



# Characterization of RNA-modifying enzymes and their roles in diseases

Dissertation

for the award of the degree

“Doctor rerum naturalium” (Dr. rer. nat.)

of the Georg-August-Universität Göttingen

within the doctoral program *Molecular Biology*

of the Georg-August University School of Science (GAUSS)

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**Göttingen 2017**

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## **Declaration**

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Göttingen, September 2017

## Acknowledgments

I would like to express my sincere gratitude and appreciation to my supervisor, Prof. Dr. Markus Bohnsack, for the guidance, advice and motivation he has provided me throughout the last four years. I have been extremely lucky to have his support when I faced challenges, and to receive his continuous encouragement to tackle exciting and competitive projects, which made me experience various topics in Molecular and Cell Biology.

I would also like to thank the members of my thesis committee, Prof. Dr. Dirk Görlich and Prof. Dr. Jörg Stülke, for their insightful advice and stimulating discussions throughout my time as a PhD student. My sincere thanks go to the members of the extended thesis committee, Prof. Dr. Peter Rehling, Prof. Dr. Detlef Doenecke and Prof. Dr. Claudia Höbartner for their time and care.

I am very grateful to Dr. Katherine Sloan for sharing her theoretical and practical experience in various topics in Molecular Biology with me on daily basis, which boosted the progress of my work, and I would also like to thank her for critically reading this dissertation and providing her valuable opinions on it. I am also grateful to Dr. Sara Haag for supervising me when I started my PhD work and for teaching me new methods and providing me with essential literature. I have also been blessed with a collaborative environment from current and previous members of the Bohnsack lab, Philipp Hackert, Jens Kretschmer, Charlotte Blessing, Benedikt Hübner, Manuel Günningmann, Lukas Brüning, Pryianka Choudhury, Indira Memet, Gerald Aquino and Jimena Davila.

I would also like to thank our collaborators, Prof. Dr. Dirk Görlich, Prof. Dr. Claudia Höbartner, Prof. Dr. Peter Rehling, Prof. Dr. Marina Rodnina and Prof. Dr. Henning Urlaub, and the members of their groups that contributed to this work, Dr. Bernard Freytag, Jan Seikowski, Dr. Sven Dennerlein, Dr. Namit Ranjan and Dr. Cristoph Lenz.

My thanks also go to the coordinators of the International Max Planck Research School of Molecular Biology in Göttingen, Dr. Steffen Burkhardt and Kerstin Grüniger for their constant support during my master studies and my PhD time.

Finally, a huge thanks to my family members, who have been supporting and encouraging me throughout my entire life, Dr. Samir Warda, Sanaa Abdelgawad, Dr. Noha Farag, Mohamed Elsaadany and Hana Elsaadany, and just like my family, Maria Silva Garcia, Mohamed Mostafa and Magdy Nasser were always present for me and provided me with continuous support.

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## List of publications

**Warda AS\***, Kretschmer J\*, Hackert P, Lenz C, Urlaub H, Höbartner C, Sloan KE, Bohnsack MT (2017) METTL16 is a  $N^6$ -methyladenosine ( $m^6A$ ) methyltransferase that targets pre-mRNAs and various non-coding RNAs. *EMBO Rep*, in press.

\* These authors contributed equally to this work.

Haag S, **Warda AS**, Kretschmer J, Günningmann MA, Höbartner C, Bohnsack MT (2015) NSUN6 is a human RNA methyltransferase that catalyzes formation of  $m^5C72$  in specific tRNAs. *RNA* **21**: 1532-1543.

Haag S\*, Sloan KE\*, Ranjan N\*, **Warda AS\***, Kretschmer J, Blessing C, Hübner B, Seikowski J, Dennerlein S, Rehling P, Rodnina MV, Höbartner C, Bohnsack MT (2016) NSUN3 and ABH1 modify the wobble position of mt-tRNA<sup>Met</sup> to expand codon recognition in mitochondrial translation. *EMBO J* **35**: 2104-2119.

\* These authors contributed equally to this work.

**Warda AS**, Freytag B, Haag S, Sloan KE, Görlich D, Bohnsack MT (2016) Effects of the Bowen-Conradi syndrome mutation in EMG1 on its nuclear import, stability and nucleolar recruitment. *Hum Mol Genet* **25**: 5353-5364.

## List of Abbreviations

3'UTR	3' untranslated region
5-aza	5-azacytidine
A-site	Aminoacylated tRNA site
ac <sup>4</sup> C	N <sup>4</sup> -acetylcytidine
acp	3-amino-3-carboxypropyl
Am	2'-O-methyladenosine
ASL	Anticodon stem loop
BCS	Bowen-Conradi syndrome
CAC	Citric acid cycle
cDNA	Complementary DNA
CRAC	Cross linking and analysis of cDNA
Cm	2'-O-methylcytosine
CMCT	N-cyclohexyl-N'-(2-morpholinoethyl)carbodiimide methyl-p-toluenesulfonate
D	Dihydrouridine
DEAF	Maternally inherited deafness
DMAPP	Dimethylallylpyrophosphate
DNA	Deoxyribonucleic acid
DBA	Diamond Blackfan anaemia
DNMT2	DNA methyltransferase 2
DUS	Dihydrouridine synthetase
ERISQ	Excess ribosomal protein quality control
ETS	External transcribed spacer
f <sup>5</sup> C	5-formylcytosine
FD	Familial dysautonomia
Gm	2'-O-methylguanosine
GNAT	Gcn5-related N-acetyltransferase
HCLA	Hypertrophic cardiomyopathy and lactic acidosis
hm <sup>5</sup> C	5-hydroxymethylcytosine
I	Inosine
i <sup>6</sup> A	N <sup>6</sup> -Isopentenyladenosine
IMP	Importin
ITS	Internal transcribed spacer
lncRNA	Long non-coding RNA
LSU	Large subunit
m <sup>1</sup> A	N <sup>1</sup> -methyladenosine



m <sup>1</sup> acp <sup>3</sup> Ψ	<i>N</i> <sup>1</sup> -methyl- <i>N</i> <sup>3</sup> -aminocarboxypropylpseudouridine
m <sup>1</sup> G	<i>N</i> <sup>1</sup> -methylguanosine
m <sup>1</sup> I	1-methylinosine
m <sup>2,2</sup> G	<i>N</i> <sup>2</sup> , <i>N</i> <sup>2</sup> -dimethylguanosine
m <sup>2</sup> G	<i>N</i> <sup>2</sup> -methylguanosine
m <sup>3</sup> C	<i>N</i> <sup>3</sup> -methylcytosine
m <sup>3</sup> U	<i>N</i> <sup>3</sup> -methyluridine
m <sup>5</sup> C	<i>C</i> <sup>5</sup> -methylcytosine
m <sup>5</sup> U	<i>C</i> <sup>5</sup> -methyluridine
m <sub>2</sub> <sup>6</sup> A	<i>N</i> <sup>6</sup> , <i>N</i> <sup>6</sup> -dimethyladenosine
m <sup>6</sup> A	<i>N</i> <sup>6</sup> -methyladenosine
m <sup>6</sup> Am	<i>N</i> <sup>6</sup> ,2'- <i>O</i> -dimethyladenosine
m <sup>7</sup> G	<i>N</i> <sup>7</sup> -methylguanosine
mcm <sup>5</sup> s <sup>2</sup> U	5-methoxycarbonylmethyl-2-thiouridine
mcm <sup>5</sup> U	5-methylcarbonylmethyluridine
ME	Myoclonic epilepsy
MELAS	Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episode
MERRF	Myoclonus epilepsy associated with ragged-red fibers
METTL	Methyltransferase-like
miRNA	MicroRNA
MLASA	myopathy, lactic acidosis and sideroblastic anemia
MM	Mitochondrial myopathy
mRNA	Messenger RNA
ms <sup>2i</sup> <sup>6</sup> A	2-methylthio- <i>N</i> <sup>6</sup> -isopentenyladenosine
ms <sup>2t</sup> <sup>6</sup> A	2-methylthio- <i>N</i> <sup>6</sup> -threonylcarbamoyladenosine
mt	Mitochondrial
ncm <sup>5</sup> U	5-carbamoylmethyluridine
ncm <sup>5</sup> Um	5-carbamoylmethyl-2'- <i>O</i> -methyluridine
ND2	NADH dehydrogenase 2
NES	Nuclear export signal
NGS	Next generation sequencing
NPC	Nuclear pore complex
NSUN	Nol1/Nop2/SUN
OXPHOS	Oxidative phosphorylation complex
P-site	Peptidyl tRNA site
PCM	Pericentriolar matrix
PDB	Protein Data Bank

PN	Poikiloderma with neutropenia
pre-rRNA	Precursor ribosomal RNA
pri-miRNA	Primary microRNA
PUA	Pseudouridine synthase and archaeosine transglycosylase
PUS	Pseudouridine synthase
ψ	Pseudouridine
PTC	Peptidyltransferase centre
Q	Queuosine
RIRCD	reversible infantile respiratory chain deficiency
RNA	Ribonucleic acid
RNAi	RNA interference
RNP	Ribonucleoprotein
RPL	Ribosomal protein of the large subunit
RPS	Ribosomal protein of the small subunit
RRM	RNA recognition motif
rRNA	Ribosomal RNA
RT	Reverse transcription
s <sup>2</sup> U	2-thiouridine
SAM	S-adenosylmethionine
scaRNAs	Small Cajal body-specific RNA
snRNA	Small nuclear RNA
snRNP	Small nuclear ribonucleoprotein
snoRNA	Small nucleolar RNA
snoRNP	Small nucleolar ribonucleoprotein
SSU	Small subunit
t <sup>6</sup> A	6-threonylcarbamoyl adenosine
TAP	Tandem affinity purification
TCS	Treacher Collins syndrome
TGTase	tRNA-guanine transglycosylase
τm <sup>5</sup> s <sup>2</sup> U	5-taurinomethyl-2-thiouridine
τm <sup>5</sup> U	5-taurinomethyluridine
TMG	2,2,7 trimethylguanosine
tRNA	Transfer RNA
Um	2'-O-methyluridine
vtRNA	Vault RNA
X-DC	X-linked dyskeratosis congenita
yW	Wybutosine

## Abstract

More than 150 types of chemical modifications have been identified in cellular RNAs (collectively called “the epitranscriptome”), and such modifications have emerged as important regulators of gene expression. Despite the recent progress in studying RNA modifications, knowledge of the enzymes that install many individual modifications and the cellular functions of these modifications is lacking. Furthermore, an increasing number of diseases are linked to defects in RNA modifications or RNA modification enzymes but the molecular basis of these diseases often remains unknown.

The human genome encodes numerous putative RNA methyltransferases, such as the methyltransferase-like (METTL) proteins and the Nol1/Nop2/SUN domain (NSUN) proteins, however, the molecular targets and functions of several of these enzymes remain unknown. We applied crosslinking and analysis of cDNA (CRAC) to identify the RNA binding sites of METTL16, NSUN6 and NSUN3 in a transcriptome-wide manner and used *in vivo* and *in vitro* assays to study their methylation activities. We showed that METTL16 is an  $N^6$ -methyladenosine ( $m^6A$ ) methyltransferase that targets A43 in the U6 snRNA, which lies within an evolutionarily conserved sequence that base pairs with 5' splice sites in pre-mRNAs, suggesting that this modification contributes to the regulation of pre-mRNA splicing. Furthermore, our results indicated that while NSUN6 introduces  $m^5C72$  on some cytoplasmic tRNAs during a late step of their biogenesis, NSUN3 installs  $m^5C34$  on the mitochondrial (mt)-tRNA<sup>Met</sup>. We have also identified ALKBH1 as the dioxygenase responsible for the oxidation of  $m^5C34$  to  $f^5C34$  of mt-tRNA<sup>Met</sup> and shown that these modifications are important for expanding codon recognition by mt-tRNA<sup>Met</sup> to enable efficient mitochondrial translation.

We studied how these proteins recognize their substrates and showed that the CCA nucleotides at the 3' end of tRNAs are important for recognition by NSUN6. Moreover, we found that the stability of the anticodon stem loop (ASL) is required for recognition by NSUN3, explaining why pathogenic mutations in mt-tRNA<sup>Met</sup> that disrupt the ASL impair methylation by NSUN3 and lead to disease. We further explored the link between RNA modification enzymes and disease by analysing effects of an aspartate 86 to glycine exchange (D86G) in the nucleolar ribosomal (r)RNA methyltransferase EMG1, which has been observed in patients with Bowen-Conradi syndrome (BCS). We discovered that EMG1<sub>D86G</sub> is chaperoned by the IMP $\beta$ /7 heterodimer in the cytoplasm and, upon the disassembly of the import complex in the nucleus, EMG1<sub>D86G</sub> aggregates and is degraded by the proteasome, leading to ribosome biogenesis defects.

Taken together, our studies characterized substrates of novel RNA-modifying enzymes and provided insights into their cellular functions and the link between defects in these enzymes and diseases.

## Chapter One: Introduction

### 1.1 Overview of RNA modifications

RNAs from all domains of life can be co- or post-transcriptionally modified by a collection of more than 150 distinct chemical moieties, ranging from simple methylations to complex modifications that are installed by the co-ordinated action of several, often highly conserved, modification enzymes (Cantara *et al.*, 2011; Machnicka *et al.*, 2013). Such modifications expand the chemical and topological properties of the four RNA nucleotides, therefore influencing the structure, molecular interactions and biological roles of the RNAs that carry them (Motorin and Helm, 2011).

Modified nucleotides are present in almost all types of cellular RNAs, and the most highly modified species are transfer RNAs (tRNAs), with up to 17% of tRNA nucleotides being modified, and ribosomal RNAs (rRNAs), which contain approximately 2% modified nucleotides (reviewed in Jackman and Alfonzo, 2013; Sloan *et al.*, 2017). Recently, high-throughput sequencing approaches have been used to generate transcriptome-wide maps of specific RNA modifications and this has revealed modified sites in messenger RNAs (mRNAs) and several classes of non-coding RNA, such as long non-coding RNAs (lncRNAs), small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs) and microRNAs (miRNAs; reviewed in Roundtree *et al.*, 2017). This complex landscape of RNA modifications (collectively termed “the epitranscriptome”) represents an important layer of gene expression regulation, and mutations in genes encoding RNA modification enzymes are implicated in various human diseases such as malignancies and metabolic disorders (reviewed in Sarin and Leidel, 2014). Nevertheless, many open questions remain on the identity of the enzymes involved and the detailed mechanisms by which RNA modifications are installed, regulated and exert their biological functions.

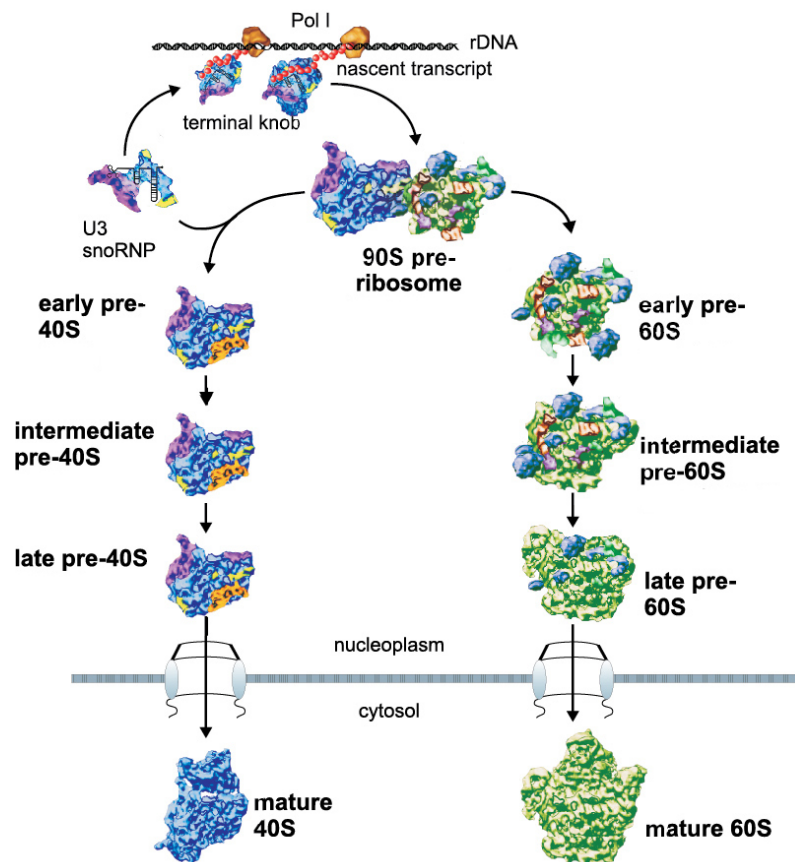
### 1.2 rRNA modifications

#### 1.2.1 Ribosome biogenesis in eukaryotes

Ribosomes are evolutionarily conserved molecular machines that are responsible for cellular protein synthesis. The eukaryotic cytoplasmic, 80S ribosome is a complex ribonucleoprotein (RNP) that comprises two subunits containing four different ribosomal RNA (rRNA) molecules and around 80 ribosomal proteins (reviewed in Melnikov *et al.*, 2012). The large subunit (LSU; 60S) contains the 28S (in metazoans)/ 25S (in the yeast *Saccharomyces cerevisiae*), 5S and 5.8S rRNAs assembled with 47 ribosomal proteins of the large subunit (RPLs), and the small subunit (SSU; 40S) comprises the 18S rRNA associated with 33 ribosomal proteins of the small subunit (RPSs; Ben-Shem *et al.*, 2011; Anger *et al.*, 2013). The biogenesis of such complex molecular machines is one of the

most crucial and energy consuming processes in the cell (Warner *et al.*, 1999). Our current knowledge of ribosome biogenesis has been mainly obtained from studies in the yeast *Saccharomyces cerevisiae*, due to the combination of powerful genetics and biochemical methods and the relative simplicity of this organism compared to humans. However, recent RNAi-based screens to identify human ribosome biogenesis factors and individual studies on human ribosome biogenesis factors have revealed that the core features of ribosome biogenesis are conserved from yeast to humans, but that several conserved factors have extra or different functions (see for example, Badertscher *et al.*, 2015; Tafforeau *et al.*, 2013; Wild *et al.*, 2010). Furthermore, these screens have uncovered the requirement for many additional factors for human ribosome assembly compared to yeast. In humans, the process starts in the nucleolus with the RNA polymerase I-mediated transcription of a long precursor ribosomal RNA (pre-rRNA) that contains the mature 18S, 5.8S, and 28S rRNA sequences, separated by the internal transcribed spacers 1 and 2 (ITS1 and ITS2) and flanked by the 5' and 3' external transcribed spacers (5'-ETS and 3'-ETS; Mullineux and Lafontaine, 2012). These additional pre-rRNA sequences are removed by an ordered series of endonucleolytic cleavages and exonucleolytic processing steps to generate the mature 5' and 3' ends of the rRNAs (Tomecki *et al.*, 2017; Henras *et al.*, 2015). The hierarchical assembly of ribosomal proteins and biogenesis factors on the pre-rRNA forms a series of pre-ribosomal particles, in which pre-rRNA processing steps and the modification of the rRNA sequences take place (Fig. 1; reviewed in Henras *et al.*, 2008). These maturation steps require the assistance of small nucleolar ribonucleoprotein (snoRNP) complexes and more than 200, mostly essential, *trans*-acting factors, such as RNA-modifying enzymes (discussed below), nucleases, kinases, GTPases and RNA-remodelling enzymes, which catalyse irreversible steps in the pathway (reviewed in Kressler *et al.*, 2010). RNA-remodelling enzymes include AAA-ATPases and DExD/H-box RNA helicases, which are suggested to modulate the unidirectional transitions of pre-ribosomal structures by unwinding or annealing RNA helices and/or facilitating the recruitment or release of RNA-binding proteins during ribosome biogenesis (reviewed in Martin *et al.*, 2013). Additionally, ribosome assembly requires the import of most ribosomal proteins from the cytoplasm to their incorporation sites on the nuclear pre-ribosomal particles. However, this is a challenging task for the cell because of specific features of ribosomal proteins: they contain unstructured extensions and highly basic regions that may form non-specific interactions when not assembled into pre-ribosomes, leading to insolubility (Jäkel *et al.*, 2002). Therefore, there are several mechanisms for preventing aggregation of newly synthesized ribosomal proteins in the cell (Pillet *et al.*, 2016). Besides the general ribosome-associated chaperones that assist the *de novo* folding of ribosomal proteins,

import receptors have been shown to protect ribosomal proteins from precipitation in the cytoplasm by shielding their basic regions (Jäkel *et al.*, 2002; Albanese *et al.*, 2006, 2010). Moreover, recent studies have uncovered a set of dedicated chaperones that bind ribosomal proteins, often co-translationally, and escort them to appropriate pre-ribosomal complexes (Pausch *et al.*, 2015). Non-(pre-)associated ribosomal proteins that are prone to aggregation are targeted for degradation by the excess ribosomal protein quality control (ERISQ), which specifically ubiquitinates lysine residues that are otherwise not accessible after the assembly into pre-ribosomes (Sung *et al.*, 2016).



