-terminal methyltransferase domain (Doll and Grzeschik, 2001; Merla et al., 2002), and probably moves through nuclear pore complexes (NPC) by active carrier-mediated transport (Marfori et al., 2011). In addition, because of its small size (~32 kDa), WBSCR22

can also enter the nucleus by passive diffusion (Marfori et al., 2011). Small variations in the results of indirect immunofluorescence analyses can be explained by the properties of the antibodies used – different antibodies can either recognize different epitopes of WBSCR22, or, furthermore, antibodies can also be unspecific and bind to epitopes of other proteins. One alternative to immunofluorescence analysis is to take advantage of recombinant proteins composed of an in-frame fusion between a fluorescent protein, such as EGFP, and the protein of interest. The use of fluorescent fusion proteins enables one to study the localisation of proteins and protein complexes in live cells. As seen in live cell microscopy analysis (Paper **II, Fig. 3A**), the fusion protein EGFP-WBSCR22 can be detected in the nucleoplasm as well as in nucleoli. The C-terminal domain of WBSCR22 (EGFP-WBSCR22-CTD), which contains the NLS, can be detected in both the nucleus and nucleoli. In contrast, the methyl-transferase domain of WBSCR22 (EGFP-WBSCR22-MTD), which lacks the NLS, is enriched in the cytoplasm. The nucleolar accumulation of WBSCR22 indicates that WBSCR22 is probably involved in both nucleolar as well as nucleoplasmic processes. In *Sacchamyces cerevisiae*, Bud23-GFP localizes mainly in the nucleolus (White et al., 2008). Compared to WBSCR22, the nucleolar accumulation of Bud23 is more pronounced and corresponds with its involvement in earlier pre-rRNA processing steps (Sardana et al., 2013; White et al., 2008). In *Arabidopsis thaliana*, RID2-GFP expressed from the *RID2* promoter localizes in the nucleus with accumulation in the nucleolus, which is in accordance with its role in early and intermediate pre-rRNA processing steps (Ohbayashi et al., 2011).

5.2. A principal interaction partner of WBSCR22 in human cells is TRMT112 (Paper II)

5.2.1. WBSCR22 interacts with TRMT112

For a better understanding of the cellular functions of WBSCR22, it is important to know its interaction partners. In order to investigate the protein complexes that contain WBSCR22, a SILAC (stable isotope labelling by amino acids in the cell culture) coupled co-immunoprecipitation analysis followed by identification by mass-spectrometry was performed. For this experiment, cells that stably express BPV1 E2-tagged WBSCR22 were generated from the U2OS cell line. As seen in Paper II, **Fig. 1A**, the expression level of epitope-tagged WBSCR22 in the U2OS-E2Tag-WBSCR22 cell line is similar to the endogenous protein, which enables one to study its function close to the physiological expression level. The same cell line was used for co-immunoprecipitation analysis and here the intensity ratio of heavy to light peptides reflects the relative abundance of peptides enriched in WBSCR22 pull-down fraction compared to control.

As seen in Fig. 1C within Paper II, the peptides of ribosomal proteins, as well as YBX1 (Y-box binding protein 1), C1QBP (complement C1q binding protein), TRMT112 (tRNA methyltransferase 11–2 homolog (*S. cerevisiae)*) and WBSCR22 itself were enriched in WBSCR22 pull-down fractions. C1QBP is a multifunctional protein involved in the complement pathway (Ghebrehiwet et al., 1994), mRNA splicing (Petersen-Mahrt et al., 1999), transcription regulation (Chattopadhyay et al., 2004), apoptosis (Itahana and Zhang, 2008) and also pre-rRNA processing (Yoshikawa et al., 2015). C1QBP localizes mainly in the cytoplasm and mitochondria; however, a fraction of this protein is found in nucleoli, where it interacts with proteins involved in ribosome biogenesis, including FBL and NOP52, and affects the early steps in pre-rRNA processing (Yoshikawa et al., 2011). Although WBSCR22 is involved in the late steps of pre-rRNA processing, the enrichment of C1QBP in WBSCR22 pull-down fractions suggests that WBSCR22 might also associate with 90S pre-ribosomes. The interaction between C1QBP and WBSCR22 has been detected in another mass-spectrometry based analysis (Zhang et al., 2013), but could not be confirmed by immunoblot analysis, which may reflect the transient nature of this interaction (Paper **II, Fig. 1D**). C1QBP interacts with and regulates the activity of YBX1 (Wang et al., 2015b), another protein found in the current study. Although the interaction between WBSCR22 and YBX1 proteins was not analysed by immunoblotting in this work, its importance for ribosome biogenesis needs to be evaluated in future studies.