**Figure 1. A simplified scheme of ribosome biogenesis in humans.**

The RNA polymerase I-mediated transcription of rDNA generates a long precursor rRNA (pre-rRNA). Association of early ribosomal proteins and ribosome biogenesis factors on the pre-rRNA leads to the formation of the 90S pre-ribosome. Processing of pre-rRNAs within pre-ribosomal complexes generates the pre-40S and pre-60S ribosomes, which undergo separate maturation pathways in the nucleoplasm before they are translocated through nuclear pore complexes to the cytoplasm, where the final maturation steps and the assembly of mature ribosomes take place (The Bohnsack lab).

In yeast, the association of the first ribosomal proteins and biogenesis factors with the nascent pre-rRNA during its transcription forms the 90S pre-ribosome/ SSU processome, which is the first stable pre-ribosomal particle (reviewed in Woolford *et al.*, 2013). Many of these biogenesis factors are recruited as part of pre-assembled subcomplexes, including

the U3 snoRNP and the UTP-A, UTP-B and UTP-C complexes, which sequentially assemble on the 5'-ETS and 18S rRNA sequence (Kornprobst *et al.*, 2016; Hunziker *et al.*, 2016). Following a pre-rRNA processing event in ITS1 (typically site A2 cleavage in yeast or site 2 cleavage in humans), the early pre-40S particle is separated from the rest of the pre-rRNA, which assembles with RPLs and biogenesis factors forming the early 60S pre-ribosomal particles (reviewed in Henras *et al.*, 2015). In yeast, it has been shown that upon separation, the protein composition of the pre-40S particle changes dramatically as most biogenesis factors are released and several RPSs are recruited (Schäfer *et al.*, 2003). This early pre-40S particle, which already displays the head, platform and body structures of the mature SSU but not the characteristic beak structure, is rapidly exported to the cytoplasm (see below; reviewed in Kressler *et al.*, 2010). Formation of the beak structure involves the incorporation of Rps3 (uS3), which is promoted by the phosphorylation-dependent release of Ltv1 (Ghalei *et al.*, 2015; Mitterer *et al.*, 2016). Prior to the final pre-rRNA processing step, Nob1-mediated cleavage to form the mature 3' end of the 18S RNA, pre-40S subunits undergo a translation-like cycle involving the GTPase Fun12 (eIF5B), in which the pre-40S subunits bind mature 60S subunits (Lebaron *et al.*, 2012; Strunk *et al.*, 2012; Turowski *et al.*, 2014). This final quality control step prevents aberrant or immature pre-40S particles from entering the pool of translating ribosomes.

In contrast to the relatively simple SSU assembly pathway in yeast, LSU assembly appears more complex as it involves the formation of several successive intermediates with different compositions (reviewed in Kressler *et al.*, 2017). Several of these intermediates have been characterised in yeast using tandem affinity purification (TAP) via pre-60S bait proteins providing insight into the timing of recruitment and dissociation of numerous *trans*-acting biogenesis factors. A key event during pre-60S biogenesis is the integration of the 5S RNP. The 5S RNP is formed by the Syo1-facilitated association of Rpl5 (uL5) and Rpl11 (uL18) with the 5S RNA, which is transcribed separately by RNA polymerase III (Ciganda and Williams, 2011; Calvino *et al.*, 2015). The incorporation of the 5S RNP into the pre-60S ribosome is then mediated by the Rpf2-Rrs1 heterodimer but in humans, several other factors have also been linked to 5S RNP recruitment to pre-ribosomal complexes (Zhang *et al.*, 2007; Sloan *et al.*, 2013; Kharde *et al.*, 2015). However, initially, the 5S RNP is rotated 180° from its final position and the peptidyltransferase centre (PTC) is occupied by the GTPases Nog1 and Nog2, preventing the recruitment of the export adaptor Nmd3 (Leidig *et al.*, 2014; Wu *et al.*, 2016). The recruitment of the Rix1 subcomplex and the AAA-ATPase Rea1 coincides with the rotation of the 5S RNP to its mature position and the activation and release of Nog2, allowing the binding of Nmd3 to the pre-60S ribosome (Barrio-Garcia *et al.*, 2016; Matsuo *et al.*, 2014). Rea1 and Nog2 are therefore considered checkpoint factors that monitor the maturation



status of pre-60S particles and licence their export to the cytoplasm. After export, the release of biogenesis factors occurs in concert with the association of the final ribosomal proteins. For example, the GTPase Nog1 is released by the AAA-ATPase Drg1, allowing the incorporation of Rpl24 (eL24; Kappel *et al.*, 2012). Moreover, the GTPase Lsg1 releases the export adaptor Nmd3 enabling the recruitment of Rpl10 (uL16; Hedges *et al.*, 2005). Finally, the release of Tif6 by the GTPase Efl1 and Sdo1 yields 60S subunits that are competent to join 40S subunits (Weis *et al.*, 2015; Ma *et al.*, 2016).

During the maturation of the pre-40S and the pre-60S particles, numerous remodelling events, nuclear export signal (NES)-containing export adaptors and additional export factors have been proposed to mediate their translocation through nuclear pore complexes (NPCs) into the cytoplasm (reviewed in Sloan *et al.*, 2015). Both pre-40S and pre-60S export rely on the exportin CRM1, which binds export adaptors associated with the pre-ribosomal subunits in the presence of RanGTP to facilitate export of these complexes (Hurt *et al.*, 1999; Stage-Zimmermann *et al.*, 2000). Additionally, the mRNA export factor Mex67-Mtr2 was shown in yeast to contribute to the export of both pre-ribosomal complexes, however, the loop insertions in the middle domain that are crucial for this function are absent from its human homolog TAP-p15, questioning whether this complex also contributes to pre-40S or pre-60S export in humans (Yao *et al.*, 2007; Faza *et al.*, 2012). Several SSU biogenesis factors that contain classical NES motifs and shuttle between the nucleus and the cytoplasm have been suggested to have a role in the export of pre-40S particles. For example, the protein kinase human RIO2 was shown to directly bind CRM1 in the presence of RanGTP via its NES and contribute to efficient pre-40S export (Zemp *et al.*, 2009). However, none of these factors is essential, suggesting the presence of unidentified pre-40S export adaptors or that multiple factors play redundant roles in the pre-40S export (reviewed in Sloan *et al.*, 2015). In contrast, the pre-60S export is dependent on the essential NES-containing export adaptor NMD3, which binds CRM1 in a RanGTP-dependent manner and facilitates the translocation of the pre-60S particles to the cytoplasm (Thomas and Kutay, 2003; Bai *et al.*, 2013). Alongside CRM1, in human cells, Exportin 5 was similarly reported to bind the pre-60S particles and facilitate their export, suggesting the presence of a second RanGTP-dependent pre-60S export pathway (Wild *et al.*, 2010). Interestingly, no role for Msn5, the yeast homolog of Exportin 5, in the export of pre-ribosomal subunits has been observed.

Taken together, assembly of the ribosomal subunits is a complex and hierarchical process that is orchestrated by a wealth of *trans*-acting factors and involves pre-rRNA processing and folding and the concomitant assembly of ribosomal proteins, forming pre-ribosomes that translocate from the nucleolus, through the nucleoplasm, to the cytoplasm as they mature.

### 1.2.2 rRNA modifications introduced by snoRNPs and stand-alone enzymes

During maturation of the pre-rRNAs, a significant portion of rRNA nucleotides (approximately 210 sites in the human rRNAs) is modified by the action of snoRNPs or stand-alone enzymes (reviewed in Sloan *et al.*, 2017). Despite the relatively large fraction of modified nucleotides in rRNA, the diversity of rRNA modifications is only limited to a set of 12 different types of modifications. In contrast to prokaryotes where base methylations are the most abundant modifications in rRNA, the majority of rRNA modifications in eukaryotes are 2'-O-methylation of the ribose of any of the four nucleotides and isomerization of uridine to pseudouridine ( $\Psi$ ; Lestrade and Weber, 2006; Pienka-Przybylska *et al.*, 2008). These modifications are mostly installed by the box C/D and box H/ACA snoRNPs respectively and, so far, approximately 100 of each modification are documented in human rRNA. The only exceptions are the stand-alone enzymes Sbp1 and Pus7, which have been reported in yeast to catalyse the 2'-O-methylation of G2922 of the 25S rRNA and pseudouridylation of U50 in the 5S rRNA respectively (Lapeyre *et al.*, 2004; Decatur and Schnare, 2008).

Eukaryotic snoRNPs, many of which are essential, contain a snoRNA that base pairs with the pre-rRNA and guides the catalytic protein component of the snoRNP to modify a specific target nucleotide (reviewed in Watkins and Bohnsack, 2012). Box C/D snoRNAs contain a C/D motif at the 5' and 3' ends of the transcript respectively and an internal C'/D' motif, and their extensive base pairing with the pre-rRNA adjacent to the D/D' box is facilitated by the association with the core proteins Nop58, Nop56 and Snu13 (15.5K in humans). The catalytic subunit of box C/D snoRNPs, fibrillarin (Nop1 in yeast) is then correctly positioned to modify the specific target nucleotide, five residues upstream of the D/D' box (Tollervey *et al.*, 1993; van Nues *et al.*, 2011). Box H/ACA snoRNAs contain a conserved H box and an ACA sequence, and form a hairpin structure that contains the "pseudouridylation pocket", where base pairing with the pre-rRNA takes place (Lafontaine *et al.*, 1998). The tertiary fold of the H/ACA box snoRNA, stabilized by the protein components Nop10, Nhp2 and Gar1, leaves the target uridine non-base-paired and correctly positioned in the pseudouridine synthase dyskerin (Cbf5 in yeast) active site (Ganot *et al.*, 1997). Interestingly, a subset of snoRNPs have been reported in yeast to guide modifications of multiple sites on the same pre-rRNA (e.g. snR60 targets A817 and G908 of the 25S rRNA) or on different pre-rRNAs (e.g. snR52 targets A420 of the 18S rRNA and U2921 of the 25S rRNA; Kiss-Laszlo *et al.*, 1996; Lowe *et al.*, 1999; Petrov *et al.*, 2014). Conversely, redundancy between snoRNAs in targeting modification of a particular site has also been documented. Examples include snoRNAs of the same class (e.g. snR39 and snR59 modify A807 of the 25S rRNA), or even different classes (e.g. the box C/D snoRNA snR65 and the box H/ACA snoRNA snR9 target U2347 of the 25S

rRNA; Taoka *et al.*, 2016). Besides their function in rRNA modification, several snoRNAs, including U14 and snR10, play additional roles in regulating pre-rRNA folding and mediating long-range interactions within pre-ribosomal particles (Enright *et al.*, 1996; Martin *et al.*, 2014).

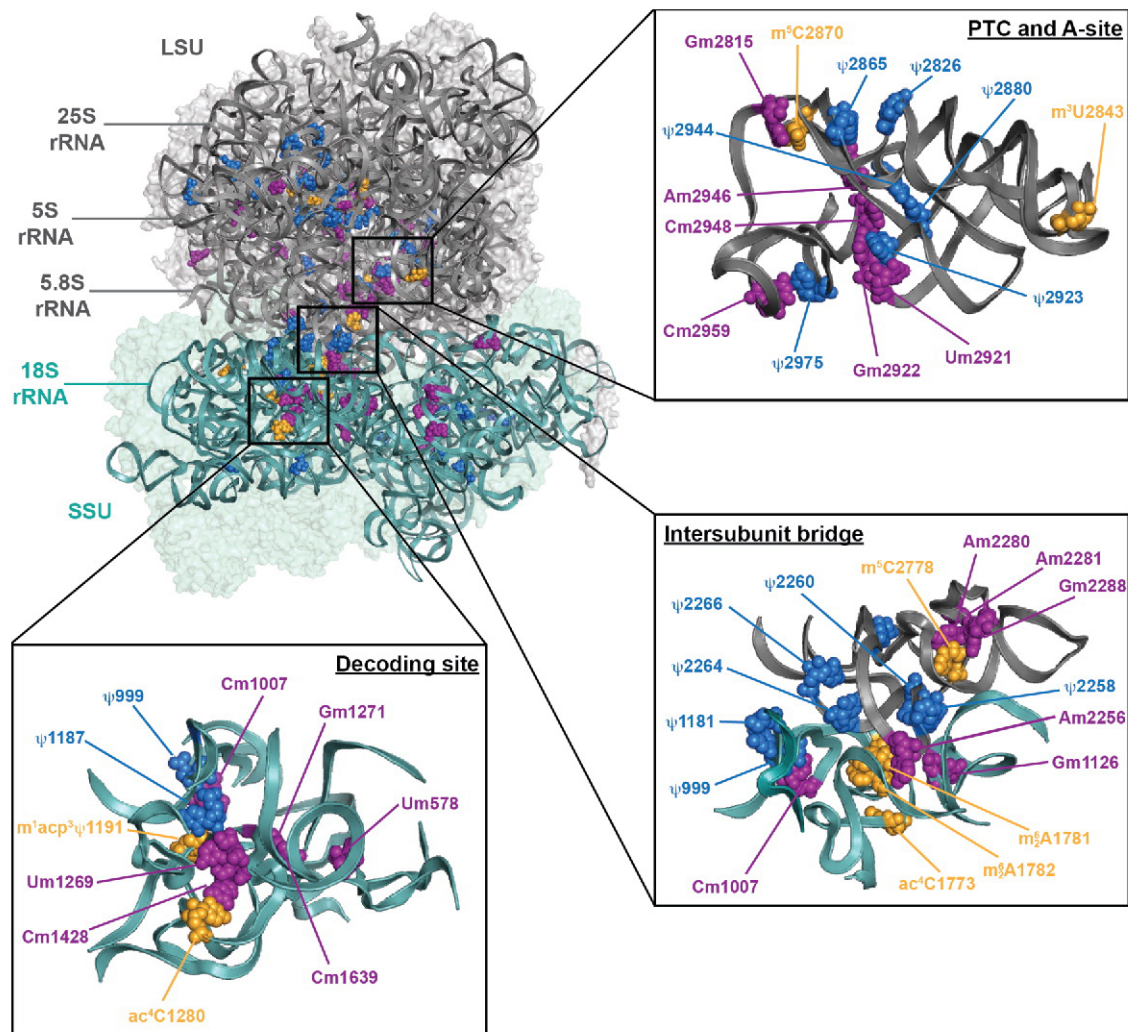
Besides uridine isomerization, several rRNA bases at sites distributed between the LSU and SSU are also modified (reviewed in Sharma and Lafontaine, 2015). In humans, the 28S rRNA of the LSU carries two C<sup>5</sup>-methylcytosine residues (m<sup>5</sup>C3761 and m<sup>5</sup>C4413/4), one N<sup>1</sup>-methyladenosine residue (m<sup>1</sup>A1309) and one N<sup>3</sup>-methyluridine residue (m<sup>3</sup>U4500). Modifications are more diverse on the 18S rRNA of the SSU, which contains two highly conserved N<sup>6</sup>,N<sup>6</sup>-dimethyladenosine residues (m<sub>2</sub><sup>6</sup>A1850 and m<sub>2</sub><sup>6</sup>A1851), two acetylated cytosine residues (ac<sup>4</sup>C1337 and ac<sup>4</sup>C1842), one N<sup>7</sup>-methylguanosine residue (m<sup>7</sup>G1639) and one hypermodified N<sup>1</sup>-methyl-N<sup>3</sup>-aminocarboxypropylpseudouridine residue (m<sup>1</sup>acp<sup>3</sup>Ψ1248; Pienka-Przybylska *et al.*, 2008). The RNA methyltransferases involved in the base modifications in human rRNA have been mostly identified: NSUN5/WBSCR20 (m<sup>5</sup>C3761; Schosserer *et al.*, 2015), NSUN1/NOL1 (m<sup>5</sup>C4413/4; Bourgeois *et al.*, 2015), NML (m<sup>1</sup>A1309; Waku *et al.*, 2016), DIMTL1 (m<sub>2</sub><sup>6</sup>A1850 and m<sub>2</sub><sup>6</sup>A1851; Zorbas *et al.*, 2015) and WBSCR22 (m<sup>7</sup>G1639; Haag *et al.*, 2015). These RNA methyltransferases use S-adenosylmethionine (SAM) as a methyl donor and display a classical Rossmann-like fold. In contrast, the RNA methyltransferase EMG1, which participates in the m<sup>1</sup>acp<sup>3</sup>Ψ hypermodification at position 1248 in the 18S rRNA, belongs to the SPOUT (alpha-beta knot fold) family (Leulliot *et al.*, 2008; Taylor *et al.*, 2008; Thomas *et al.*, 2010). The chemically complex modification of U1248 is of particular interest because it requires several strictly ordered steps by different factors in different subcellular compartments (Brand *et al.*, 1978). The first step, which has been described in yeast, is isomerization of U1248 to Ψ in the nucleolus by the H/ACA box snoRNP snR35 (ACA 13 in humans; Samarsky *et al.*, 1995). This initial step generates the substrate for subsequent N<sup>1</sup>-methylation of the residue by the essential nucleolar RNA methyltransferase EMG1 (Wurm *et al.*, 2010). Finally, TSR3, which also has a SPOUT fold and utilizes SAM, introduces the acp group in the cytoplasm (Meyer *et al.*, 2016). Besides RNA methyltransferases, the RNA acetyltransferase NAT10, which has a Gcn5-related N-acetyltransferase (GNAT) domain, has been shown to be responsible for introducing ac<sup>4</sup>C1337 and ac<sup>4</sup>C1842 in the 18S rRNA (Ito *et al.*, 2014; Sharma *et al.*, 2015). Interestingly, the yeast homolog of NAT10, Kre33 was recently suggested to be guided to its modification sites by snoRNAs snR4 and snR45 (Sharma *et al.*, 2017).

Notably, several modifications have been reported in yeast and are not conserved in humans, including one N<sup>1</sup>-methyladenosine residue (m<sup>1</sup>A2142) and two N<sup>3</sup>-methyluridine residues (m<sup>3</sup>U2634 and m<sup>3</sup>U2843) in the 25S rRNA. Interestingly, the enzymes

responsible for installing these modifications (Bmt2 (Sharma *et al.*, 2013), Bmt5 and Bmt6 (Sharma *et al.*, 2014) respectively) are non-essential, suggesting that their roles in ribosome structure or function may be less important than other enzymes. In contrast, all other evolutionarily conserved enzymes involved in base modifications in rRNAs are either essential or important for growth, and surprisingly, this is in most cases not because of their RNA-modifying catalytic activity, but because their presence in pre-ribosomal complexes is required for subunit assembly (reviewed in Sharma and Lafontaine, 2015). For example, apart from its methylation function, Emg1 plays an additional essential role in yeast ribosome biogenesis, which is proposed to be the recruitment of Rps19 (eS19) into pre-40 particles (Meyer *et al.*, 2011; Buchhaupt *et al.*, 2006).

### 1.2.3 Functions of rRNA modifications

The use of this minimal set of different modifications in rRNA in eukaryotes, despite the plethora of modified RNA nucleotides in nature, suggests that their chemical nature is particularly appropriate for their functions in rRNAs. Furthermore, rRNA modifications are not randomly distributed over the ribosome, but they cluster in evolutionarily conserved positions at the interface between the LSU and SSU and the inner cores of the subunits (Fig. 2; Decatur and Fournier, 2002). These include functionally important regions, such as the peptidyltransferase centre (PTC) in the LSU and the decoding site in the SSU, suggesting that rRNA modifications might play important roles in optimizing ribosome structure and function (Ben-Shem *et al.*, 2011). Indeed, the two most abundant rRNA modifications, 2'-O-methylation and pseudouridylation, are known to alter local conformation and folding properties of RNA. 2'-O-methylation increases RNA rigidity by promoting base stacking and can alter RNA folds (Prusiner *et al.*, 1974; reviewed in Helm, 2006), while pseudouridine has increased hydrogen bonding capability compared to uridine and stabilizes specific RNA structures (reviewed in Charette and Gray, 2000; Hayrapetyan *et al.*, 2009). However, rRNA modifications are generally thought to act in a cumulative manner since loss of individual modifications causes only subtle defects in cell growth, and significant phenotypes are only observed when clusters of modifications in certain functional regions of the ribosome are concomitantly lacking (Baxter-Roshek *et al.*, 2007; Esguerra *et al.*, 2008). For example, combined deletion of 5 box H/ACA snoRNAs that guide modifications in the PTC was shown, in yeast, to cause synergistic effects on ribosome structure and activity (King *et al.*, 2003). Similarly, the base modifications present in the rRNAs can serve to stabilise secondary and tertiary rRNA structures. For example, loss of the conserved m<sup>5</sup>C2278 base modification in combination with the ribose methylation at G2288 was shown to cause changes in the structure of the yeast 25S rRNA, leading to dramatic ribosome instability (Gigova *et al.*, 2014).



**Figure 2. Distribution of rRNA modifications on functionally important regions in the yeast ribosome.**

Positions of 2'-O-methylations (purple), pseudouridines (blue) and base modifications (orange) are shown on the yeast *Saccharomyces cerevisiae* ribosome (PDB 4V88; Ben-Shem *et al.*, 2011). The LSU is shown in grey and the SSU is shown in teal. The peptidyltransferase centre (PTC), the intersubunit bridge (eB14) and the decoding site are presented in a magnified view (taken from Sloan *et al.*, 2017).

In addition to their role in modulating rRNA structure, rRNA modifications have also been implicated in regulating translation efficiency and fidelity. 2'-O-methylation and pseudouridylation of residues at the aminoacylated tRNA site (A-site) and the peptidyl tRNA site (P-site) of the yeast LSU play a significant role in ensuring translation efficiency (Liang *et al.*, 2009). Furthermore, a cluster of modifications in the decoding site of the yeast SSU was shown to influence translation accuracy, demonstrating that modifications in different regions of the ribosome have different functions (Baudin-Baillieu *et al.*, 2009). In line with this, base modifications have also been proposed to optimize translation. For example, the dimethylation ( $m_2^6A1781$  and  $m_2^6A1782$ ) in the yeast 18S rRNA was shown to

be required for translation, and cells expressing mutant forms of Dim1 or Emg1 display hypersensitivity to aminoglycoside antibiotics, which affect translation (Lafontaine *et al.*, 1998; Liu and Thiele, 2001). Strikingly, certain rRNA modifications have been shown to influence translation of specific subsets of mRNAs. For example, the loss of Rcm1 in yeast, which introduces m<sup>5</sup>C2278 in the 25S rRNA, was shown to promote the recruitment of a specific subset of oxidative stress-responsive mRNAs into polysomes (Schosserer *et al.*, 2015). In conclusion, rRNA modifications and the enzymes introducing them play different roles in regulating ribosome biogenesis, structure and function. Interestingly, some enzymes that perform rRNA modifications have been implicated in modification of other classes of RNA. For example, NAT10 was shown to acetylate specific tRNAs (Sharma *et al.*, 2015) and fibrillarin was shown to mediate 2'-O-methylation in human snRNAs (Tycowski *et al.*, 1998; Jady and Kiss, 2001), suggesting possible crosstalk between the biogenesis of different species of RNA.

#### **1.2.4 Timing and regulation of rRNA modifications**

The observations that rRNA modifications are present in the core of the ribosomal subunits, and that pre-ribosomal complexes are highly structured imply that factors involved in rRNA modifications have limited access to their substrate residues during late stages of ribosome maturation. It was therefore expected that the majority of rRNA modifications are introduced in early stages, where pre-ribosomal complexes have more open structures. Indeed, most 2'-O-methylations were shown in yeast to occur during early stages of ribosome maturation, often co-transcriptionally (Kos and Tollervey, 2010; Birkedal *et al.*, 2015). It is similarly proposed that pseudouridylations occur early during ribosome biogenesis, however, this remains to be documented. In contrast, rRNA base modifications are thought to be introduced later during the process, however, the precise timing of most base modifications has remained unclear so far. An exception is the N<sup>3</sup>-acp modification of nucleotide 1248 of the 18S rRNA that is installed by TSR3 in the cytoplasm and can therefore be clearly defined as a “late modification” (Meyer *et al.*, 2016). Furthermore, several rRNA modification enzymes are bound to early pre-ribosomal complexes, but do not modify their substrate residues until later. For example, Dim1 associates with early nucleolar pre-ribosomes in yeast, but it only installs the m<sub>2</sub><sup>6</sup>A1850 and m<sub>2</sub><sup>6</sup>A1851 dimethylation after the export of the maturing pre-SSU to the cytoplasm (Lafontaine *et al.*, 1995). Remarkably, the corresponding dimethylation in humans is introduced by DIMTL1 in the nucleus (Zorbas *et al.*, 2015), but the significance of this temporal difference is not yet understood.

The introduction of several rRNA modifications during late stages of ribosome biogenesis suggests that RNA-remodelling enzymes, such as RNA helicases, may be required to

enable certain modifications to be installed. Late-acting snoRNPs, for example, were proposed to gain access to their target sites in the human 18S rRNA by the coordinated action of the RNA helicase DDX21 (Sloan *et al.*, 2015). Similarly, several base modification enzymes require co-factors with RNA-remodelling activities or even carry such activities within their own sequence. For instance, the acetyltransferase NAT10 has an ATPase-dependent helicase domain, which is suggested to facilitate the access of the enzyme to its target site for acetylation (Sharma *et al.*, 2015). Additionally, the yeast m<sup>7</sup>G methyltransferase Bud23 requires the coactivator Trm112, which stabilizes Bud23 by masking solvent-unfavourable hydrophobic surfaces (Figaro *et al.*, 2012). The action of the RNA helicase Dhr1 is then required for the Bud23-Trm112 complex to gain access to its target site within the pre-40S particle (Sardana *et al.*, 2014).

It has recently emerged that certain rRNA residues are differentially modified, i.e. some rRNA molecules carry specific modifications while others do not (Birkedal *et al.*, 2015). In yeast, 18 modification sites were found to be modified on less than 85% of ribosomes (Taoka *et al.*, 2016). In line with this, in several human cell lines, around one-third of 2'-O-methylation sites are partially modified (Krogh *et al.*, 2016). Together, this suggests that rRNA modifications contribute to the heterogeneity of the ribosomes. However, base modifications installed by the conserved stand-alone enzymes appear to be constitutively present. It has been speculated by Krogh and colleagues that such conserved modifications are involved in essential aspects of rRNA folding and assembly of ribosomes, while the fractionally modified residues play roles in fine-tuning translation. The extent of specific 2'-O-methylations has been suggested to be determined by the levels of the corresponding snoRNAs (Buchhaupt *et al.*, 2014), but it is also possible that alternative rRNA processing and folding pathways, or the selective removal of the methylations by demethylases, also contribute to differences in rRNA modifications (reviewed in Sloan *et al.*, 2017). Excitingly, variations in the levels of specific rRNA modifications in response to environmental signals have been reported. Diauxic shift and heat-shock, for example, were observed to alter the modification levels of specific rRNA residues in the yeast *Saccharomyces cerevisiae* (Schwartz *et al.*, 2014; Carlile *et al.*, 2014). Similarly, changes in growth conditions of *Schizosaccharomyces pombe* cells were shown to significantly affect the extent of modifications of specific sites in rRNA (Taoka *et al.*, 2015). In addition to their variation under physiological conditions, the extent of rRNA modifications is also altered in several genetic disorders and cancers (see below).

### 1.2.5 Ribosomopathies and diseases associated with rRNA modifications

Recently, a number of human diseases associated with defects in ribosome biogenesis have been identified and collectively termed “ribosomopathies” (reviewed in Narla and Ebert, 2010; Khanna-Gupta, 2013). Ribosomopathies are caused by alterations in genes encoding ribosomal proteins or ribosome biogenesis factors, and these alterations are in most cases caused by congenital haploinsufficiency of the affected gene (mutation or deletion of one copy of a gene, leaving the other copy functional; reviewed in McCann and Baserga, 2013). For example, mutations in a number of ribosomal proteins, including RPS19 (eS19), have been linked to Diamond-Blackfan anaemia (DBA; Draptchinskaia *et al.*, 1999; Gazda *et al.*, 2006; Cmejla *et al.*, 2007). Furthermore, mutations in the ribosome biogenesis factor TCOF1/treacle have been found in Treacher Collins syndrome (TCS; The Treacher Collins Syndrome Collaborative Group, 1996). Similarly, Schwachman-Diamond syndrome patients have been found to carry biallelic mutations in SBDS, which associates with late pre-60S complexes (Boocock *et al.*, 2003). To date, the only reported acquired ribosomopathy is the 5q-syndrome, which is caused by the deletion of chromosome 5q including one allele of *RPS14* (Ebert *et al.*, 2008; Pellagatti *et al.*, 2008). Generally, many ribosomopathies display a number of common symptoms (bone marrow failure, growth retardation, skeletal abnormalities and malignancies), however, each ribosomopathy is clinically distinct. For example, patients with haploinsufficiency of RPSA (uS2; isolated congenital asplenia) lack a spleen, but they have no other observable anomalies (Bolze *et al.*, 2013). Similarly, patients with a mutation in the ribosome biogenesis factor hUTP4/Cirhin (North American Indian childhood cirrhosis) show one main phenotype, which is biliary cirrhosis (Chagnon *et al.*, 2002). The mechanism by which such defects in a macromolecular complex as constitutive and essential as the ribosome cause only tissue-specific phenotypes remains unclear. One possible explanation is that certain defects in ribosomes could influence translation of specific mRNAs, which might be essential for the affected cell type (reviewed in McCann and Baserga, 2014). This model is supported by the observation that reduction of RPL40 in humans impedes translation of specific vesicular stomatitis virus mRNAs (Lee *et al.*, 2013). An alternative explanation is that ribosome variants are produced in different cell types, and ribosomes in the affected cell types are more sensitive to the defect that causes the disease (Marcel *et al.*, 2015).

Defects in ribosome biogenesis, such as those observed in ribosomopathies, cause the 5S RNP to bind the E3 ubiquitin ligase HDM2 and inhibits its activity, leading to induction of the tumor suppressor p53 (Sloan *et al.*, 2013; Donati *et al.*, 2013). Such accumulation of p53 arrests cell division and leads to apoptosis, which can be responsible for some of the symptoms (McGowan *et al.*, 2008; Fumagalli *et al.*, 2009). In line, reduction of p53



activity rescued the craniofacial abnormalities in a mouse model for TCS (Jones *et al.*, 2008) and rescued the erythroid abnormalities in zebrafish models for DBA (Boulton *et al.*, 2012). However, the accumulation of p53 when ribosome biogenesis is impeded is surprising given the increased susceptibility to cancers observed in most ribosomopathies (reviewed in Teng *et al.*, 2013). One possible explanation for this is that the elevated p53 levels may lead to mutations or downregulation of the p53 pathway, resulting in desensitizing patient cells to p53 (Pelava *et al.*, 2016). Another possible explanation is that defects in ribosomal proteins or ribosome biogenesis factors could produce ribosomes with altered translation capacities that differentially translate distinct subpopulations of mRNAs, leading to tumorigenesis. This hypothesis is supported by the finding that depletion of RPL38 in mice impairs translation of a subset of Hox mRNAs, which could lead to malignant transformation when dysregulated (Kondrashov *et al.*, 2011).

Similarly, mutations in *DKC1*, which encodes the box H/ACA pseudouridine synthase dyskerin, in patients with X-linked dyskeratosis congenita (X-DC) cause defects in IRES-dependent translation of a subset of mRNAs including the ones encoding the tumor suppressor p27 and the antiapoptotic factors Bcl-xL and XIAP (Yoon *et al.*, 2006; Bellodi *et al.*, 2010). This suggests that changes in rRNA pseudouridylation pattern may result in ribosomes with differential translation activities that promote tumorigenesis. In a similar way, altered rRNA 2'-O-methylation profiles have been associated with tumorigenesis and differences in the extent of modification at multiple sites have been reported in different cancer cell lines (Krogh *et al.*, 2016). The tumor suppressor p53 is suggested to regulate the expression levels of fibrillarin, with overexpression of fibrillarin leading to changes in the rRNA 2'-O-methylation pattern of the rRNAs and altered translation fidelity of ribosomes (Marcel *et al.*, 2013). Reciprocally, lack of fibrillarin has been reported to cause an increase in the cap-independent translation of p53, independent from the 5SRNP-HDM2 pathway (Su *et al.*, 2014). Furthermore, the levels of other components of box C/D snoRNPs including NOP56 (Cowling *et al.*, 2014) and NOP58 (Nakamoto *et al.*, 2001) are elevated in different types of cancer, making them good markers of tumorigenesis (Liao *et al.*, 2010).

Defects in stand-alone rRNA base-modifying enzymes have also been linked to cancers and genetic diseases. For example, WBSCR22 promotes survival and metastasis of tumor cells (Nakazawa *et al.*, 2011), and the high expression levels of NSUN1/NOL1 have been correlated with growth of lung adenocarcinoma (Sato *et al.*, 1999). WBSCR22 and NSUN5/WBSCR20 are deleted, along with other genes, in a developmental disorder called Williams-Beuren syndrome (Doll and Grzeschik, 2001) and NML has been associated with high fat diet-induced obesity (Oie *et al.*, 2014). More specifically, a point mutation in the gene encoding EMG1, leading to an aspartate to glycine exchange in the

protein sequence, causes a severe genetic disorder known as Bowen-Conradi syndrome (BCS), which is characterized by bone marrow failure, bone abnormalities, growth retardation and death within the first year of life (Armistead *et al.*, 2009). However, whether lack of the rRNA modifications or other effects caused by defects in the modification enzymes cause these disease phenotypes is often still unknown. Interestingly, chemical inhibition of the acetyltransferase NAT10 was shown to rescue defects of laminopathic cells, raising the possibility of using rRNA modification enzymes as drug targets (Larrieu *et al.*, 2014). In conclusion, disruptions in ribosome biogenesis are linked to multiple human diseases, and further understanding of the effects of such genetic alterations on the molecular and cellular levels is integral to finding therapeutic interventions to treat these disorders.

### **1.3 tRNA modifications**

#### **1.3.1 Overview of eukaryotic tRNA biogenesis**

tRNAs are highly structured short non-coding RNAs of approximately 70 nucleotides that decode the mRNA codons and carry the cognate amino acids to the ribosome for protein biosynthesis in the cytoplasm of all living cells, and in eukaryotic mitochondria and plastids (reviewed in Fujishima and Kanai, 2014). tRNAs display a highly conserved cloverleaf secondary structure, which comprises four distinct domains: the acceptor stem, the anticodon arm, the D loop and the T $\Psi$ C loop. Interactions between these domains are required to form the folded L-shaped tertiary structure of tRNAs (see for example, Kim *et al.*, 1973; Robertus *et al.*, 1974). The biosynthesis of cytoplasmic tRNAs starts with the RNA polymerase III-mediated transcription of tRNA genes in the nucleolus, generating precursor tRNAs (pre-tRNAs) that contain sequences that are not present in the mature tRNAs: the 5' leader sequence, the 3' trailer and, in some cases, introns (reviewed in Hopper and Phizicky, 2003; Phizicky and Hopper, 2010). Maturation of pre-tRNAs requires processing of these three sequence elements. The 5' leader sequence is removed by an endonucleolytic cleavage by RNase P, which is an RNP composed of a catalytically active RNA component, the H1 RNA, and 10 protein subunits in humans (reviewed in Walker and Engelke, 2006). Maturation of the 3' end of tRNAs requires processing of the 3' trailer by the endonuclease tRNase Z (ELAC2 in humans), and the subsequent addition of the CCA tail by the tRNA nucleotidyltransferase (CGI-47 in humans; Takaku *et al.*, 2003; Rossmann *et al.*, 2011; Reichert *et al.*, 2001). Furthermore, introns, which are found between nucleotides 37 and 38 of some eukaryotic tRNAs, are spliced by the consecutive activities of an endonuclease complex (human active subunits: HsSen2 and HsSen34) and a tRNA ligase complex (human active subunit: HSPC117; Paushkin *et al.*, 2004; Popow *et al.*, 2011). In vertebrates, tRNA splicing is thought to

occur in the nucleus, while, in yeast, the process takes place after nuclear export on the cytosolic surface of the outer mitochondrial membrane (reviewed in Leisegang *et al.*, 2012). A reason for this difference is that Exportin-T, which is the major tRNA export receptor in humans, probes the structure of the tRNAs, ensuring their proper folding and completion of nuclear maturation events (Kutay *et al.*, 1998; Lipowsky *et al.*, 1999; Cook *et al.*, 2009; Hopper *et al.*, 2010). In contrast, the yeast homolog of Exportin-T, Los1, does not distinguish between intron-containing and spliced tRNAs, allowing export of unspliced tRNAs (Yoshihisa *et al.*, 2003, 2007). Interestingly, a retrograde import pathway that is conserved in yeast and vertebrates exists by which tRNAs are returned to the nucleus (by the importin Mtr10 in yeast), and they can be re-exported to the cytoplasm (Whitney *et al.*, 2007; Shaheen *et al.*, 2007). In addition to Exportin-T, Exportin 5 (Msn5 in yeast) has also been implicated in the re-export of tRNAs to the cytoplasm (Bohnsack *et al.*, 2002; Calado *et al.*, 2002).