TRMT112 exhibits the highest enrichment (H/L ratio) in the WBSCR22 pull-down fraction (Paper **II, Fig. 1C**) and is also confirmed as a WBSCR22 interacting protein by immunoblot analysis (Paper **II, Fig. 1B**). In agreement with the results of this work, another study that investigated the interaction partners of over-expressed WBSCR22 by mass-spectrometry also identified C1QBP, YBX1, and TRMT112 in WBSCR22 pull-down fractions (Jangani et al., 2014). The budding yeast ortholog of TRMT112, Trm112, is a small zinc-finger protein (~15 kDa) that interacts with several methyltransferases, including the tRNA methyltransferases Trm9 and Trm11 (Purushothaman et al., 2005; Studte et al., 2008), the protein methyltransferase Mtq2 (Heurgué-Hamard et al., 2006) and the rRNA methyltransferases Nop2 and Rcm1 (Sardana and Johnson, 2012). Human TRMT112 protein also interacts with methyltransferases involved in translation, namely with ALKBH8 (ABH8) and HEMK2α, which methylate the wobble uridine of certain tRNAs and the translation termination factor eRF1, respectively (Figaro et al., 2008; Fu et al., 2010). Thus, in complex with various methyltransferases, budding yeast Trm112 and human TRMT112 serve as cofactors. Notably, the interaction between WBSCR22 and TRMT112 is required for pre-rRNA processing as well as facilitating the 18S rRNA m^7G 1639 methylation (Zorbas et al., 2015).

5.2.2. TRMT112 regulates the stability of WBSCR22

In order to study the functional importance of the protein-protein interactions revealed in this work, the expression levels of WBSCR22, TRMT112, and C1QBP were reduced by siRNAs, and the efficiency of depletion was determined by immunoblotting. As seen in **Fig. 2A** within Paper II, the expression of all proteins analysed was reduced upon treatment with their respective siRNAs. Furthermore, transfection with siRNA that targets *TRMT112* reduced the expression level of TRMT112 itself and also decreased the expression level of WBSCR22 protein in HeLa cells. This indicates that the presence of TRMT112 is important for the stability of WBSCR22 protein. In contrast to TRMT112 depletion, downregulation of C1QBP expression by RNA interference did not alter the expression level of WBSCR22. In order to determine if the decrease in WBSCR22 protein level is specific to a reduced level of TRMT112 protein rather than an off-target effect of the particular siRNA sequence, alternative siRNA sequences that target *TRMT112* mRNA were designed and used in a comparable study. As with the original TRMT112 siRNA, other siRNA sequences were effective in decreasing the TRMT112 mRNA expression level and all led to a decrease in WBSCR22 expression in the human cell lines HeLa and U2OS (Paper **II, Fig. 2B and C**). In addition, in the African green monkey cell line COS-7, siRNAs that target *TRMT112* also reduce the expression of both TRMT112 and WBSCR22 (Paper **II, Fig. 2D**).

siRNAs complementary to the 3´ untranslated region (3´UTR) of human *TRMT112* mRNA decrease the expression of endogenous TRMT112; however, these siRNAs do not target exogenously expressed TRMT112 that lacks the native 3´UTR, which makes it possible to perform rescue experiments. In complementation analysis, the expression of exogenous TRMT112 is able to restore the normal protein level of WBSCR22 in cells where the expression of endogenous TRMT112 is silenced by siRNA transfection (Paper **II, Fig. 2E**). These results reveal that the expression level of WBSCR22 protein is positively regulated by the expression of TRMT112 and indicate that the availability of TRMT112 is crucial for the stability of WBSCR22.

Unpublished results from our research group demonstrate that in *Escherichia coli*, recombinant histidine-tagged WBSCR22 protein is insoluble and forms inclusion bodies. On the other hand, co-expression of WBSCR22 and its interaction partner TRMT112 increases the solubility of WBSCR22 in bacterial cells which implies that TRMT112 regulates folding and thereby the stability of the WBSCR22 protein (Zorbas et al., 2015). Similarly, the budding yeast Bud23, Mtq2, and Trm9 proteins are largely insoluble in *E. coli*, and the co-expression of these methyltransferases with Trm112 increases their solubility (Figaro et al., 2012; Heurgué-Hamard et al., 2006; Mazauric et al., 2010). Crystal structure analysis has revealed that Bud23 interacts with Trm112 through the formation of a parallel β-zipper involving the Bud23 strand β3 and the Trmt112 strand β4, thereby covering the hydrophobic residues of Bud23 (Létoquart et al., 2014). Thus, shielding the hydrophobic amino acid residues of Bud23 increases the

solubility and stability of this protein (Létoquart et al., 2014). In budding yeast cells, Trm112 is required for the stability of Bud23 and Mtq2, but not for Trm11, Nop2, and Rcm1 (Sardana and Johnson, 2012). The interaction mode of other complexes (Trm11-Trm112, Mtq2-Trm112, and Trm9-Trm112 heterodimers) is similar to the Bud23-Trm112 complex and only the tRNA methyltransferase Trm11 can be expressed and purified in *E. coli* without Trm112 co-expression (Bourgeois et al., 2017b).

5.2.3. WBSCR22 regulates the subcellular localization of TRMT112

We and others have previously shown by indirect immunofluorescence analysis that endogenous WBSCR22 localizes diffusely in the cell nucleus (Paper **I, Fig. 5A**; Nakazawa et al., 2011; Zorbas et al., 2015). *In silico* predictions indicate that ~50% of human TRMT112 protein localizes to the cytoplasm (Towns and Begley, 2012). In accordance with this, indirect immunofluorescence analyses reveal that human TRMT112 distributes to both the nucleus and the cytoplasm of HeLa cells, and, moreover, is excluded from the nucleolus (Paper **II, Fig. S1**). In another publication, human TRMT112 was found to be enriched in the nucleus and excluded from the nucleoli, with only a fraction of the protein found in the cytoplasm (Zorbas et al., 2015). Comparing the work of our group and D. Lafontaine's group, the sub-nuclear distribution of TRMT112 protein is identical; however, the amount and distribution of cytoplasmic TRMT112 protein is different. The same antibody against human TRMT112 protein as in our work was also used for the Human Protein Atlas database; however, in their work, TRMT112 protein co-localizes with microtubules, while only a small fraction of the protein is nuclear (Uhlén et al., 2015). Thus, the results of different groups are in partial disagreement and, therefore, the use of another method for studying the subcellular localization of TRMT112 would be beneficial. In order to further examine the subcellular distribution of TRMT112, a construct that encodes the fluorescent fusion protein TRMT112-mCherry was generated. As demonstrated in **Fig. 3A** of Paper II, the control protein mCherry and TRMT112-mCherry can be detected throughout the entire cell, both in the nucleus and in the cytoplasm. These data are in agreement with immunofluorescence analysis of the TRMT112 protein and indicate that TRMT112 can shuttle between the nucleus and cytoplasm of the cell.

In order to analyse the subcellular distribution of the WBSCR22 and TRMT112 complex, EGFP-WBSCR22 and TRMT112-mCherry fusion proteins were co-expressed in human cells and analysed using live cell microscopy. The fluorescence signals of EGFP-WBSCR22 and TRMT112-mCherry were detected in the cell nucleus, indicating that WBSCR22-TRMT112 complex localizes mainly in nucleoplasm (Paper **II, Fig. 3B**). When WBSCR22 is exogenously expressed together with TRMT112, the latter protein redistributes to the cell nucleus, thereby implying that WBSCR22 affects the subcellular localization of TRMT112. However, while TRMT112-mCherry in complex with EGFP-

WBSCR22 can be detected in the nucleolus it does not display enrichment towards this compartment.

Based on co-immunoprecipitation analysis, WBSCR22 interacts with TRMT112 via its N-terminal methyltransferase domain (MTD) (Paper **II, Fig. 3D**). The fusion protein EGFP-WBSCR22-MTD, which lacks the C-terminally located NLS, localizes within the cytoplasm of HeLa cells, and, crucially, when co-expressed with TRMT112-mCherry, both proteins localize within the cytoplasm (Paper **II, Fig. 3B**). On the other hand, the chimeric protein EGFP-WBSCR22-CTD, which is comprised of the EGFP protein fused with 38 amino acids from the C-terminus of WBSCR22, does not interact with TRMT112 (Paper **II, Fig. 3E**). In agreement with the other experiments, co-expression of EGFP-WBSCR22-CTD and TRMT112-mCherry does not change the sub-cellular localization of the latter protein – EGFP-WBSCR22-CTD is a nuclear protein that is enriched in the nucleoli, whereas in the same cells, TRMT112-mCherry can be detected in the cytoplasm and nucleus of the cell and is excluded from the nucleoli (Paper **II, Fig. 3B**). These results demonstrate that the methyltransferase domain of WBSCR22 protein is important for its interaction with TRMT112, and, furthermore, the localization of the WBSCR22-TRMT112 complex is determined by the WBSCR22 protein.