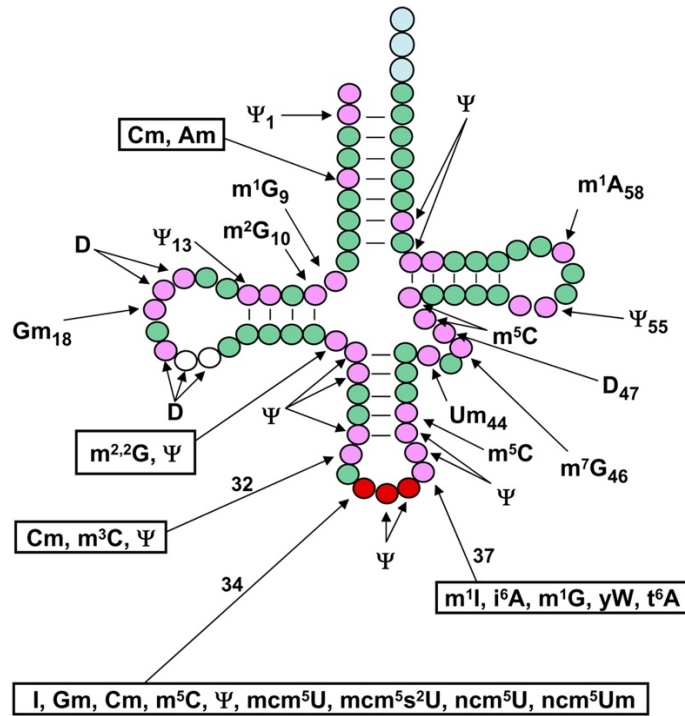
Besides cytoplasmic tRNAs, which are encoded by the nuclear genome, the mitochondrial (mt) genome encodes 22 mt-tRNAs in humans, alongside 13 mt-mRNAs and 2 mt-rRNAs (Anderson *et al.*, 1981). Transcription of mt-tRNAs in humans is mediated by the mitochondrial RNA polymerase POLMRT, and, akin to the maturation of cytoplasmic tRNAs, the generated transcripts are further processed at the 5' and 3' ends of each tRNA (reviewed in Suzuki *et al.*, 2011). The 5' end of mt-tRNA is processed by mitochondrial RNase P (MRPP1, 2 and 3), which is a protein complex devoid of a catalytically active RNA, while the 3' end is cleaved by mitochondrial tRNase Z (ELAC2; Holzmann *et al.*, 2008; Brzezniak *et al.*, 2011). Finally, the CCA tail is added to the 3' end of mt-tRNAs by a mitochondrial CCA-adding enzyme (TRNT1; Nagaike *et al.*, 2001).

Alongside these maturation steps, a myriad of chemical modifications is introduced to both cytoplasmic and mitochondrial tRNAs, resulting in mature tRNAs that can be aminoacylated by cognate tRNA aminoacyl synthetases and can function in translation (Hopper *et al.*, 2010).

### **1.3.2 Diversity of tRNA modifications**

A striking feature of tRNAs from all living organisms is that they carry the most numerous and chemically diverse post-transcriptional modifications. On average, each tRNA carries 14 modifications, and the type and extent of these modifications vary between different tRNAs and in some cases between similar isoacceptors (Fig. 3; reviewed in Nachtergaele and He, 2017). While some tRNA modifications at specific positions are conserved among the three phylogenetic domains of life, other modifications vary between tRNAs from different organisms within the same domain of life (reviewed in Phizicky and Alfonzo, 2010; Sprinzl and Vassilenko, 2005). The chemical nature of the evolutionarily conserved

tRNA modifications is generally simple. Examples include pseudouridylation, reduction of uridine to dihydrouridine, base and 2'-O-methylation, replacement of oxygen with sulfur, acetylation and threonylation. In contrast, tRNA modifications that are unique to specific domains of life typically display larger and more complex chemical structures (reviewed in Jackman and Alfonzo, 2013).



**Figure 3. Overview of cytoplasmic tRNA modifications in eukaryotes.**

A schematic *Saccharomyces cerevisiae* cytoplasmic tRNA is shown in its secondary structure. Green circles show residues that are not modified; pink circles show residues that are modified in some or all yeast cytoplasmic tRNAs; white circles indicate additional residues, 20a and 20b, which are present in some yeast cytoplasmic tRNAs and are sometimes modified; red circles indicate the anticodon residues; light blue circles indicate the CCA tail. Lines represent base pairs. The RNA modifications present at specific positions (numbered) are indicated using conventional abbreviations, according to the abbreviations list (adapted from Phizicky and Hopper, 2010).

The two uridine derivatives pseudouridine ( $\Psi$ ) and dihydrouridine (D) are amongst the most common modifications in tRNAs. Although the T $\Psi$ C and the D loops have been named after these modifications,  $\Psi$  and D residues can also be found at other positions (reviewed in Hur and Stroud, 2007). Unlike rRNA, where the majority of pseudouridylations are introduced by box H/ACA snoRNPs, cytoplasmic and mt-tRNAs are mostly pseudouridylated by stand-alone pseudouridine synthases (PUS enzymes; see for example, Czudnochowski *et al.*, 2013; McCleverty *et al.*, 2007). Similarly, uridine is mostly reduced to D by stand-alone enzymes, known as dihydrouridine synthetases (DUS1-4) in both cytoplasmic and mt-tRNAs (Xing *et al.*, 2004). Although D is highly abundant in cytoplasmic tRNAs, it has only been reported at one position in mt-tRNA<sup>Ser</sup> and mt-tRNA<sup>Leu</sup> in humans (reviewed in Suzuki *et al.*, 2014).

Additionally, tRNAs also undergo base methylation at multiple positions. A classic example is the conserved  $N^1$ -methyladenosine ( $m^1A$ ) at position 58 of many cytoplasmic and mitochondrial tRNAs (Anderson *et al.*, 1998). In humans, these modifications are installed by the TRMT61A/TRMT6 complex on cytoplasmic tRNAs and by TRMT61B on mt-tRNAs (Anderson *et al.*, 2000; Ozanick *et al.*, 2005). Furthermore, cytosines can be methylated at nitrogen 3 and carbon 5, generating  $N^3$ -methylcytosine ( $m^3C$ ) and  $C^5$ -methylcytosine ( $m^5C$ ) modifications on tRNAs respectively.  $m^3C$  modifications can be installed on tRNA<sup>Thr(UGU)</sup> and tRNA<sup>(CCU)</sup> at position 32 by methyltransferase-like (METTL)2 and on tRNA<sup>Ser(AGA)</sup> and tRNA<sup>Ser(GCU)</sup> at the same position by METTL6 (Xu *et al.*, 2017).  $m^5C$  modifications can be installed on tRNA, as well as other classes of RNA, in humans by members of the Nol1/Nop2/SUN domain (NSUN) family of putative methyltransferases (NSUN1-7) and by DNA methyltransferase 2 (DNMT2; Tuorto *et al.*, 2012). For example, NSUN2 and DNMT2 introduce  $m^5C$  modifications at position 34 of tRNA<sup>Leu</sup> and at position 38 of tRNA<sup>Asp</sup> respectively (Brzezicha *et al.*, 2006; Goll *et al.*, 2006). The enzymes installing  $m^5C$  modifications on mt-tRNA are not well characterized but the known and putative  $m^5C$  methyltransferases NSUN2, NSUN3 and NSUN4 have all been detected in human mitochondria, making them candidates for catalysing such reactions. Other examples of conserved base methylations that occur in specific cytoplasmic or mitochondrial tRNAs, include  $N^1$  or  $N^2$  or  $N^7$ -methylguanosine ( $m^1G$ ,  $m^2G$  or  $m^7G$ ) and  $N^3$  or  $C^5$ -methyluridine ( $m^3U$  or  $m^5U$ ). Dimethylation of tRNA residues has also been reported and examples include  $N^2,N^2$ -dimethylguanosine ( $m^{2,2}G$ ) and  $N^6,N^6$ -dimethyladenosine ( $m^{2,2}A$  or  $m_2^6A$ ; reviewed in Jackman and Alfonzo, 2013).

The diverse and abundant nature of tRNA modifications, and the evolutionary conservation of many individual modifications implies that they play important roles in ensuring proper structure and function of tRNAs.

### 1.3.3 Functions of tRNA modifications

It is generally thought that modifications in the core of tRNAs influence folding and stability, whereas modifications in or around the anticodon affect efficiency or accuracy of translation (reviewed in Phizicky and Hopper, 2010). For example, the lack of  $m^5U$  at position 54 in tRNA<sup>fMet</sup> and tRNA<sup>Phe</sup> lowers the melting temperature of the tRNA by 2-6°C *in vitro* and the lack of  $m^1A$  at position 9 of mt-tRNA<sup>Lys</sup> causes misfolding of the tRNA (Sengupta *et al.*, 2000; Helm *et al.*, 1999). Interestingly, many tRNAs contain both  $\psi$ , which increases RNA rigidity, and D, which promotes RNA flexibility, and it has been suggested that the balance between the opposite effects caused by these two modifications is important for maintaining the optimal structure of some tRNAs (reviewed in Zagryadskaya *et al.*, 2004; Giege *et al.*, 2012). The lack of specific modifications can

also lead to degradation of the tRNA. For example, the lack of m<sup>1</sup>A modifications at position 58 in *trm6* temperature-sensitive mutants increases the turnover of the initiator tRNA<sup>Met</sup> (Anderson *et al.*, 1998). Additionally, tRNA modifications at various positions serve as identity elements; for example, the 2'-O-ribosyl phosphate modification at position 64 in tRNA<sup>Met</sup> allows discrimination between the initiator and elongator tRNAs<sup>Met</sup> in yeast (Astrom *et al.*, 1994).

In the anticodon loop, two positions are modified in almost every tRNA, position 37 and the wobble nucleotide at position 34, and the modifications present in these positions show the largest chemical diversity. In most cytoplasmic and mt-tRNAs, position 37 is a modified purine (reviewed in Jackman and Alfonzo, 2013). Modifications at this position typically reduce its Watson-Crick base pairing potential, thereby preventing unspecific interactions with nearby tRNA or mRNA nucleotides and also helping maintain an open structure of the anticodon loop. Consistent with this, the lack of m<sup>1</sup>G37 formation in tRNA<sup>Arg</sup> or tRNA<sup>Leu</sup> results in increased frameshifting by the ribosome and consequently affects translation fidelity and cellular growth (Bjork *et al.*, 2001; Urbonavicius *et al.*, 2001). Notably, m<sup>1</sup>G37 is also present in mt-tRNAs and mutations in the enzyme installing the modification (TRMT5) have been linked to multiple mitochondrial respiratory chain deficiencies highlighting the importance of the modification at this position (Powell *et al.*, 2015). In some cases, m<sup>1</sup>G37 is further modified into wybutosine (yW), which prevents -1 frameshifting by allowing base stacking interactions that stabilize codon-anticodon base pairing in the A site of the ribosome (Waas *et al.*, 2007; de Crecy-Lagard *et al.*, 2010). When position 37 is an adenosine, it can be further modified in a number of different ways. In both cytoplasmic and mt-tRNAs, threonylcarbamoyl adenosine (t<sup>6</sup>A) at position 37 maintains an open conformation of the anticodon loop and promotes codon-anticodon interactions, which maintain the speed and accuracy of translation (Morin *et al.*, 1998; Thiaville *et al.*, 2016). Alternatively, adenosine at position 37 can be isopentenylated forming a bulky modification known as isopentenyl adenosine (i<sup>6</sup>A), which is found in a subset cytoplasmic and mitochondrial tRNAs. In yeast, i<sup>6</sup>A has been shown to enhance translation efficiency of specific codons (Lamichhane *et al.*, 2011). Coupled with these modifications at position 37, various modifications at the wobble position (34) ensure efficient, accurate and flexible decoding. One of the best-understood examples is the thiolation of U34 forming 2-thiouridine (s<sup>2</sup>U), which enhances anticodon rigidity, and, combined with other modifications at carbon 5, serves as a translation efficiency and fidelity determinant (Johansson *et al.*, 2008). Another prevalent modification at the wobble position in various cytoplasmic and mt-tRNAs is the replacement of guanosine with the 7-deaza guanosine derivative, queuosine (Q; Katze *et al.*, 1984). It has been suggested that Q regulates the strength of specific codon-anticodon interactions, however, the exact

function of the modification is still unknown (Morris *et al.*, 1999). tRNA modifications at the wobble position are particularly important for decoding the non-universal mitochondrial genetic code in mammals, where they allow non-canonical base pairing with the third nucleotide of the mRNA codon (reviewed in Bohnsack and Sloan, *in press*). For example, the 5-formylcytosine modification (f<sup>5</sup>C34) in the mt-tRNA<sup>Met</sup> has been proposed to increase the decoding capacity of the tRNA by influencing the thermodynamic and structural features of the anticodon (Bilbille *et al.*, 2011). Such effects could possibly contribute to the ability of the mt-tRNA<sup>Met</sup> to decode AUG, AUU and AUA codons during translation initiation and AUG and AUU codons during translation elongation.

Through regulation of codon usage, modifications in the anticodon loop have also been suggested to influence translation of specific mRNAs (reviewed in Gustilo *et al.*, 2008). For example, the 5-methylcarbonylmethyluridine modifications (mcm<sup>5</sup>U) at the wobble position of tRNA<sup>ARG(UCU)</sup> and tRNA<sup>GLU(UUC)</sup> increase the translation of mRNAs enriched in these codons upon DNA damage (Begley *et al.*, 2007). Strikingly, the mcm<sup>5</sup>U34 modification enhances the translation of two mRNAs highly enriched in these codons, RNR1 and RNR3, which encode key proteins involved in the DNA damage response, suggesting a link between tRNA modifications and cellular responses. Similarly, m<sup>5</sup>C modifications installed in tRNAs by NSUN2 and DNMT2 have been implicated in stress responses, as the lack of these modifications leads to endonucleolytic cleavage of the tRNAs and accumulation of tRNA fragments, which downregulate protein translation and promote apoptosis (Blanco *et al.*, 2014). However, the mechanisms by which tRNA modifications are regulated in response to different cellular conditions remain not fully understood. One exciting means by which tRNA modifications have been shown to be dynamically regulated is through the presence of demethylases (so called “eraser” proteins), which selectively remove the modification in certain conditions. Indeed, the alpha-ketoglutarate and Fe(II)-dependent dioxygenase ALKBH1 (ABH1) has been recently identified as a tRNA demethylase that catalyses the selective removal of m<sup>1</sup>A modifications at position 58 of selected cytoplasmic and mitochondrial tRNA, thereby regulating the utility of these tRNAs in translation (Liu *et al.*, 2016).

Although defects in tRNA structure and translation due to lack of individual modifications have been observed, it has also been suggested that tRNA modifications act in concert. This model is supported by the findings that the combinatory depletion of m<sup>7</sup>G and m<sup>5</sup>C modifications at positions 46 and 49 respectively in *trm8 trm4* yeast mutants causes specific degradation of tRNA<sup>Val(ACC)</sup> (Alexandrov *et al.*, 2006) and that the combinatory depletion of non-essential genes encoding tRNA modification enzymes cause growth defects in yeast (Chernyakov *et al.*, 2008). Furthermore, the installation of tRNA modifications at different positions have been shown in some cases to be coordinated. For

example, the introduction of an m<sup>3</sup>C modification at position 32 of cytoplasmic tRNAs<sup>Ser</sup> is dependent on the presence of an i<sup>6</sup>A modification at position 37, suggesting that the functions of these tRNA modifications may be interconnected (Arimbasseri *et al.*, 2016).

In short, this network of diverse, and often complex, chemical modifications plays significant roles in maintaining the structure, stability and function of cytoplasmic and mt-tRNAs, which modulate gene expression and regulate cellular processes. Therefore, it has been proposed that defects in tRNA modifications may play crucial roles in human diseases.

### 1.3.4 tRNA modifications and disease

Growing evidence links defects in tRNA modifications to complex human pathologies, such as neurological disorders, metabolic diseases, cancer and mitochondrial-linked disorders (reviewed in Sarin and Leidel, 2015). Such defects can result from alterations in genes encoding tRNA modification enzymes, or mutations in tRNA sequences that prevent the installation of modifications. For example, mutations in the human *IKBKAP* gene, which encodes IKAP, a subunit of the human Elongator complex, have been linked to a complex genetic neuropathy affecting the autonomic nervous system, called familial dysautonomia (FD; reviewed in Slaugenhaupt and Gusella, 2002). The Elongator complex has been shown to play a role in the formation of the mcm<sup>5</sup>U and 5-methoxycarbonylmethyl-2-thiouridine (mcm<sup>5</sup>s<sup>2</sup>U) modifications in U34 of several tRNAs (Huang *et al.*, 2005). The most prevalent mutation identified in FD patients causes tissue-specific exon skipping and reduced IKAP levels, leading to reduced levels of mcm<sup>5</sup>U and mcm<sup>5</sup>s<sup>2</sup>U modifications (Anderson *et al.* 2001; Slaugenhaupt *et al.*, 2001). Another tRNA modification enzyme that is associated with a neurological condition is FTSJ1, a homolog of the yeast Trm7, which is required for the 2'-O-methylation at several positions of different tRNAs (Pintard *et al.*, 2002). Mutations in *FTSJ1* are implicated in non-syndromic X-linked mental retardation (Freude *et al.*, 2004). Furthermore, mutations in *NSUN2*, which installs an m<sup>5</sup>C modification at the wobble position of tRNA<sup>Leu(CCA)</sup>, have been linked to autosomal recessive intellectual disability (Abbasi-Moheb *et al.*, 2012; Khan *et al.*, 2012).

Lack of specific tRNA modifications have also been associated with metabolic disorders such as type 2 diabetes, which is of particular interest because it affects health and economies on a global scale (reviewed in Zimmet *et al.*, 2001). Mutations in *CDKAL1*, which encodes the methylthiotransferase that is required for the formation of 2-methylthio-N<sup>6</sup>-threonylcarbamoyladenosine (ms<sup>2</sup>t<sup>6</sup>A) at position 37 of tRNA<sup>Lys(UUU)</sup>, leads to reduced modification levels, perturbed proinsulin processing and reduced insulin secretion in  $\beta$

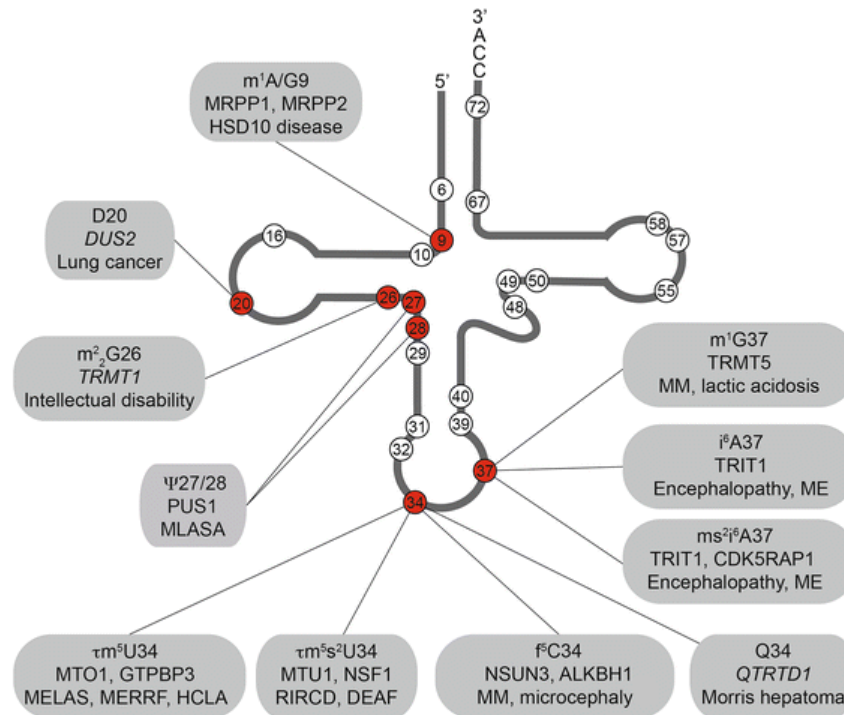


cells (Ohara-Imaizumi *et al.*, 2010; Wei *et al.*, 2011). Notably, CDKAL1 has also been identified as a risk factor for Psoriasis and Crohn's disease (Quaranta *et al.*, 2009).