5.2.4. WBSCR22 mutants defective in TRMT112 binding are less stable than the wild-type protein

In order to gain more knowledge about the biological function of the WBSCR22-TRMT112 interaction, WBSCR22 mutants with substitutions in amino acids located on the interface of the WBSCR22-TRMT112 complex were generated. A crystal structure analysis of the budding yeast Bud23-Trm112 complex indicates that the interaction interface consists of a large hydrophobic core surrounded by polar residues (Létoquart et al., 2014). A sequence alignment between Bud23 and WBSCR22 proteins demonstrates that the amino acid residues involved in complex formation are conserved between budding yeast and human (Létoquart et al., 2014).

Based on the conservation data, two putative TRMT112 binding deficient mutants of WBSCR22 protein – WBSCR22-KT/AA and WBSCR22-D117A – were constructed in the current work (Paper II). In the WBSCR22-D117A mutant, the conserved aspartic acid at position 117 of WBSCR22 is replaced with alanine, and in the WBSCR22-KT/AA double-mutant, the lysine and threonine at positions 112 and 115, respectively, are replaced with alanines. In addition, these mutants contained either an E2Tag epitope tag in their N-terminus or were expressed as fusion proteins in-frame with EGFP.

The amino acid D112 of Bud23, corresponding to D117 in WBSCR22, is involved in the formation of a salt bridges between Bud23 and Trm112, and the Bud23 D112A and D112G mutants exhibit a slow-growth phenotype (Létoquart et al., 2014). The amino acids R107 and S110 of Bud23 are both involved in the

formation of hydrogen bonds in the Bud23-Trm112 complex, and correspond to K112 and T115 in the WBSCR22 protein (Létoquart et al., 2014). As shown by two hybrid assays in yeast, single-mutant R107L, S110P, and D112G forms of Bud23, as well as triple-mutant Bud23, have reduced binding to Trm112 (Sardana and Johnson, 2012; Sardana et al., 2014). Much less is known about the interaction between human WBSCR22 and TRMT112 proteins.

With the aim of tracking the expression of the WBSCR22 protein variants described above, HeLa cells were transiently transfected with plasmids that either encode for EGFP-WBSCR22, or one of its mutants KT/AA and D117A, and the number of EGFP positive cells was monitored up to three days after transfection using flow cytometry analysis. As shown in Fig. 4A of Paper II, 24 hours after transfection approximately 50% of the cells expressed the EGFP and EGFP-WBSCR22 proteins, whereas only about 40% of the cells expressed the mutant proteins EGFP-WBSCR22-D117A and EGFP-WBSCR22-KT/AA. After 48 hours, the percentage of cells expressing EGFP and EGFP-WBSCR22 proteins was still close to 50%, whereas the number of cells expressing mutant proteins EGFP-WBSCR22-D117A and EGFP-WBSCR22-KT/AA had declined to 15–20%. 72 hours after transfection, the difference between WBSCR22 and its mutants was even more pronounced. Similar results were seen in other human cell lines.

The expression of WBSCR22 protein and its mutants using flow cytometry was also confirmed using immunoblot analysis. For this experiment, the epitope-tagged WBSCR22 protein was expressed in HeLa cells, which were lysed 24 hours after transfection. As seen in Fig. 4B of Paper II, the expression level of wild-type WBSCR22 protein was elevated compared to the mutant proteins WBSCR22-KT/AA and D117A, which were barely detectable. The ectopic expression of epitope-tagged TRMT112 protein increased the abundance of wild-type WBSCR22 protein as well as the expression levels of the mutant proteins, which indicates that the mutants have possibly retained some ability to bind to TRMT112. Nevertheless, over-expression of TRMT112 had more influence on the protein level of wild-type WBSCR22 protein compared to its impact on the expression levels of the WBSCR22 mutants. The protein level of TRMT112 was also increased by co-expression with WBSCR22, indicating that WBSCR22 and TRMT112 proteins stabilize each other within the cells.

In order to investigate how strongly the WBSCR22 point-mutations affect the interaction with TRMT112 protein, a co-immunoprecipitation analysis was performed in HeLa cells. Epitope-tagged wild-type WBSCR22 protein was able to pull-down the endogenous TRMT112 protein (Paper **II, Fig. 4C**), and, reciprocally, epitope-tagged TRMT112 co-immunoprecipitated with the endogenous WBSCR22 protein (Paper **II, Fig. 4D**). Unfortunately, due to the low expression levels of the WBSCR22 mutants in HeLa cells, the results of the pull-down experiments did not provide insight into whether the interaction with TRMT112 was affected in these cells. Therefore, a similar co-immunoprecipitation analysis was performed in COS-7 cells. COS-7 cells express the large T-antigen of simian virus 40 (SV40), allowing plasmids containing the