It has been long known that cancer cells often contain more methylated tRNA nucleotides than tRNAs derived from non-tumor cells and elevated tRNA methyltransferase activity has been observed during tumorigenesis, however subsequent studies have shown that also hypomodification of tRNAs is associated with cancer (Tsutsui *et al.*, 1966; Dirheimer *et al.*, 1995). For example, NSUN2 is highly expressed in a range of human and mice tumor types including breast cancer, colorectal cancer and squamous cell carcinoma, although it is expressed at low levels in non-tumor cells and in benign papillomas (Frye and Watt, 2006). Similarly, TRM12, which is the human homolog of the yeast enzyme that catalyses the formation of wybutosine at position 37 of tRNA<sup>Phe</sup>, is overexpressed in multiple breast cancer tumors (Rodriguez *et al.*, 2007). In contrast, reduced levels of Q34 modifications in tRNAs have been observed in leukaemia, lymphoma and other types of tumors (Shindo-Okada *et al.*, 1981; Huang *et al.*, 1992). It has been suggested that Q deficiency is due to reduced activity of the enzyme that installs the modification, tRNA-guanine transglycosylase (TGTase), in tumor cells (Morris *et al.*, 1999; Costa *et al.*, 2004). In a similar way, expression of TRMT5, which catalyses the m<sup>7</sup>G modification at position 37 of several tRNAs, is downregulated in colorectal cancers (reviewed in Sarin and Leidel, 2015).

Besides alterations in the tRNA modification enzymes, mutations in the genes encoding tRNAs can also prevent the installation of specific modifications and lead to serious pathologies. This is particularly important in mitochondria, where modifications in the anticodon of mt-tRNAs influence their decoding capacity and enable the use of the non-conventional mitochondrial genetic code. For example, mutations in mt-tRNA<sup>Leu(UAA)</sup> and mt-tRNA<sup>Lys(UUU)</sup> lead to mitochondrial encephalomyopathy with lactic acidosis and stroke-like episode (MELAS) and myoclonus epilepsy associated with ragged-red fibers (MERRF) syndromes respectively (Fig. 4; Yasukawa *et al.*, 2000a, 2000b). In both syndromes, the mutations disrupt the tertiary structure of tRNAs and prevent their recognition by the taurine transferases, which are suggested to be MTO1 and GTPBP3 (Tischner *et al.*, 2015; Chen *et al.*, 2016), leading to 5-taurinomethyluridine ( $\tau\text{m}^5\text{U}$ ) hypomodification of U34 in MELAS patients and 5-taurinomethyl-2-thiouridine ( $\tau\text{m}^5\text{s}^2\text{U}$ ) hypomodification of U34 in MERRF patients (Kirino *et al.*, 2005). The lack of  $\tau\text{m}^5\text{U}$  impedes mt-tRNA<sup>Leu(UAA)</sup> decoding UUG codons, but it does not affect reading of UUA codons. In MELAS patients, this leads to a specific reduction in ND6, which is a component of the respiratory chain complex I that is translated from a transcript enriched in UUG codons. However, the absence of  $\tau\text{m}^5\text{s}^2\text{U}$  prevents mt-tRNA<sup>Lys(UUU)</sup> from reading

both of its cognate codons (AAG and AAA), leading to reduced overall mitochondrial translation in MERRF patients (reviewed in Suzuki *et al.*, 2011).



**Figure 4. Defects in enzymes installing chemical modifications on mitochondrial tRNAs lead to human diseases.**

A schematic mitochondrial (mt)-tRNA is shown in its secondary structure. Circles show residues that can be modified. Red circles show modified residues that are associated with human diseases and the types of the modifications, the enzyme(s) installing them and the associated diseases are given using conventional abbreviations, according to the abbreviations list (taken from Bohnsack and Sloan, 2017).

The dysfunction of the tRNA modification machinery in multiple human disorders raises the possibility of developing therapeutic strategies based on correcting the levels of tRNA modifications or targeting the enzymes installing them. Modulating tRNA modification enzymes has been suggested to be a promising therapeutic strategy to treat cancer (reviewed in Torres *et al.*, 2014). For example, the proposed human homolog of Trm9, which installs  $mcm^5U$  at position 34 of  $tRNA^{Arg}$  and  $tRNA^{Glu}$  in yeast, HTRM9L, is downregulated in breast, testicular, cervical and colorectal carcinomas, and re-expression of the protein reduced tumor growth *in vivo* (Begley *et al.*, 2013). In contrast, downregulation of NSUN2, which is overexpressed in different cancers, decreases the growth of human squamous cell carcinoma xenografts (Frey and Watt, 2006). Additionally, small molecules can also be used to target tRNA modification enzymes. For example, Kinetin has shown positive clinical results in the treatment of FD, which is caused by the lack of  $mcm^5s^2U$  modification in several tRNAs (see above; Axelrod *et al.*, 2011). Moreover, the anti-cancer drug Azacytidine has been shown to reduce  $m^5C$  levels at DNMT2 target sites, suggesting that tRNA demethylation may contribute to the efficacy of

the drug (Schaefer *et al.*, 2009). Besides their potential as therapeutic tools, changes in tRNA modifications could also be used as diagnostic markers for certain diseases. For example, the human homolog of Trm10, which installs m<sup>1</sup>G modifications at position 9 in several tRNAs in yeast, HRG9MTD2, is a good candidate to diagnose early onset and late onset colorectal cancer patients (Berg *et al.*, 2010).

Despite the increasing number of reports linking tRNA modification to disease, we still lack understanding of the mechanisms by which many tRNA modifications are installed or regulated, and their precise biological roles.

## **1.4 Modifications in other RNA species**

### **1.4.1 Transcriptome-wide mapping approaches**

Although RNA modifications have been recognized from the early days of molecular biology (examples include Cohn and Volkin, 1951; Davis and Allen, 1957), the majority of early research was limited to modifications in highly abundant RNAs, such as tRNAs, rRNAs and snRNAs (reviewed in Schwartz, 2016). This is because detection of modifications in RNAs expressed at low levels, such as mRNAs, is technically challenging for classical biochemical methods. However, recently, transcriptome-wide studies of RNA modifications using next generation sequencing (NGS) technologies allowed the identification and mapping of several modification types in all classes of RNA, including mRNAs and long non-coding RNAs (reviewed in Roundtree *et al.*, 2017). NGS technologies involve the reverse transcription of RNAs into cDNAs, which are ligated to adaptors for sequencing, and mapping of the obtained sequence reads to the genome and read-end mapping enables the sites of the modifications to be determined in a transcriptome-wide manner. Such RNA modification mapping techniques require a means by which nucleotides carrying specific modification can be differentiated from non-modified nucleotides. One approach by which this can be achieved is based on distinctive reverse transcription signatures (RT signature) resulting from nucleotides misincorporation or abortive cDNA products caused by large RNA modifications or chemical adducts on the Watson-Crick face of the modified residues (Ebhardt *et al.*, 2009; Findeiss *et al.*, 2011). This approach has been used in transcriptome-wide mapping of m<sup>1</sup>A modifications after antibody-mediated enrichment (Li *et al.*, 2016; Dominissini *et al.*, 2016). Interestingly, RNA modifications, such as pseudouridine ( $\Psi$ ), which do not themselves cause reverse transcriptase stalling, can also be analysed by such approaches as, chemical treatments of RNA can enhance the RT signature of specific RNA modifications. For example, *N*-cyclohexyl-*N'*-(2-morpholinoethyl)carbodiimide methyl-*p*-toluenesulfonate (CMCT) treatment of RNAs leads to formation of RT-arresting adducts at  $\Psi$  residues, allowing their detection and the generation of transcriptome-wide maps of the modification (Carlile *et al.*,

2014; Li *et al.*, 2015). Furthermore, the different reactivities of modified and unmodified nucleotides to specific chemical treatments can be exploited to enable detection of modification sites in cellular RNAs. This is the basis of the well-known bisulfite sequencing approach, which can be applied to both DNA and RNA. Here, bisulfite treatment deaminates unmodified cytidines to uridines but does not affect 5-methylcytidines, and this conversion can be detected by sequencing after reverse transcription (Schaefer *et al.*, 2009).

An alternative approach for the detection of modified sites in RNAs is through the use of antibodies that specifically recognise modified nucleotides to enrich modification-containing RNA fragments, which are then converted into a cDNA library for sequencing. This approach is used for transcriptome-wide mapping of several RNA modifications, including *N*<sup>6</sup>-methyladenosine (m<sup>6</sup>A; Dominissini *et al.*, 2012; Meyer *et al.*, 2012). m<sup>6</sup>A mapping combined with RNAi-mediated knockdowns of METTL3 and METTL14 allowed identification of the METTL3-METTL14 heterodimer as a methyltransferase complex that installs m<sup>6</sup>A modifications on multiple RNAs (Liu *et al.*, 2014). Moreover, the antibody-mediated immunoprecipitation of 5-hydroxymethylcytosine (hm<sup>5</sup>C)-containing RNA fragments uncovered the transcriptome-wide distribution of the modification in *Drosophila melanogaster* cells (Delatte *et al.*, 2016). However, these antibody-based techniques enable mapping of the modifications to RNA regions of approximately 100 nucleotides but do not provide their exact nucleotide positions. For m<sup>6</sup>A mapping, a significant improvement in the resolution of the mapping approaches was obtained by crosslinking the antibodies to the modified sites in RNA, which induced unique signature mutations upon reverse transcription, allowing the generation of the first single-nucleotide resolution map of m<sup>6</sup>A (Linder *et al.*, 2015). In short, this rapidly expanding spectrum of techniques for the detection of RNA modifications in cellular RNAs has led to a resurgence in the field of 'epitranscriptomics' through generating transcriptome-wide maps of different modifications on all classes of RNA. These methods allow studying the dynamic occurrence of RNA modifications under different conditions and their potential roles in regulation of gene expression and cellular behaviour (reviewed in Helm and Motorin, 2017).

#### **1.4.2 Modifications in other non-coding RNAs**

Besides tRNAs and rRNAs, modifications on other classes of non-coding RNAs that play crucial roles in cellular homeostasis have been identified (reviewed in Esteller and Pandolfi, 2017). For example, the long non-coding RNA (lncRNA) XIST, which mediates transcriptional repression of multiple genes on the X chromosome during female mammalian development, has been recently shown to be highly methylated and the

METTL3-METTL14 complex was shown to be responsible for many of these modifications (Linder *et al.*, 2015). In fact, it is suggested that XIST carries more m<sup>6</sup>A modifications than any other RNA with 78 mapped m<sup>6</sup>A residues, and these modifications were later shown to be critical for its gene silencing activity (Patil *et al.*, 2016). Additionally, XIST also carries m<sup>5</sup>C modifications, which regulate its function by affecting the binding to the chromatin-modifying complex PCR2 (Amort *et al.*, 2013). Another well-studied modified lncRNA is MALAT1, which is known to regulate alternative splicing (Tripathi *et al.*, 2010). MALAT1 has at least four m<sup>6</sup>A modifications that are suggested to regulate its hairpin stem structure (Liu *et al.*, 2013; Zhou *et al.*, 2016).

Primary microRNAs (pri-miRNAs) can also carry m<sup>6</sup>A modifications, which allow their recognition and processing by DGCR8 (Alarcon *et al.*, 2015). Lack of the METTL3-METTL14 complex, which installs m<sup>6</sup>A modifications on pri-miRNAs, results in accumulation of unprocessed pri-miRNAs and a general reduction of mature miRNAs. Additionally, some miRNAs also carry m<sup>5</sup>C modifications, however, in contrast to the role of m<sup>6</sup>A in pri-miRNAs, the NSUN2-mediated m<sup>5</sup>C modification of pri-miRNA125 inhibits its processing, therefore repressing its function in silencing gene expression (Yuan *et al.*, 2014). Interestingly, NSUN2 also introduces m<sup>5</sup>C modifications on vault non-coding RNAs, and lack of these modifications causes aberrant processing producing small RNA fragments that can function as miRNAs (Hussain *et al.*, 2013).

Finally, the spliceosomal small nuclear RNAs (snRNAs) are also heavily modified. In eukaryotes, five snRNAs (U1, U2, U4, U5 and U6) participate in pre-mRNA splicing by the major spliceosome as small nuclear ribonucleoprotein (snRNP) complexes (reviewed in Matera and Wang, 2014). The U1, U2, U4 and U5 snRNAs have a 2,2,7 trimethylguanosine (TMG) cap at the 5' end, which can function as a nuclear localization signal (Fischer and Lührmann, 1990). In contrast, the 5' guanosine triphosphate cap of the U6 snRNA, which remains in the nucleus during its maturation, is monomethylated at the  $\gamma$  position and does not contribute to its nuclear localization (Singh *et al.*, 1989). Moreover, the 3' end of the U6 snRNA has a 2',3'-cyclic phosphate, which has been recently shown to be generated by the exonuclease/phosphodiesterase USB1 (Mroczek *et al.*, 2012; Shchepachev *et al.*, 2012). Internally, the most abundant modifications in snRNAs are, similar to rRNA, 2'-O-methylation and  $\Psi$  (reviewed in Roundtree *et al.*, 2017). The majority of these modifications are guided by specialized snoRNAs that reside in Cajal bodies, called small Cajal body-specific RNAs (scaRNAs; reviewed in Meier *et al.*, 2017). Exceptions include the  $\Psi$  modifications at positions 44 and 35 of the yeast U2 snRNA, which are introduced by the stand-alone pseudouridine synthases Pus1 and Pus7 respectively (Massenet *et al.*, 1999; Ma *et al.*, 2003). Besides  $\Psi$  and ribose methylation, snRNAs also carry several other types of RNA modifications. For example, the U6 snRNA

has been reported to carry one m<sup>6</sup>A modification at position 43 and one N<sup>2</sup>-methylguanosine (m<sup>2</sup>G) at position 72 (Shimba *et al.*, 1995; Massenet *et al.*, 1999). Recently, an additional m<sup>6</sup>A site in the U6 snRNA at position 76 has been proposed (Sun *et al.*, 2016). Moreover, the U2 snRNA, which is the most modified snRNA, was also suggested to carry an m<sup>6</sup>A modification (Bringmann and Lührmann, 1987). However, most of the enzymes that introduce snRNA modifications remain unidentified.

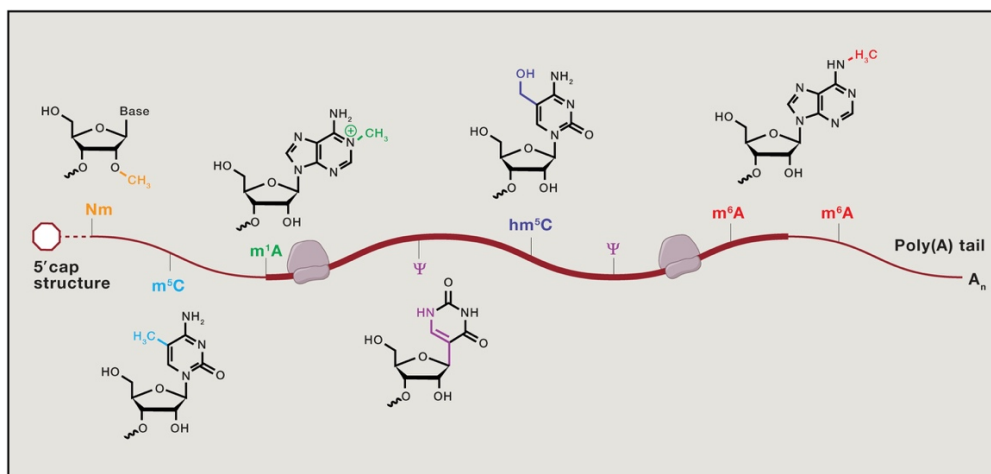
Interestingly, snRNA modifications are present in evolutionarily conserved sequences that are functionally important, suggesting that these modifications may play important roles in regulating the structure or function of the snRNPs (reviewed in Karijolich *et al.*, 2010). For example, the m<sup>6</sup>A43 in the U6 snRNA is present in the conserved ACAGAGA sequence, which directly interacts with pre-mRNA 5' splice sites during splicing (Wassarman and Steitz, 1992). Despite the potential of snRNA modifications to influence numerous aspects of pre-mRNA splicing, most functional studies have focused on the U2 snRNA since it has the highest number of modifications. For example, Ψ modifications within the U2 snRNA sequence that base pairs with pre-mRNA branch-points during splicing influence the catalytic phase of pre-mRNA splicing (Lin and Kielkopf, 2008). Moreover, several 2'-O-methylations at the 5' end of human U2 snRNA have been shown to be individually required for pre-mRNA splicing, and although individual Ψ modifications in the same region are not essential, they have a cumulative effect on U2 function (Dönmez *et al.*, 2004). Modifications at the 5' end of U2 were also later shown to modulate the dynamic equilibrium of the U2-U6 complex, which is the catalytic core of the spliceosome (Karunatilaka and Rueda, 2014). Besides their well-studied functions in the U2 snRNA, modifications in other snRNAs have also been suggested to impact pre-mRNA splicing. For example, modifications at the U4/U6 snRNA base pairing region have been suggested to influence the rate of unwinding the duplex, and modifications in the U1 snRNA can contribute to its interaction with the 5' splice site during the initial step of mRNA splicing (Freund 2003; reviewed in Karijolich *et al.*, 2010). Recently, defects in snRNA modifications have been linked to disease, as the absence of Usb1, which modifies the 3' end of the U6 snRNA, leads to a genetic disease known as poikiloderma with neutropenia (PN; Mroczek *et al.*, 2012; Shchepachev *et al.*, 2012). In addition to snRNA modifications, pre-mRNA splicing can also be influenced by modifications in the pre-mRNAs themselves.