SV40 origin of replication to replicate in these cells, thereby augmenting the level of plasmid-driven gene expression. The vector that encodes EGFP protein replicates in COS-7 cells (Paper **II, Fig. 4A**) and the expression levels of WBSCR22 protein and its mutants are high in COS-7 cells (Paper **II, Fig. 4E**). Furthermore, the number of cells expressing EGFP-WBSCR22 and its mutant variants remained constant for three days (Paper **II, Fig 4A**). Fig. 4E within Paper II shows that the wild-type WBSCR22 protein and also both mutants (KT/AA and D117A) were able to pull-down endogenous TRMT112 protein. However, the resulting amount of TRMT112 protein was different between WBSCR22 and its mutants. Mutating WBSCR22 lysine 112 and threonine 115 results in about a 2-fold decrease of co-precipitated TRMT112, while mutating the highly conserved aspartic acid 117 results in an almost complete loss of interaction between WBSCR22 and TRMT112. This confirms that the residues K112, T115, and D117 within WBSCR22 are all important for interacting with TRMT112.

The subcellular localization of EGFP-WBSCR22-D117A is similar to the wild-type protein, i.e., the D117A mutant localises to the nucleus and nucleoli (Paper **II, Fig. 3B**). Surprisingly, the co-expression of EGFP-WBSCR22-D117A and TRMT112-mCherry caused an enrichment of TRMT112-mCherry in the nucleus. This result points to the possibility that the nuclear accumulation of TRMT112-mCherry is caused by endogenous WBSCR22 protein. In support of this, the depletion of endogenous WBSCR22 by RNA interference decreased the amount of WBSCR22-D117A and TRMT112 in the nucleus and increased the amount of cytoplasmic TRMT112-mCherry (Paper **II, Fig. S2**). Nevertheless, these results indicate that the WBSCR22-D117A mutant has retained some ability to bind to TRMT112.

5.2.5. The level of WBSCR22 protein is regulated by the ubiquitin-proteasome pathway

The stability of WBSCR22 protein is controlled by its interaction partner TRMT112, and WBSCR22 mutants with reduced affinity towards TRMT112 are less stable than wild-type WBSCR22. Furthermore, ectopically expressed EGFP-WBSCR22 wild-type seems to be less stable than the control protein EGFP. In eukaryotic cells, the turnover of cellular proteins is chiefly regulated by the ubiquitin-proteasome pathway. In order to find out whether the amount of WBSCR22 protein and its mutants are controlled by proteasomal degradation, HeLa cells were transfected with plasmids encode for EGFP, EGFP-WBSCR22, and its mutants, and the number of cells expressing EGFP was monitored in the presence and absence of MG132, an inhibitor of the proteasome. MG132 was added to the cells 24 hours after transfection, and cells were subsequently incubated for 16 hours before analysis by flow cytometry (Paper **II, Fig. 6A**). Inhibition of the ubiquitin-proteasome pathway slightly increased the percentage of cells expressing EGFP-WBSCR22; however, the proportion

of cells expressing EGFP protein did not change. The percentage of cells expressing EGFP-WBSCR22-D117A and EGFP-WBSCR22-KT/AA increased to the level of wild-type WBSCR22. These results indicate that the turnover of wild-type WBSCR22 as well as its mutants is regulated by proteasomal degradation. Immunoblot analysis confirmed that the inhibition of proteasomes with MG132 increased the amount of ectopically expressed WBSCR22, whereas the amount of endogenous tubulin did not change (Paper **II, Fig. 6B**). In the same experiment, a slight increase in the expression level of endogenous TRMT112 protein was also detected, implying that the degradation of TRMT112 protein is also under the control of the ubiquitin-proteasome pathway. As shown in Fig. 6C within Paper II, the downregulation of TRMT112 expression decreased the amount of WBSCR22 protein, whereas the inhibition of proteasomes is able to increase the amount of WBSCR22 in TRMT112-depleted cells. This indicates that unbound WBSCR22 is degraded by the ubiquitin-proteasome pathway.

Proteasome-dependent degradation involves the addition of poly-ubiquitin chains to the target molecule. In order to study whether ubiquitin chains are added to WBSCR22 protein, a co-immunoprecipitation experiment was performed. In this experiment, HeLa cells were transfected with plasmids that encode HA-tagged ubiquitin and E2-tagged WBSCR22, and the antibody against E2 Tag was used to precipitate WBSCR22 complexes. As shown in Fig. 6D within Paper II, WBSCR22 and WBSCR22-D17A pull-down fractions contained poly-ubiquitinylated molecules, which again confirm that the amount of WBSCR22 is controlled by proteasome-dependent degradation.

5.2.6. WBSCR22 complements the growth and ribosome biogenesis defect of budding yeast *ΔBud23* mutant strain

The budding yeast Δ*Bud23* strain is characterized by a slow grow growth phenotype, which is caused by defects in ribosome biogenesis and pre-rRNA processing (White et al., 2008). The human WBSCR22 protein is also involved in the formation of 18S rRNA and the ribosome small subunit, and the decreased expression of WBSCR22 protein also affects the rate of cell growth (Paper **I, Fig. 1 and 2**). In order to investigate whether the functions of these two proteins are conserved, a complementation experiment was performed in the Δ*Bud23* yeast strain. As demonstrated in Paper I, Fig. 4A, the expression of the budding yeast protein Bud23 is able to restore a normal growth phenotype and defects in ribosome biogenesis. The expression of WBSCR22 protein in this yeast strain partially complements both defects, which indicates that the function of human WBSCR22 and budding yeast Bud23 are conserved, at least in part. In comparison, others have shown that the expression of wild-type RID2 protein of *Arabidopsis thaliana* does not rescue the slow growth phenotype of Δ*Bud23* (Ohbayashi et al., 2011). Compared to the negative control, the expression of WBSCR22 mutants D117A and KT/AA also enhanced the slow growth of Δ*Bud23*, although to a much lesser extent than the expression of wild-type

WBSCR22 (Paper **II, Fig. 5A**). Further, the expression of WBSCR22 mutants with reduced TRMT112 binding is not able to restore the ribosome biogenesis defects of Δ*Bud23* (Paper **II, Fig. 5B**). These mutants may have lost the ability to bind yeast Trm112, which can reduce their stability in yeast cells in a similar manner to what was observed in human cells.

5.3. Ribosomal proteins and tRNA synthetases are incorporated into MLV Gag induced extracellular vesicles (Paper III)

Recombinant virus-like particles (VLPs) can be used to transfer biological information between cells and tissues. In the second part of this dissertation, the protein content of retrovirus Gag protein induced VLPs was analysed. This information is important to avoid unwanted side-effects, when using these VLPs in biological systems. This work is part of a larger project that aimed to produce VLPs that express melanoma antigens with the purpose of using them for immunotherapy of melanoma patients.

In mammalian cells, the expression of retroviral Gag protein causes the spontaneous formation and release of virus-like particles to the tissue culture media (Larson et al., 2005). In the current work, the budding properties of Gag protein of murine leukemia virus (MLV) were used to produce a family of chimeric VLPs that each carry one of the following cancer/testis antigens: MART1, TRP1, MAGEA4, MAGEA10, or MCAM. The results of dynamic light scattering and transmission electron microscopy experiments indicate that the average size of MLV Gag VLPs is ~140 nm and the size of VLPs that carry melanoma antigens are very similar to each other. The assembled particles have a spherical structure and regular shape.

In order to characterize the protein content of Gag VLPs and VLPs carrying melanoma antigens, LC-MS/MS-based label-free quantitative proteomics analysis was performed. The results of these proteomics tests are presented in Supplementary Dataset 1 and in Fig. 5 within Paper III. The results indicate that all six types of VLPs contained the MLV Gag protei, and in addition, the melanoma antigens were incorporated into their respective VLPs. An immunoblot analysis of the MLV Gag protein further confirms that this protein is included in all particles (Paper **III, Fig. 5E**). However, in addition to exogenously expressed proteins, the mass-spectrometry analysis enabled to detect that VLPs contained more than 500 proteins acquired from the host cells. Probably, most of these cellular proteins are encapsulated into VLPs. Nonetheless, some of the cellular proteins might bind to the surface of VLPs after the assembly of VLPs.

In order to better understand which proteins form the components of these VLPs, the identified cellular proteins were divided into several classes based on their function. Among the proteins identified by LC-MS/MS analysis were Tsg101, Alix, and Nedd4, which are all proteins that directly interact with MLV

Gag protein and are involved in the budding of VLPs (Segura-Morales et al., 2005). Tsg101 and Alix are components of extracellular vesicles and are considered as markers of exosomes (Colombo et al., 2014). In addition, all VLPs contained the clathrin adaptor complex subunit AP-1, which is involved in retroviral release (Camus et al., 2007).