### 1.4.3 mRNA modifications

The revived interest in the epitranscriptomics field over recent years is mainly due to novel findings from studies on mRNA modifications, particularly m<sup>6</sup>A, which is the most abundant internal modification in eukaryotic mRNAs (Fig. 5; reviewed in Roundtree and He, 2016). In humans, m<sup>6</sup>A is reported to be present at more than 10,000 sites in the

transcriptome and is suggested to occur in at least a fourth of all mRNA transcripts (Domonissini *et al.*, 2012; Meyer *et al.*, 2012). Notably, m<sup>6</sup>A modifications are typically enriched in the 3' untranslated regions (3'UTRs) and in close proximity to stop codons. m<sup>6</sup>A modifications are often installed co-transcriptionally by a multicomponent methyltransferase ('writer') complex containing METTL3, METTL14 and the regulatory subunits WTAP and KIAA1429 (Liu *et al.*, 2014; Ping *et al.*, 2014; Schwartz *et al.*, 2014). Although both METTL3 and METTL14 have methyltransferase domains, structural and functional data have suggested that METTL3 is the catalytic core of the complex, while METTL14 serves as an inactive RNA-binding platform (Wang *et al.*, 2016). Strikingly, some m<sup>6</sup>A modifications have been shown to be selectively removed by demethylases ('erasers'), called FTO and AlkB family member 5 (ALKBH5), indicating that m<sup>6</sup>A modifications can be dynamically regulated and that changes in RNA modifications could be a means to alter the biological functions of specific RNAs under certain conditions (Jia *et al.*, 2011; Zheng *et al.*, 2013). m<sup>6</sup>A can affect the functions of RNAs by altering RNA folding and structure, or via 'reader' proteins, which directly recognize and bind the modification and thereby regulate the fate of the RNA (reviewed in Wu *et al.*, 2016). m<sup>6</sup>A-mediated structural effects (collectively termed 'm<sup>6</sup>A switches') can directly affect codon anti-codon base pairing, thus influencing translation dynamics of mRNAs, or indirectly recruit functional proteins that recognize the formed RNA structures (Choi *et al.*, 2016; Liu *et al.*, 2015). In contrast, the best characterised group of m<sup>6</sup>A readers all contain a YTH domain, which contains a conserved hydrophobic pocket that can specifically bind m<sup>6</sup>A with a much higher affinity than A (Luo and Tong, 2014). During pre-mRNA splicing, YTHDC1 binds m<sup>6</sup>A-containing transcripts and recruits SRSF3, leading to exon inclusion (Xiao *et al.*, 2016). Although m<sup>6</sup>A has been suggested to play a role in facilitating pre-mRNA export to the cytoplasm, the mechanistic details remain to be reported (Zheng *et al.*, 2013). In the cytoplasm, YTHDF1 binds specific m<sup>6</sup>A-modified mRNAs and recruits translation initiation factors, promoting translation (Wang *et al.*, 2015). YTHDF3, another cytoplasmic m<sup>6</sup>A reader, was shown to facilitate translation and decay of a subset of m<sup>6</sup>A-modified mRNAs (Li *et al.*, 2017; Shi *et al.*, 2017). Similarly, YTHDC2 was recently reported to enhance translation of some m<sup>6</sup>A-modified mRNAs and to decrease their abundance (Hsu *et al.*, 2017). Finally, YTHDF2 localizes m<sup>6</sup>A-methylated mRNAs to cytoplasmic processing bodies, accelerating their degradation (Wang *et al.*, 2014). Collectively, m<sup>6</sup>A modifications are proposed to regulate almost every stage of RNA metabolism and modulate the expression of specific groups of mRNAs during complex cellular events, such as differentiation, development, cell cycle regulation, circadian rhythm and stress responses (reviewed in Zhao *et al.*, 2016).

Interestingly,  $N^6$ -methylation of adenosine can also occur in the second nucleotide of mRNA if it is 2'-*O*-methylated, forming  $N^6,2'$ -*O*-dimethyladenosine ( $m^6A_m$ ; Wei *et al.*, 1975, 1976). Interestingly,  $m^6A_m$  renders the transcripts resistant to the mRNA-decapping enzyme DCP2 and therefore less susceptible to miRNA-mediated mRNA degradation (Mauer *et al.*, 2017). Moreover, FTO was shown in the same study to selectively remove the modification, suggesting that  $m^6A_m$  is another dynamic epitranscriptomic mark in mRNA.



**Figure 5. Summary of modifications reported in eukaryotic mRNAs.**

The line represents a eukaryotic mRNA, which includes the 5' cap structure, the 5' untranslated region (UTR), the coding sequence (bold), the 3' UTR and the poly(A)tail. The types of modifications detected in mRNAs are shown at the positions where they have predominantly been detected and are abbreviated as described in the abbreviations list (taken from Roundtree *et al.*, 2017).

$N^1$ -methylation of adenosine can also take place in mRNAs, forming  $N^1$ -methyladenosine ( $m^1A$ ), which carries a positive charge that can alter RNA structure or influence RNA-protein interactions (Zhou *et al.*, 2016). Recently, transcriptome-wide mapping of  $m^1A$  sites showed its enrichment in proximity to translation start sites and the first splice site, suggesting a role in regulating mRNA translation that has yet to be documented (Dominissini *et al.*, 2016; Li *et al.*, 2016). Mapping of  $m^5C$  sites in mRNAs was also recently performed, uncovering its enrichment in the vicinity of the Argonaute protein-binding regions within 3'UTRs and downstream of translation initiation sites (Squires *et al.*, 2012). To date, NSUN2, which installs  $m^5C$  on several tRNAs, is the only characterized  $m^5C$  writer on mRNA (Hussain *et al.*, 2013; Khoddami *et al.*, 2013). Excitingly,  $m^5C$  modifications in mRNAs have been recently shown to be 'read' by the mRNP export factor ALYREF, facilitating mRNA export to the cytoplasm (Yang *et al.*, 2017).

$\Psi$  and 2'-*O*-methylation, which are the most abundant modifications in several classes of RNA in eukaryotes, have been also identified in mRNAs. Recently, the distribution of  $\Psi$  on mRNA has been reported, revealing thousands of modified sites in human mRNAs (Li *et*



*al.*, 2015; Carlile *et al.*, 2014; Lovejoy *et al.*, 2014). Members of the pseudouridine synthetase (PUS) family have been shown to mediate pseudouridylation of mRNAs (Karijolich *et al.*, 2011). Although the precise roles of  $\Psi$  in mRNA have not been shown in detail yet, in *E. coli*, it has been suggested that  $\Psi$  modification of the first nucleotide of stop codons affects base pairing during decoding, leading to increased read-through (Fernandez *et al.*, 2013). The distribution of 2'-O-methylation sites on mRNAs was recently reported, uncovering the presence of more than 7000 2'-O-methylation sites (Dai *et al.*, 2017). It has been suggested that certain snoRNAs bind to mRNAs where it is possible that they install 2'-O-methylations, however, experimental evidence of these interactions is lacking and the exact mechanism of installing the modification on mRNA is still not clear (Bachellerie *et al.*, 2002; Cavaille *et al.*, 2000). Similarly, the exact functions of non-cap 2'-O-methylations in mRNAs are not fully understood, but *in vitro* studies have suggested that it can inhibit A to I RNA editing (Beal *et al.*, 2007).

Taken together, the last decade has witnessed great progress in the field of RNA modifications, unravelling transcriptome-wide maps of different modifications on different RNA species and their dynamic occurrence. However, we are still "scratching the surface" of the field, since we still need to understand the mechanisms by which different RNA modifications are installed as well as their contribution to signalling pathways, human physiology and disease.

## Chapter Two: Aims of this work

Recent studies have shown that chemical modifications are widespread in almost all classes of RNA and these modifications are emerging as key players in the regulation of gene expression. Modifications expand the features of RNA residues, thereby influencing RNA secondary structures, base pairing properties and/ or RNA-protein interactions. These functions enable RNA modifications to regulate the fates of the modified RNAs and affect cellular processes that involve ribonucleoprotein (RNP) complexes, such as ribosome biogenesis, mRNA splicing and translation. Interestingly, some RNA modifications were recently shown to be selectively removed by 'eraser' proteins, suggesting that they represent a dynamic layer of regulation of gene expression. Moreover, defects in RNA-modifying enzymes have been linked to human diseases, such as cancer and metabolic disorders.

The enzymes that install many RNA modifications and the biological functions of these modifications remain uncharacterized and knowledge of the molecular mechanisms of most diseases caused by defects in RNA modification enzymes is often lacking. Here, we aimed to characterise several putative RNA methyltransferases for which no functions were known. We set out to determine the RNA substrates of the putative m<sup>6</sup>A and m<sup>5</sup>C methyltransferases METTL16, NSUN6 and NSUN3 in a transcriptome-wide manner using the crosslinking and analysis of cDNA (CRAC) approach. In addition, we aimed to characterize the modification activity of these enzymes using *in vivo* and *in vitro* methylation assays and to identify recognition elements that are required for recruitment of the RNA-modifying enzymes to their specific substrate RNAs. Furthermore, to gain insight into the cellular functions of the modifications installed by these enzymes, we analysed the effects of lack of these modifications/modification enzymes on the target pathways identified by CRAC. Another important aspect of this work was to better understand how defects in RNA modification enzymes can cause disease, and we studied Bowen-Conradi syndrome (BCS), which is caused by a point mutation in the gene encoding the rRNA methyltransferase EMG1, to elucidate the molecular basis of this disease.

Collectively, our studies aimed to characterize RNA-modifying enzymes by providing insights into their modification substrates, cellular functions and their involvement in human diseases.

## Manuscript 1

**Warda AS\***, Kretschmer J\*, Hackert P, Lenz C, Urlaub H, Höbartner C, Sloan KE, Bohnsack MT (2017) METTL16 is a  $N^6$ -methyladenosine ( $m^6A$ ) methyltransferase that targets pre-mRNAs and various non-coding RNAs. *EMBO Rep*, in press.

\* These authors contributed equally to this work.

URL: <http://embor.embopress.org/content/18/11/2004.long>

doi: 10.15252/embr.201744940

### Author contributions:

**Figure 1:** Generation of HEK293 cell line expressing METTL16-His-Prc-Flag (AW); UV- and PAR-CRAC experiments for METTL16 (PH); bioinformatics analysis of METTL16 CRAC data (JK, KS, MB); RNA-IP after crosslinking and northern blotting for snRNAs (AW).

**Figure 2:** RNAi against METTL16 (AW); Bioinformatics analysis of METTL16 CRAC data (JK, KS, MB); anti- $m^6A$  RNA-immunoprecipitation and northern blotting for snRNAs (AW).

**Figure 3:** Native RNA-immunoprecipitation and northern blotting for snRNAs (AW); immunoprecipitation and western blotting for La, LARP7 and MEPCE (AW, PH); mass spectrometry of immunoprecipitation eluates (CL, HU).

**Figure 4:** Bioinformatics analysis of METTL16 CRAC data (JK, MB); immunoprecipitation of METTL16-associated RNAs (AW); quantitative RT-PCR analysis of METTL16-associated RNAs (JK).

**Figure EV1:** Generation of HEK293 cell line expressing METTL16-GFP and immunofluorescence analysis of METTL16 (AW).

**Figure EV2:** Bioinformatics analysis of METTL16 CRAC data (JK, KS, MB).

**Non-experimental contributions:** Markus Bohnsack, Claudia Höbartner and Katherine Sloan conceived the study and supervised the work. Katherine Sloan, Markus Bohnsack and Ahmed Warda wrote the manuscript and prepared the figures. All authors analysed data and contributed to preparation of the manuscript.

## Manuscript 2

Haag S, **Warda AS**, Kretschmer J, Günnigmann MA, Höbartner C, Bohnsack MT (2015) NSUN6 is a human RNA methyltransferase that catalyzes formation of m<sup>5</sup>C72 in specific tRNAs. *RNA* **21**: 1532-1543.

URL: <http://rnajournal.cshlp.org/content/21/9/1532.long>

doi: 10.1261/rna.051524.115

### Author contributions:

**Figure 1:** Generation of HEK293 cell line expressing NSUN6-His-Proc-Flag (SH); UV- and 5-azaC-CRAC experiments for NSUN6 (SH); bioinformatics analysis of NSUN6 CRAC data (JK, SH).

**Figure 2:** Bioinformatics analysis of NSUN6 CRAC data (JK, SH); RNA-IP after crosslinking and northern blotting for tRNAs (SH).

**Figure 3:** RNAi against NSUN6 (SH); purification of recombinant NSUN6 (SH); preparation of RNA substrates by *in vitro* transcription and RNA synthesis (SH, CH); *in vitro* methylation assays using recombinant NSUN6 (SH, MG).

**Figure 4:** Cell fractionation and western blotting (SH, AW); generation of cell line expressing NSUN6-GFP (SH); immunofluorescence of NSUN6, GOPC, GM130; PCM1 and tubulin (AW).

**Non-experimental contributions:** Markus Bohnsack and Sara Haag conceived the study and wrote the manuscript. Markus Bohnsack supervised the work. All authors analysed data and contributed to preparation of the manuscript.

### Manuscript 3

Haag S\*, Sloan KE\*, Ranjan N\*, **Warda AS\***, Kretschmer J, Blessing C, Hübner B, Seikowski J, Dennerlein S, Rehling P, Rodnina MV, Höbartner C, Bohnsack MT (2016) NSUN3 and ABH1 modify the wobble position of mt-tRNA<sup>Met</sup> to expand codon recognition in mitochondrial translation. *EMBO J* **35**: 2104-2119.

\* These authors contributed equally to this work.

URL: <http://emboj.embopress.org/content/35/19/2104.long>

doi: 10.15252/emj.201694885

#### Author contributions:

**Figure 1:** Generation of HEK293 cell line expressing NSUN3-GFP (SH); fluorescence microscopy of NSUN3-GFP and a mitotracker (AW); purification of mitochondria, mitochondrial fractionation and western blotting (AW, SD).

**Figure 2:** Generation of HEK293 cell line expressing NSUN3-His-PrC-Flag (SH); bioinformatics analysis of NSUN3 CRAC data (JK, MB, SH); RNA-IP after crosslinking and northern blotting for mt-tRNAs (SH, KS).

**Figure 3:** Preparation of RNA substrates by *in vitro* transcription and RNA synthesis (SH, CH); *in vitro* methylation assay (SH).

**Figure 4:** Preparation of RNA substrates and *in vitro* methylation assays (SH, AW).

**Figure 5:** Immunofluorescence against ABH1 (AW); RNA-IP after crosslinking and northern blotting for mt-tRNAs (SH, KS, BH).

**Figure 6:** Recombinant expression of ABH1, ABH1<sub>R338A</sub>, ABH1<sub>D233A</sub> and FTO (BH, SH); *in vitro* methylation/oxidation assays (BH, KS, CH, SH); preparation and RP-HPLC analysis of m<sup>5</sup>C-, hm<sup>5</sup>C- and f<sup>5</sup>C-containing ASL (CH, KS).

**Figure 7:** Purification of recombinant human mitochondrial translation factors (KS, CB, MB); preparation of m<sup>5</sup>C-, hm<sup>5</sup>C- and f<sup>5</sup>C-containing mt-tRNA<sup>Met</sup> (CH, KS); ribosome-binding studies using bacterial *in vitro* translation system (NR).

**Figure 8:** RNAi against NSUN3 and ABH1 (SH, KS, BH, AW); TMI-labelling and primer extension analysis of f<sup>5</sup>C34 in mt-tRNA<sup>Met</sup> (KS, CH); Bisulfite sequencing of mt-tRNA<sup>Met</sup> after RNAi (SH, CB, JK, MB).

**Figure 9:** <sup>35</sup>S-labelling of RNAi-treated cells and analysis of mitochondrial protein levels (AW, SD).

**Figure EV1:** Bioinformatics analysis of NSUN3 CRAC data (JK, SH); *in vitro* methylation assays (SH); RNA-IP after crosslinking and northern blotting for mt-tRNAs (KS, SH).

**Figure EV2:** Western blotting for sub-mitochondrial localisation of ABH1 (AW, SD).

**Figure EV3:** Analysis of cell viability of RNAi against NSUN3 or ABH1 (KS)

**Non-experimental contributions:** Markus Bohnsack, Claudia Höbartner, Sara Haag and Katherine Sloan conceived the study. Peter Rehling, Marina Rodina, Markus Bohnsack, Claudia Höbartner, Katherine Sloan and Sara Haag supervised the research. Markus Bohnsack, Katherine Sloan and Claudia Höbartner wrote the manuscript.

## Manuscript 4

**Warda AS**, Freytag B, Haag S, Sloan KE, Görlich D, Bohnsack MT (2016) Effects of the Bowen-Conradi syndrome mutation in EMG1 on its nuclear import, stability and nucleolar recruitment. *Hum Mol Genet* **25**: 5353-5364.

URL: <https://academic.oup.com/hmg/article/25/24/5353/2593613/Effects-of-the-Bowen-Conradi-syndrome-mutation-in>

doi: 10.1093/hmg/ddw351.

### Author contributions:

**Figure 1:** Generation of HEK293 cell line expressing EMG1 and EMG1<sub>D86G</sub> (AW); RNAi-mediated depletion of EMG1 (AW); Northern blot analysis of pre-rRNA levels (AW).

**Figure 2:** Immunofluorescence analysis of EMG1 and EMG1<sub>D86G</sub> localisation (AW).

**Figure 3:** Purification of recombinant EMG1 (AW, SH); Binding assays between EMG1 and import receptors (AW); Immunoprecipitation of EMG1-containing complexes for mass spectrometry analysis (SH); RNAi against NOP14, NOC4L and UTP14 (AW); northern blot and pulse-chase labelling analysis of pre-rRNA levels (AW, KS).

**Figure 4:** Immunofluorescence analysis of EMG1, NOP14, NOC4L and UTP14 after RNAi (AW).

**Figure 5:** Nuclear import assays in permeabilised cells using recombinant EMG1 and importins (AW, BF).

**Figure 6:** *In vitro* chaperone assays using recombinant EMG1 and importins (AW, BF).

**Figure S1:** Proteasome inhibitor treatment and western blotting for EMG1 (AW).

**Figure S2:** Immunoprecipitation assays and western blotting (AW); *in vitro* methylation assay using recombinant EMG1 and EMG1<sub>D86G</sub> (AW).

**Figure S3:** *In vitro* chaperone assays using recombinant EMG1 and importins (AW, BF).

**Non-experimental contributions:** Markus Bohnsack conceived the study, and Katherine Sloan, Markus Bohnsack and Ahmed Warda wrote the manuscript and prepared the figures. Markus Bohnsack, Dirk Görlich, Katherine Sloan and Sara Haag supervised the work. All authors analysed data and contributed to preparation of the manuscript.

## Chapter Four: Discussion

More than 150 distinct chemical modifications have been reported in RNAs from diverse organisms. In recent years, our knowledge of RNA modifications has substantially advanced with transcriptome-wide studies showing that the landscape of different RNA modifications represents an important dynamic layer of regulation of gene expression. However, many enzymes responsible for installing particular RNA modifications, their biological roles in cellular processes and their contributions to human diseases remain to be uncovered.