In addition to proteins that facilitate the budding of particles, these VLPs contained cellular proteins from various groups, including cell adhesion, membrane and cytoskeleton proteins, ribosomal proteins, translation factors, tRNA synthetases, metabolic enzymes, RNA binding proteins, and RNA helicases. The results of immunoblot analyses (Paper **III, Fig. 5E**) support these LC-MS/MS findings and demonstrate that cytoskeleton protein α-tubulin and ribosomal protein Rps6 are components of the MLV Gag based VLPs. Others have also reported that ribosomal proteins and rRNA can be incorporated into MLV VLPs (Muriaux et al., 2002); however, the significance of incorporating components of translational machinery (e.g., ribosomal proteins, tRNA synthetases, and translation factors) into VLPs remains unknown. Mutations in the MLV Gag protein cause the assembly of immature particles, where the viral genomic RNA is replaced with ribosomal RNA (Muriaux et al., 2002). These authors suggest that intact ribosomes or ribosomal subunits are packaged into the VLPs (Muriaux et al., 2002). Similarly, other proteomic characterizations of endogenous extracellular vesicles demonstrate that both microvesicles and exosomes contain ribosomal proteins and other proteins involved in translation (Kowal et al., 2016; Willms et al., 2016). The majority of proteins found in the exosomes of neuroblastoma cells are either cytoplasmic (~40%) or membrane proteins (~16%) (Marimpietri et al., 2013). It is possible that ribosomal proteins that naturally locate within the cytoplasm are passively incorporated into VLPs and other types of EVs. To support this, a comparison of the proteome of host cell membrane and HIV Gag induced particles found that the protein composition of VLPs and the plasma membrane is similar and the bulk of the host proteins in the plasma membrane are passively incorporated into the VLPs (Hammarstedt and Garoff, 2004).

6. CONCLUSIONS

Based on the results of Paper I and Paper II presented in the first part of this dissertation, the following conclusions can be made:

- The expression level of WBSCR22 protein is decreased in cell lines derived from Williams-Beuren syndrome patients. This result is in agreement with a reduced level of WBSCR22 mRNA in these cells.
- WBSCR22 is a nuclear protein that exhibits a diffuse signal throughout the nucleoplasm in human cells. When overexpressed, some of the WBSCR22 protein accumulates within the nucleoli, which reveals that WBSCR22 might have an affinity to bind with pre-ribosomes.
- WBSCR22 is important for cell growth, ribosome small subunit biogenesis, and pre-rRNA processing. Depletion of WBSCR22 causes the nuclear accumulation of the last precursor of 18S rRNA, the 18S-E pre-rRNA, thereby leading to reduced production of mature 18S rRNA and ribosomal 40S subunits.
- WBSCR22 interacts with TRMT112 protein in human cells and this interaction is important for the stability of WBSCR22 protein.
- The subcellular localization of WBSCR22-TRMT112 complexes are determined by WBSCR22.
- The expression level of WBSCR22 protein is tightly controlled. Under normal conditions, WBSCR22 protein is either bound to TRMT112 in the nucleoplasm or localizes to nucleolus where it may be associated with nucleolar proteins or rRNA. Under these conditions, when the amount of TRMT112 is limited, e.g., WBSCR22 is overexpressed or the expression level of TRMT112 is decreased, unbound WBSCR22 is tagged with ubiquitin and degraded by the 26S ubiquitin-proteasome pathway.

The results of the second part of this dissertation enabled to conclude that virus-like particles, induced by the exogenous expression of murine leukemia virus Gag protein, contain several groups of cellular proteins, including ribosomal proteins, tRNA synthetases, and translation initiation factors. It is currently unknown whether the incorporation of ribosomal proteins into VLPs are functionally relevant or are present by chance.

SUMMARY IN ESTONIAN

Williams-Beureni sündroomi kromosoomiregiooni valk WBSCR22 kui ribosoomi biogeneesifaktor

Ribosoomide biogenees on kompleksne protsess, milles osaleb ligi 300 valku ning neist valkudest umbes kolmandik on seotud genoomsete haiguste ja kasvajate tekkega. Williams-Beureni sündroom (WBS) on multisüsteemne arenguhäire, mida põhjustab on umbes 30 geeni puudumine seitsmenda kromosoomi pikast õlast, lookusest 7q11.23. WBS patsientidel esineb rida erinevaid probleeme, millest kõige sagedasemateks on kaasasündinud südame-veresoonkonna haigused, aga ka sidekoe arenguhäired, neuroloogilised probleemid, spetsiifiline kognitiivne profiil, kasvuprobleemid ja iseloomulikud näojooned. Genoomsed ümberkorraldused nagu deletsioonid, duplikatsioonid ja inversioonid WBS kromosoomiregioonis tekivad selle genoomipiirkonna kompleksuse tõttu. Nimelt ümbritsevad 7q11.23 piirkonna geenidega ala madalakoopiaarvulised kordusjärjestused, mis oma kõrge sarnasuse tõttu põhjustavad valepaardumisest tingitud ümberkorraldusi. Ehkki mehhanismid, mis viivad 7q11.23 piirkonna genoomsete häirete tekkimiseni, on suhteliselt hästi iseloomustatud, on vähem teada, kuidas üksikute geenide koopiaarvu muutus ja sellest tulenev avaldumistaseme erinevus põhjustab haigustunnuseid. Hetkel arvatakse, et paljud geenid on olulised WBS-i ning sama piirkonna duplikatsioonisündroomi kujunemisel, mistõttu on vajalik nendelt geenidelt ekspresseerivate valkude funktsioonide uurimine. Üheks WBS lookusest ekspresseeritavaks valguks, mille funktsioon enne antud doktoritöö tegemist oli teadmata, on WBSCR22. Nimetatud valgu iseloomustamine on oluline ka seetõttu, et on näidatud tema kõrget avaldumistaset mitmete kasvajate korral ning on teada, et WBSCR22 on oluline kasvajarakkude elulemuse ja metastaatilise potentsiaali reguleermisel.

Oma struktuurilt on WBSCR22 valk metüültransferaas ning bioinformaatiliste meetoditega on talle ennustatud DNA metüleerimise aktiivsust. Uuritud on ka WBSCR22 valgu rolli histoonide metüleerijana, kuid alles hiljuti avastati, et tegelikult on WBSCR22 ensüüm, mis katalüüsib 18S ribosomaalse RNA (18S rRNA) metüleerimist. Antud doktoritöö esimese ning peamise osa eesmärgiks oli WBSCR22 funktsiooni uurimine imetaja rakkudes.

Antud töö raames leiti, et WBSCR22 valk asub hajusalt rakutuumas, kuid tema kõrgema avaldumistaseme juures on näha valgu kogunemist tuumakesse. Selles tuumasiseses struktuuris toimub rRNA süntees ja algab ribosoomide biogenees. WBSCR22 valgu paiknemine tuumakeses viitab, et ta võib seonduda varajaste pre-ribosoomidega. Eukarüootide ribosoomide biogenees hõlmab pre-rRNA ja ribosoomivalkude sünteesimist, pre-rRNA protsessimist, rRNA-de modifitseerimist, ribosoomi subühikute kokkupanemist, nende eksoprti tuumast tsütoplasmasse ning sünteesitavate ribosoomide kvaliteedi kontrollimist. Antud doktoritöös leiti, et WBSCR22 on oluline rakkude jagunemiseks, ribosoomi väikese subühiku biogeneesil ja pre-rRNA protsessingul. Täpsemalt, WBSCR22 ekspressiooni mahasurumine põhjustab 18S-E pre-rRNA kuhjumist raku tuumas.

18S-E pre-rRNA on viimane 18S rRNA eellane, mida leidub nii raku tuumas kui tsütoplasmas. Pärast 18S-E pre-rRNA eksporti toimub tema viimane endoribonuklelüütiline lõikamine, mille tulemusel moodustub küps 18S rRNA. WBSCR22 valgu avaldumistaseme vähenemine toob kaasa 18S rRNA hulga languse tsütoplasmas, mistõttu pärsitakse ribosoomi 40S subühikute teke ning see omakorda võib põhjustada rakkude aeglasemat jagunemist.

Selleks et uurida, millised on WBSCR22 valgu interaktsioonipartnerid, konstrueeriti inimese rakuliin, mis püsivalt tootis epitoopmärgisega WBSCR22 valku ning leiti, et üheks WBSCR22-ga seonduvaks valguks on TRMT112. Lisaks näidati, et TRMT112 paiknemine rakus on määratud WBSCR22 poolt, WBSCR22-TRMT112 kompleks paikneb raku tuumas ning WBSCR22-TRMT112 kompleksi teke on vajalik WBSCR22 stabiilsuse tagamiseks. WBSCR22 valgu hulk rakus on kontrollitud – tuumaplasmas on WBSCR22 seotud TRMT112-ga ning tuumakese piirkonnas ilmselt tuumakese valkude või rRNA-ga. Juhul kui rakkudes ei ole piisavalt TRMT112, näiteks WBSCR22 üleekspressiooni või TRMT112 vaigistamise korral, siis WBSCR22 valk märgistatakse ubikvitiini molekulidega ja lagundatakse 26S proteasoomi rajas.

Selle doktoritöö teises osas analüüsiti retroviiruste Gag valgu poolt indutseeritud viiruslaadsete partiklite (VLP-de) valgulist koostist ning avastati, et nendes VLP-des esineb suur hulk rakulisi valke, nende seas ka suur hulk ribosoomivalke. Paljude rakuliste valkude, sealhulgas ribosoomivalkude roll, viiruslaadsetes partiklites ei ole teada.

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