### 4.1 Characterization of the novel m<sup>6</sup>A writer METTL16

N<sup>6</sup>-methyladenosine (m<sup>6</sup>A) is the most abundant internal modification in mRNAs and lncRNAs (reviewed in Roignant and Soller, 2017). Mapping of m<sup>6</sup>A has revealed its enrichment within a RRACH sequence motif (R=A or G; H=A or U or C) around stop codons in mRNAs (Dominissini *et al.*, 2012; Meyer *et al.*, 2012). Recent studies showed that m<sup>6</sup>A modifications can regulate almost all stages of RNA metabolism, including (pre-) mRNA splicing, processing, nuclear export, translation and degradation, and therefore, m<sup>6</sup>A influences multiple cellular processes, such as differentiation and development (reviewed in Roundtree *et al.*, 2017). Initial studies showed that m<sup>6</sup>A can be reversed by the dioxygenases FTO and ALKBH5, indicating that the modification is highly dynamic and can be regulated in different cellular conditions (Jia *et al.*, 2011; Zheng *et al.*, 2013). However, recently, m<sup>6</sup>A was shown to be not the preferred target of FTO *in vivo* and the dynamic regulation of m<sup>6</sup>A was proposed to occur on nascent pre-mRNAs at the level of the m<sup>6</sup>A writers, and it was suggested that m<sup>6</sup>As are relatively stable after the release of pre-mRNAs into the nucleoplasm (Mauer *et al.*, 2017; Ke *et al.*, 2017). Mis-regulation of the installation or removal of m<sup>6</sup>A modifications can lead to human diseases, such as cancers and metabolic disorders (reviewed in Batista, 2017).

m<sup>6</sup>A can be installed by a 'writer' methyltransferase complex containing METTL3 and METTL14, as well as the regulatory subunit WTAP (Liu *et al.*, 2014; Ping *et al.*, 2014). Although the binding sites of the METTL3-METTL14 complex show a significant overlap with previously mapped m<sup>6</sup>A sites and the RRACH sequence motif, numerous m<sup>6</sup>A modifications do not map within the METTL3-METTL14 binding sites and lie within different sequence contexts, raising the exciting possibility that other m<sup>6</sup>A writers may exist in human cells. Based on its homology to the *Escherichia coli* protein YbiN, which introduces an m<sup>6</sup>A modification at position 1618 in the 23S rRNA (Sergiev *et al.*, 2008), we hypothesised that METTL16 could possess such activity in human cells.



Using crosslinking and analysis of cDNA (CRAC; Bohnsack *et al.*, 2012), we identified the RNA binding sites of METTL16 in a transcriptome-wide manner. Our CRAC analysis identified numerous lncRNAs, pre-mRNAs and the U6 snRNA as direct interaction partners of METTL16 *in vivo*. Consistent with a previous report, our data confirms the association of METTL16 with a triple helix close to the 3'-end of the MALAT1 lncRNA, which is required for its nuclear expression (Brown *et al.*, 2016). Moreover, we observed the association of METTL16 with the MAT2A mRNA, which encodes the S-adenosylmethionine (SAM) synthetase. The binding of METTL16 to MAT2A mRNA was recently reported to promote MAT2A splicing under SAM-limiting conditions, therefore METTL16 increases the synthesis of the SAM synthetase and maintains SAM homeostasis (Pendleton *et al.*, 2017).

Collectively, our CRAC data identified 355 pre-mRNAs associated with METTL16 *in vivo*. Interestingly, and in contrast to the binding of the METTL3-METTL14 complex in proximity to stop codons (Liu *et al.*, 2014), the majority of METTL16 binding sites in pre-mRNAs lie within introns, indicating that METTL16 installs a different subset of m<sup>6</sup>A modifications that likely have different functions. Together with the results of a recent study, where m<sup>6</sup>A modifications were suggested to be installed on chromatin-bound pre-mRNAs (Ke *et al.*, 2017), and the nuclear localization of METTL16, this suggests that METTL16 installs m<sup>6</sup>A modifications on nascent pre-mRNAs during their synthesis and processing in the nucleus. Although the precise functions of intronic m<sup>6</sup>A modifications installed by METTL16 are not yet clear, in *Drosophila melanogaster*, intronic m<sup>6</sup>A modifications have been shown to be required for female-specific alternative splicing of *Sxl* (sex-lethal gene) (Hausmann *et al.*, 2016). However, our analysis showed that the majority of the pre-mRNA introns bound by METTL16 are constitutively spliced, suggesting that the m<sup>6</sup>A modifications installed by METTL16 in pre-mRNA introns are likely involved in other functions than regulating alternative splicing of these introns. It is possible that intronic m<sup>6</sup>A modifications affect the RNA secondary structure or long-distance interactions. Structural changes at conserved sequences, such as the splice sites or the branch point, could affect the binding of splicing factor proteins or influence base pairing with spliceosomal snRNAs, thereby affecting splicing efficiency (reviewed in Buratti and Baralle, 2004). Changes in RNA secondary structures of intronic regions other than the conserved splicing sequences can vary the relative distance between these conserved elements, which could similarly affect splicing efficiency (Deshler and Rossi, 1991). Furthermore, intronic m<sup>6</sup>A modifications in pre-mRNAs could be bound by 'reader' proteins that influence pre-mRNA splicing or processing, for example, by the recruitment of splicing factors in a similar way to the m<sup>6</sup>A reader YTHDC1, which recruits SRSF3 and promotes exon inclusion (Xiao *et al.*, 2016).

The direct binding of various lncRNAs, pre-mRNAs and the U6 snRNA by METTL16 suggested that they may be modification targets. We tested this hypothesis on the U6 snRNA, as it has been previously shown to carry an m<sup>6</sup>A modification at position 43, installed by an unknown enzyme (Shimba *et al.*, 1995). Interestingly, the amount of the m<sup>6</sup>A-modified U6 snRNA was reduced to around 50% upon the knockdown of METTL16, compared to the amount of the m<sup>6</sup>A-modified U2 snRNA, demonstrating that METTL16 is an m<sup>6</sup>A writer that installs m<sup>6</sup>A43 in human U6 snRNA. The finding that the modification level falls only to 50% may be explained by the high stability of the U6 snRNA (Sauterer *et al.*, 1988), which could lead to the detection of U6 snRNAs that are modified prior to the depletion of METTL16, or by the detection of the recently proposed m<sup>6</sup>A at position 76 of the U6 snRNA (Sun *et al.*, 2016). Importantly, m<sup>6</sup>A76 lies in a binding site of the METTL3-METTL14 complex and within a RRACH sequence, suggesting that it is introduced by METTL3-METTL14 rather than METTL16 (Liu *et al.*, 2014).

Although the precise function of the METTL16-mediated m<sup>6</sup>A43 in the U6 snRNA remains unknown, the location of the modification within the conserved ACm<sup>6</sup>AGAGA sequence, which base pairs with the 5' splice site of pre-mRNAs during splicing, suggests an important role in the regulation of pre-mRNA splicing. This is supported by the finding that mutating this sequence is lethal in yeast (Peebles *et al.*, 1995; Keating *et al.*, 2010). Based on recent structural studies of human pre-spliceosomal particles, it is most likely that m<sup>6</sup>A43 exerts an effect on the RNA structure or base pairing properties rather than functioning through the recruitment of a reader protein (Agofonov *et al.*, 2016; Bertram *et al.*, 2017). The U6 snRNA undergoes conformational changes during its assembly into spliceosomal complexes (Fortner *et al.*, 1994) and m<sup>6</sup>A43 may influence local RNA secondary structures that are required to accommodate these conformational changes. Furthermore, m<sup>6</sup>A43 may affect U6-pre-mRNA interactions, which are important for 5' splice site recognition and the splicing reaction (McPheeters, 1996). Interestingly, during splicing, the ACm<sup>6</sup>AGAGA sequence is contacted by the U5 snRNP protein PRP8, which plays crucial roles in the formation of the catalytic core of the spliceosome (Galej *et al.*, 2013). It is possible that m<sup>6</sup>A43 influences the interaction of PRP8 with the U6 RNA sequence and consequently affects the function of PRP8 in splicing. This model is supported by the observation that the expression levels of METTL16 and PRP8 are co-regulated in human cells (Kohn *et al.*, 2014). Recently, frameshift mutations in the gene encoding METTL16 have been suggested to play a role in the tumorigenesis of colorectal cancer, but whether this phenotype is related to its methyltransferase activity or not remains unclear (Yeon *et al.*, 2017).

The U6 snRNA is present in cells as part of three distinct snRNP complexes: a U6 mono-snRNP, the U4/U6 di-snRNP or the U4/U6.U5 tri-snRNP (reviewed in Patel *et al.*, 2008).

Our finding that METTL16 associates with the U6 snRNA, but not with the U4 or the U5 snRNAs, in native immunoprecipitation experiments indicates that the m<sup>6</sup>A43 modification is installed in the context of the U6 mono-snRNP, during early maturation stages of the U6 snRNA. Maturation steps of the U6 snRNA include synthesis by RNA polymerase III in the nucleus, addition of a 5' monomethylated guanosine triphosphate cap structure and formation of a 3' uridine tract, which is initially bound by the chaperone-like protein La that plays a role in stabilizing the transcript (reviewed in Mroczek and Dziembowski, 2013). Further maturation of the 3' end of the U6 snRNA leads to the replacement of the La protein with the LSM2-8 proteins, which also stabilize the transcript (Mayes *et al.*, 1999). The LSM-chaperoned U6 snRNP enters the nucleoli where it undergoes snoRNP-guided 2'-O-methylation and pseudouridylation, before it is returned to the nucleoplasm where additional proteins associate to form the mono-U6 snRNP (reviewed in Meier *et al.*, 2017). In order to gain further insights into the timing of m<sup>6</sup>A43 installation, we identified protein interaction partners of METTL16. Immunoprecipitation experiments followed by mass spectrometry revealed the La protein, the La-associated protein LARP7 and the guanosine triphosphate capping enzyme MEPCE as interaction partners of METTL16. Our finding that these interactions are RNA-dependent suggests that they are likely mediated by the U6 snRNA, however, we cannot exclude that the interactions are mediated by other RNAs. For example, the 7SK snRNA, which we also found to interact with METTL16, similarly binds La, LARP7 and MEPCE (Uchikawa *et al.*, 2015) suggesting that these interactions may also be formed in the context of the 7SK RNP. The interaction of METTL16 with the U6 snRNA and MEPCE and LARP7 suggests that METTL16 binds 5'-capped U6 snRNA, and since La and LARP7 favour binding to oligo-U sequences, it was anticipated that METTL16 binds 3'-oligouridylated U6 snRNA (Pannone *et al.*, 1998; Markert *et al.*, 2008). Further analysis of our CRAC data revealed the association of METTL16 with U6 containing non-genomically-encoded U's, further supporting this hypothesis. Together with the prominent nuclear localization of METTL16, our data indicate that the METTL16-mediated m<sup>6</sup>A43 modification is one of the earliest events during the biogenesis of the U6 snRNP, more specifically, METTL16 binds to nuclear 5'-capped oligouridylated U6 snRNA while it is chaperoned by the La protein, before the installation of 2'-O-methylation and pseudouridylation modifications in the nucleolus. It is possible that the presence of m<sup>6</sup>A43 is a pre-requisite for the installation of the snoRNP-guided modifications on the U6 snRNA. While this has not yet been demonstrated, the close proximity of m<sup>6</sup>A43 to snoRNA-guided modifications, together with the extensive base pairing interactions snoRNAs form with their substrates and the known role of m<sup>6</sup>A in regulating RNA-RNA base pairing interactions, makes this a strong possibility. Such

coordination of installing different modifications has been previously reported in tRNA<sup>Ser</sup>, where i<sup>6</sup>A37 is required for the installation of m<sup>3</sup>C32 (Arimbasseri *et al.*, 2016).

Besides the U6 snRNA, it is possible that METTL16 installs m<sup>6</sup>A modifications on other *in vivo* RNA targets identified in our CRAC results. Sequencing of m<sup>6</sup>A-containing RNA fragments, with or without the knockdown of METTL16, has been recently performed, and showed reduced m<sup>6</sup>A modification levels on multiple RNAs upon METTL16 knockdown (Pendleton *et al.*, 2017). However, since the lack of METTL16 causes a reduction of SAM levels, it is difficult to differentiate RNAs that are directly bound and modified by METTL16 from RNAs that have reduced m<sup>6</sup>A modification as a secondary effect caused by the lack of SAM. Indeed, Pendleton and colleagues showed that the GNPTG and GMIP mRNAs, which contain m<sup>6</sup>As that are highly sensitive to depletion of METTL16, are not bound by METTL16. However, comparison of the transcriptome-wide mapping of the binding sites of METTL16 by our CRAC experiments with this m<sup>6</sup>A-seq dataset allowed the identification of METTL16-dependent m<sup>6</sup>A modification sites that are directly bound by METTL16, strongly suggesting that these modifications are introduced by METTL16. Together, the identification of METTL16 as an m<sup>6</sup>A methyltransferase that targets a range of RNA substrates provides important insights into the installation of the m<sup>6</sup>A landscape in different classes of RNA. In the future, characterization of the METTL16-mediated m<sup>6</sup>A modifications will lead to understanding their functional roles and how different their modes of actions are from the m<sup>6</sup>A modifications installed by the METTL3-METTL14 complex.

The identification of METTL16 as a second active m<sup>6</sup>A methyltransferase in human cells raises the question of why multiple m<sup>6</sup>A writer proteins are required. The presence of more than one m<sup>6</sup>A writer may allow recognition of different modification substrates. In contrast to the METTL3-METTL14 complex, which recognizes the RRACH sequence motif in its substrate RNAs, the sequence and/or structural elements that allow METTL16 to recognize its targets remain unknown. Interestingly, the UACAGAGAA sequence is methylated by METTL16 in both the U6 snRNA and the MAT2A pre-mRNA (Pendleton *et al.*, 2017). However, this sequence is absent from other METTL16 targets, showing that it is not the only recognition element for METTL16. Therefore, it is more likely that METTL16 recognizes secondary structures, which is in line with the binding of METTL16 to hairpin 1 in the MAT2A pre-mRNA and to the highly structured U6 snRNA as well as other structured (l)ncRNAs, e.g. MALAT1, vault RNAs, Y-RNAs, etc. Crystal structures of METTL16 with different RNA binding partners may provide insights into the required elements for substrate recognition. Another possible explanation for the need for multiple m<sup>6</sup>A writers is that m<sup>6</sup>A modifications that are installed by one writer on specific RNAs need to be co-regulated during a certain cellular response and this is achieved by

modulating the activity of the corresponding writer protein. For example, the METTL3-METTL14 complex installs m<sup>6</sup>A modifications on pluripotency-promoting transcripts, such as mRNAs encoding NANOG and other Yamanaka factors, reducing their stability during differentiation of embryonic stem cells (Geula *et al.*, 2015). Interestingly, the presence of known m<sup>6</sup>As that do not appear to be installed by either the METTL3-METTL14 complex or METTL16, such as in the 28S rRNA (Linder *et al.*, 2015), raises the possibility that more m<sup>6</sup>A writers remain to be identified in human cells. The speculation that more m<sup>6</sup>A writers exist in human cells is also supported by the detection of m<sup>6</sup>A modifications in mitochondrial RNAs (Sun *et al.*, 2016), where neither of the known m<sup>6</sup>A writers has so far been reported to localise.

#### **4.2 Characterization of the putative m<sup>5</sup>C methyltransferases NSUN6 and NSUN3**

Similar to m<sup>6</sup>A, 5-methylcytosine (m<sup>5</sup>C) is an evolutionarily conserved modification that is present in RNAs from the three domains of life (reviewed in Motorin *et al.*, 2010). Recently, transcriptome-wide studies have suggested that m<sup>5</sup>C modifications are widespread in mRNAs as well as several classes of non-coding RNA, including tRNAs and rRNAs (Squires *et al.*, 2012; Edelheit *et al.*, 2013). In these RNAs, m<sup>5</sup>C has been shown to play important roles, including modulating RNA structural stability, translation, RNA processing and degradation (see for example, Blanco *et al.*, 2016; Alexandrov *et al.*, 2006; Tuorto *et al.*, 2012). Excitingly, m<sup>5</sup>C modifications in mRNA have been recently shown to be specifically recognised by a reader protein, ALYREF, which can specifically bind the modification and influence export of m<sup>5</sup>C-modified mRNAs to the cytoplasm (Yang *et al.*, 2017).

m<sup>5</sup>C modifications in RNA can be installed by DNMT2 or by one of the members of the Nol1/Nop2/SUN domain (NSUN) family (Goll *et al.*, 2006). The human genome encodes seven NSUN proteins (NSUN1-7), which can target distinct classes of RNA in different cellular compartments (Chi and Delgado-Olguin, 2013). Examples include modification of mRNA (NSUN2; Hussain *et al.*, 2013), tRNA (NSUN2; Schaefer *et al.*, 2010) or rRNA in the cytoplasm (NSUN1, NSUN5; Sloan *et al.*, 2013; Schrosser *et al.*, 2015) or in the mitochondria (NSUN4; Metodiev *et al.*, 2014). Furthermore, NSUN7, which is highly expressed in testes, has been suggested to install m<sup>5</sup>C modifications in enhancer RNAs and affect their stability (Chi and Delgado-Olguin, 2013; Aguilo *et al.*, 2016). Here, we identified the RNA substrates of NSUN6 and NSUN3, providing insights into their cellular functions.

Transcriptome-wide mapping of NSUN6 binding sites *in vivo* using UV CRAC revealed binding to tRNA<sup>Cys</sup> and tRNA<sup>Thr</sup>. Interestingly, CRAC using 5-azacytidine as a crosslinking reagent (5-aza CRAC), which traps m<sup>5</sup>C methyltransferases on their RNA substrates in a

covalent intermediate that is formed during the methylation reaction (Khoddami and Carins, 2013), showed binding of NSUN6 to the same tRNAs, demonstrating that NSUN6 is an m<sup>5</sup>C tRNA methyltransferase that modifies these tRNA. This finding was confirmed *in vitro* using methylation assays (Haag *et al.*, 2017), and these assays further revealed that NSUN6 methylates cytosine C72 at the 3' end of the acceptor stems of tRNA<sup>Cys</sup> and tRNA<sup>Thr</sup>. This was in line with the CRAC data, where most of the sequence reads in the NSUN6 sample map to the 3' ends of these two tRNAs, and a transcriptome-wide mapping study that showed the presence of an m<sup>5</sup>C modification at this nucleotide in tRNA<sup>Thr</sup> *in vivo* (Squires *et al.*, 2012). Although the precise functions of the NSUN6-mediated m<sup>5</sup>C modifications remain unknown, based on their presence at the 3' acceptor stems of the tRNAs, it is tempting to speculate that the m<sup>5</sup>C72 modifications may affect the aminoacylation of the tRNAs and therefore their function in translation.

Modifications can be introduced at various stages during the maturation of tRNAs. For example, the nucleolar localization of NSUN2 suggests that it methylates the substrate tRNAs at early stages in their maturation, likely co-transcriptionally (Frye and Watt, 2006). In contrast, the cytoplasmic localization of NSUN6 suggests that it modifies its substrate tRNAs at a late step in their biogenesis. Interestingly, we observed that NSUN6, unlike most other tRNA-modifying enzymes, co-localizes with the pericentriolar matrix (PCM) and the *cis*-golgi. Although there is no evidence that tRNAs function at the Golgi apparatus and the association with the Golgi apparatus is not a typical step of tRNA biogenesis, such tethering of proteins to cytoplasmic structures might play a role in preventing them from entering the nucleus by diffusion or while the nuclear envelope is dis-/re-assembled during mitosis (Wang *et al.*, 2000). The cytoplasmic localization of NSUN6 likely ensures that NSUN6 only modifies its tRNA substrates after their nuclear maturation but since NSUN6 can methylate unmodified tRNA<sup>Cys</sup> and tRNA<sup>Thr</sup> transcripts *in vitro*, it is unlikely that other nucleotide modifications installed on these tRNAs during the early stages of their maturation are a pre-requisite for NSUN6-mediated m<sup>5</sup>C72 modification.

In contrast to NSUN6, which methylates cytoplasmic tRNAs, NSUN3 CRAC results showed an association of NSUN3 with mitochondrial tRNAs, which is in line with the localization of NSUN3 in the mitochondrial matrix. Detailed analysis of the CRAC data showed the association of NSUN3 with the mitochondrial (mt-)tRNA<sup>Met</sup>. Furthermore, we demonstrated *in vivo* and *in vitro* that NSUN3 installs an m<sup>5</sup>C at cytosine 34, the wobble position, of mt-tRNA<sup>Met</sup>. However, this position was previously reported to contain a 5-formylcytosine (f<sup>5</sup>C; Moriya *et al.*, 1994), therefore we hypothesized that m<sup>5</sup>C34 installed by NSUN3 can be further oxidized by a specific dioxygenase to form f<sup>5</sup>C34. We focused on the AlkB-like Fe(II)/ alpha-ketoglutarate-dependent dioxygenases (ALKBH) as candidates for catalysing such a reaction, since members of this protein family have been

previously reported to perform oxidative reactions on RNA modifications (reviewed in Shen *et al.*, 2014). Interestingly, we found that ALKBH1/ABH1, similar to NSUN3, is localized to the mitochondrial matrix and binds mt-tRNA<sup>Met</sup>, suggesting that it could perform such activity. Indeed, oxidation assays have revealed that ABH1 oxidises m<sup>5</sup>C34, generating f<sup>5</sup>C34 in mt-tRNA<sup>Met</sup>. Using bisulfite sequencing, we showed that C34 in mt-tRNA<sup>Met</sup> is almost fully modified *in vivo*, and although the majority of the modification is f<sup>5</sup>C, in line with previous reports of f<sup>5</sup>C34 in mt-tRNA<sup>Met</sup> (Moriya *et al.*, 1994), a portion of cytosines appear to carry m<sup>5</sup>C modifications introduced by NSUN3. This is consistent with results of a parallel study that reported the presence of an m<sup>5</sup>C modification at position 34 of mt-tRNA<sup>Met</sup> *in vivo* (Van Haute *et al.*, 2016). However, we cannot exclude that the detection of m<sup>5</sup>C34 may result from partial reduction of f<sup>5</sup>C34 during the bisulfite sequencing protocol. Moreover, it remains unclear if m<sup>5</sup>C34 is just an intermediate during the formation of f<sup>5</sup>C34 or it has a cellular function itself. However, our finding that m<sup>5</sup>C34-containing mt-tRNA<sup>Met</sup> binds AUG and AUA codons in the P site stronger than mt-tRNAs<sup>Met</sup> containing different modification states of C34, suggests a potential role for m<sup>5</sup>C34 in codon recognition by mt-tRNA<sup>Met</sup>. In DNA, oxidation of m<sup>5</sup>C by TET enzymes can form 5-hydroxymethylcytosine (hm5C) as an intermediate product (Ito *et al.*, 2011). Recently, it was found that ABH1 first hydroxylates m<sup>5</sup>C34 to form hm<sup>5</sup>C34, and then oxidizes hm<sup>5</sup>C34 generating f<sup>5</sup>C34 in mt-tRNA<sup>Met</sup> (Kawarada *et al.*, 2017). However, in our *in vitro* assays, the conversion of m<sup>5</sup>C34 to f<sup>5</sup>C34 was highly efficient and the presence of hm<sup>5</sup>C34 as an intermediate was not significantly detected. Together with our finding that hm<sup>5</sup>C34 does not show a significant effect in the codon recognition experiments, it seems unlikely that hm<sup>5</sup>C34 plays an important function on mt-tRNA<sup>Met</sup>.

Human mitochondria contain their own genome, which encodes 13 mRNAs that are translated by mitochondrial ribosomes to form the essential subunits of the oxidative phosphorylation complex (OXPHOS; reviewed in Powell *et al.*, 2015). The mitochondrial protein synthesis machinery uses a minimalistic set of 22 mt-tRNAs, which are responsible for decoding 60 sense codons. Therefore, mitochondria use a non-conventional genetic code, where all mt-tRNAs recognise at least two different codons (reviewed in Suzuki & Suzuki, 2014). The expanded decoding capacity of several mt-tRNAs has been attributed to chemical modifications in the wobble position (reviewed in Bohnsack and Sloan, 2017). For example, the 5-taurinomethyluridine at the wobble position of mt-tRNA<sup>Trp</sup> enables UGA codons, which are normally read as stop codons during cytoplasmic translation, to encode Tryptophan (Suzuki *et al.*, 2002). Similarly, f<sup>5</sup>C34, which is installed by the consecutive action of NSUN3 and ABH1, has been suggested to expand the codon recognition capacity of mt-tRNA<sup>Met</sup> (Moriya *et al.*, 1994). In contrast to cytoplasmic translation, where the initiator tRNA<sub>i</sub><sup>Met</sup> decodes methionine during

translation initiation and the elongator tRNA<sub>e</sub><sup>Met</sup> decodes methionine during translation elongation, mitochondria contain only one mt-tRNA<sup>Met</sup> that functions during both initiation and elongation (Takeuchi *et al.*, 2001). In addition to decoding the standard AUG codon for the incorporation of methionine, mt-tRNA<sup>Met</sup> can decode AUA codons during translation initiation and elongation and AUU codons during the initiation of NADH dehydrogenase 2 (ND2) translation (Tekamoto *et al.*, 1995). f<sup>5</sup>C34 was shown to allow mt-tRNA<sup>Met</sup> to decode AUA codons, as well as AUG codons, as it is able to pair with both A and G at the third position of the codon (Bilbille *et al.*, 2011; Cantara *et al.*, 2013). Consistent with this, our *in vitro* codon recognition experiments with mitochondrial translation factors demonstrate that the different modification states of C34 in mt-tRNA<sup>Met</sup> can influence the ability of the tRNA to decode multiple codons during translation initiation and elongation, suggesting that the modification plays a role in mitochondrial translation. Indeed, we found that the depletion of NSUN3 or ABH1 leads to a general reduction in translation of all mitochondrial proteins, in line with the presence of AUA codons encoding methionine in all of the mitochondrial mRNAs. This is further supported by the findings that oxidative phosphorylation and respiratory coupling of mitochondria are impaired in NSUN3 knockout cells (Nakano *et al.*, 2016), and that depletion of NSUN3 or ABH1 reduces cellular growth. Together, our data describe the biosynthetic pathway of installing f<sup>5</sup>C34 in the wobble position of mt-tRNA<sup>Met</sup> by NSUN3 and ABH1, and show the importance of the modification in expanding the decoding capacity of the tRNA, which is essential for the mitochondrial translation machinery.

Taken together with the previously described functions of NSUN1, NSUN2, NSUN4 and NSUN5 in modification of cytoplasmic and mitochondrial rRNAs and cytoplasmic tRNAs, these data show that members of the NSUN protein family of m<sup>5</sup>C methyltransferases target diverse cellular RNAs. Therefore, an interesting question is how these different proteins recognize their specific substrates in the complex cellular environment. NSUN proteins can localize in different subcellular compartments via localization signals in their sequences or via recruitment into a complex. For example, NSUN1 contains a nuclear targeting signal that was shown to be required for its nuclear and nucleolar localization (Valdez *et al.*, 1994) and NSUN4 was shown to form a complex with MTERF4, which is required for its recruitment to the mitochondrial large ribosomal subunit (Spahr *et al.*, 2012). The recruitment of NSUN proteins to their localization sites may allow their presence in the vicinity of their substrates or regulate their sequence of action in conjunction with other proteins. For example, the tethering of NSUN6 to cytoplasmic structures may facilitate the modification of tRNAs after their export to the cytoplasm. Based on its sequence and prediction of its domain structure, NSUN6 contains an N-terminal pseudouridine synthase and archaeosine transglycosylase (PUA) domain and C-



terminal methyltransferase domain that includes an RNA recognition motif (RRM) and the catalytic core. In our study, we showed that NSUN6 methylates C72 at the 3' end of the acceptor stems of tRNA<sup>Cys</sup> and tRNA<sup>Thr</sup> and is exclusively recruited to tRNAs that contain a cytosine at position 72. Consistent with this, a recently published structure of NSUN6 revealed extensive interactions between C72 and the catalytic core of NSUN6 (Liu *et al.*, 2017). Interestingly, we found that the CCA tail of the substrate tRNA is essential for recognition by NSUN6, as deletion or exchange of one of the three nucleotides abolishes the methylation reaction. This is consistent with the finding that the surface of the PUA domain of NSUN6 precisely recognizes the CCA tail of the substrate tRNAs (Liu *et al.*, 2017). Binding of NSUN6 to CCA-tailed tRNA substrates might represent a quality control mechanism ensuring that the m<sup>5</sup>C72 modification is installed on tRNA substrates that contain an appropriate 3' end and therefore will be functional. However, the observation that NSUN6 only methylates a subset of CCA-containing tRNAs indicates that the CCA-tail and C72 are not sufficient for NSUN6 to recognize its substrate tRNAs. Indeed, mutational analysis has revealed more recognition elements including U73, which acts as a discriminator base that directly binds the RRM motif, and more distant nucleotides in the D stem that form extensive electrostatic interactions with the PUA domain (Long *et al.*, 2016; Liu *et al.*, 2017). Consistent with this, we found that NSUN6 does not methylate the C72-containing tRNA<sup>Arg</sup>, which lacks the discriminator base U73. Taken together, NSUN6 requires a delicate network for recognition of tRNA substrates, involving both primary sequence elements and tertiary structural features. Recently, NSUN6 was found to form a complex with LLGL2 and the lncRNA MAYA for the methylation of Hippo/MST at lysine 59 (Li *et al.*, 2017). Based on the structure of NSUN6, it is likely that these interactions are mediated by the RNA-binding PUA domain (Liu *et al.*, 2017).

In our study of NSUN3, we gain insight into how the protein recognizes tRNA<sup>Met</sup> in order to methylate C34 in the anticodon loop. We observed that mutation of the neighbouring cytosines C33 and C32 reduces the extent of methylation of C34 by NSUN3 *in vitro*, suggesting that these residues might contribute to the binding or methylation by NSUN3. Furthermore, mutation of C39, which base pairs with G31 at the base of the anticodon stem, abolished NSUN3-mediated methylation of C34, suggesting that NSUN3 might recognise the anticodon stem loop (ASL) of mt-tRNA<sup>Met</sup>. Interestingly, swapping the nucleotides at positions 31 and 39 of the anticodon stem, which results in a stable stem structure but a different sequence, did not affect C34 methylation, further supporting that NSUN3 requires the ASL for substrate recognition. Furthermore, NSUN3 can methylate a chemically synthesized ASL, indicating that the ASL is sufficient for recognition and methylation by NSUN3. This is in contrast to NSUN6, which requires a correctly folded, full-length tRNA as a substrate (Long *et al.*, 2016).

The NSUN3-mediated methylation of C34 of mt-tRNA<sup>Met</sup> is a pre-requisite for subsequent oxidation by ABH1. Interestingly, ABH1 was previously reported to recognize different substrates other than mt-tRNA<sup>Met</sup>. For example, ABH1 can demethylate 3-methylcytosine (m<sup>3</sup>C) in single-stranded RNA and DNA *in vitro* (Westbye *et al.*, 2008), and is suggested to act as a histone demethylase and a DNA lyase at abasic sites (Müller *et al.*, 2010; Ougland *et al.*, 2012). Moreover, ABH1 was recently suggested to demethylate m<sup>1</sup>A at position 58 in a subset of cytoplasmic tRNAs (Liu *et al.*, 2017). Although structural information on how ABH1 recognizes its substrates is lacking, it is tempting to speculate that ABH1 might recognize structural elements that are shared between its diverse substrates. It is possible that different protein co-factors recruit ABH1 to its target sites, in a similar way to the rRNA/tRNA acetyltransferase NAT10, which requires the adaptor protein THUMP1 for its role in tRNA modification (Sharma *et al.*, 2015) but is targeted to its modification sites in the 18S rRNA by snoRNAs (Sharma *et al.*, 2017).

Aberrant expression or mutations of several NSUN proteins have been linked to human diseases. NSUN5 is among the completely deleted genes in Williams-Beuren syndrome (Merla *et al.*, 2002). Moreover, mutations in NSUN2 cause intellectual disability (Khan *et al.*, 2012; Abbasi-Moheb *et al.*, 2012) and mutations in NSUN7 are associated with reduced sperm motility and infertility (Harris *et al.*, 2007; Khosronezhad *et al.*, 2015). Recently, most of the disease-related mutations in NSUN proteins were suggested to impair m<sup>5</sup>C methylation of RNA, implying the direct association between defects of m<sup>5</sup>C modifications and these diseases (Liu *et al.*, 2017). In contrast, NSUN6 was reported to play a role in bone metastasis through the methylation of the Hippo/MST1 protein complex and the activation of YAP in tumour cells, showing that NSUN proteins could also play a role in human diseases through other functions than m<sup>5</sup>C modification of RNA (Li *et al.*, 2017).

In mitochondria, point mutations in mt-tRNAs can interfere with the installation of modifications and cause diseases (see for example, Brule *et al.*, 1999; Yasukawa *et al.*, 2005; Yarham *et al.*, 2014). To date, eight pathogenic mutations have been reported in mt-tRNA<sup>Met</sup> and suggested to lead to mitochondrial disorders that manifest with a broad range of symptoms (Lott *et al.*, 2013). Interestingly, we found that one of these pathogenic mutations in mt-tRNA<sup>Met</sup> (C39U), which destabilizes the ASL structure, abolishes the methylation at C34 due to disruption of the ASL. Moreover, another pathogenic mutation in mt-tRNA<sup>Met</sup>, A37G, was shown to abolish the methylation by NSUN3, further supporting that defects in modification at C34 and consequently, mitochondrial translation and function are the underlying cause of these diseases (Nakano *et al.*, 2016). Consistent with this, a patient with mitochondrial disease symptoms was recently shown to lack f<sup>5</sup>C modification of C34 of mt-tRNA<sup>Met</sup> (Van Haute *et al.*, 2016). Furthermore, exome

sequencing of DNA derived from patient cells identified loss-of-function variants of the *NSUN3* gene and that lead to a lack of functional NSUN3 protein. Taken together, lack of the modifications installed by the NSUN proteins can lead to diseases, and our data provide insights into the basic mechanistic aspects of these modifications, which might help in explaining the molecular mechanisms of these diseases.

### **4.3 Understanding the molecular basis of Bowen-Conradi syndrome**

A growing number of human disorders have been linked to ribosome dysfunction. Such disorders, which are known as 'ribosomopathies', are generally characterized by developmental defects, haematological dysfunction, craniofacial anomalies, and increased incidence of cancer (reviewed in Narla and Erbert, 2010). Although many of the mutations in ribosomal proteins or biogenesis factors that cause these diseases have been identified, the precise functions of these factors in human ribosome assembly often remain uncharacterized and moreover, the molecular basis of most of these diseases are not thoroughly understood. Bowen-Conradi syndrome (BCS) is a severe autosomal recessive disorder that is characterized by bone marrow failure, craniofacial abnormalities and early infant death (Lowry *et al.*, 2003). BCS is caused by a point mutation in the gene encoding the rRNA methyltransferase EMG1, leading to an aspartate to glycine exchange at position 86 (D86G; Armistead *et al.*, 2009). In yeast, Emg1 is an essential nucleolar protein that plays a role in the maturation of the small ribosomal subunit, where it participates in the unique hypermodification of uridine 1191 of the 18S rRNA (Meyer *et al.*, 2011). The hypermodification begins with the isomerization of uridine 1191 to pseudouridine ( $\Psi$ ) by the action of the H/ACA box snoRNP snR35, which allows the subsequent  $N^1$  methylation by Emg1 (Wurm *et al.*, 2010). Recently, the cytoplasmic enzyme Tsr3 was shown to catalyse the addition of a 3-amino-3-carboxypropyl (acp) moiety, generating the  $N^1$ -methyl- $N^3$ -aminocarboxypropylpseudouridine ( $m^1acp^3\Psi$ ) modification (Meyer *et al.*, 2016). Although the presence of Emg1 within its binding site in pre-ribosomal complexes is required for small ribosomal subunit biogenesis, the methyltransferase activity was shown to be not essential (Meyer *et al.*, 2011).

While the functions of Emg1 in biogenesis of the small ribosomal subunit are relatively well understood, characterizing the effects of the BCS mutation in EMG1 on its function was necessary in order to understand the molecular basis of the syndrome and thereby, potentially enable the development of therapeutic agents to treat this disorder in the future. We discovered that the BCS mutation destabilizes the EMG1 protein, which is consistent with the previous finding that EMG1 protein levels are dramatically reduced in BCS patient fibroblasts (Armistead *et al.*, 2009). Structural analysis of EMG1 suggests that the destabilization of EMG1<sub>D86G</sub> may be explained by the high conformational

flexibility of glycine compared to aspartate, which likely mitigates the stabilizing effect of proline 85 in EMG1, or that the BCS mutation disrupts a stabilizing salt-bridge between aspartate 86 and arginine 84 (Leulliot *et al.*, 2008; Armistead *et al.*, 2009). Interestingly, the reduced levels of EMG1<sub>D86G</sub> could be rescued by inhibiting the proteasome, implying that EMG1<sub>D86G</sub> is largely degraded in BCS cells.

In line with a conserved role for Emg1 in the biogenesis of the small ribosomal subunit (Liu and Thiele, 2001), the reduced expression of EMG1<sub>D86G</sub> compared to EMG1 lead to defects in maturation of the 18S rRNA. This is consistent with the finding that the rate of the 18S rRNA processing was reduced in BCS patient cells (Armistead *et al.*, 2014). Interestingly, overexpression of EMG1<sub>D86G</sub> to protein levels that were comparable to the endogenous levels of wildtype EMG1 could rescue the pre-rRNA processing defects, implying that the BCS mutation in EMG1 does not directly impair the function of EMG1 in ribosome biogenesis, but the reduced EMG1<sub>D86G</sub> protein levels cause the small ribosomal subunit biogenesis defects. Consistent with this, our *in vitro* methylation assays demonstrated that EMG1<sub>D86G</sub> still has methylation activity, implying that the lack of the m1acpΨ1248 modification is not the main reason for the molecular defect observed in BCS.

In order to obtain further insights into the effects of the BCS mutation, we monitored the localization of EMG1<sub>D86G</sub>. In contrast to the nucleolar localization of wildtype EMG1, only a portion of EMG1<sub>D86G</sub> is recruited to the nucleolus, whereas the majority of the protein forms foci in the nucleoplasm. Together with the previously observed increased insolubility and dimerization of EMG1<sub>D86G</sub> (Armistead *et al.*, 2009; Meyer *et al.*, 2011), these nuclear foci likely present protein aggregates. The mislocalization of EMG1<sub>D86G</sub> to the nucleoplasm suggested that the mutation prevents its specific recruitment to the nucleolus, leading to destabilization. Our finding that a sub-complex containing NOP14, NOC4L and UTP14A recruits EMG1 to the nucleolus allowed us to address whether the nucleolar fraction of EMG1<sub>D86G</sub> is specifically recruited to the nucleolus or formed by non-specific accumulation of the protein. In line with the observations that EMG1<sub>D86G</sub> can perform its function in ribosome biogenesis and methylate its substrate residue, our data showed that the nucleolar fraction EMG1<sub>D86G</sub> is recruited to the nucleolus by its interaction partners, implying that it the BCS mutation does not directly affect nucleolar recruitment of EMG1.

The presence of EMG1<sub>D86G</sub> in foci in the nucleoplasm raised the question of how EMG1 is imported to the nucleus after its translation in the cytoplasm and why it does not aggregate in the cytoplasm or during import. The import of proteins to the nucleus occurs via nuclear pore complexes (NPCs), which contain, in their central channels, numerous nucleoporins possessing phenylalanine-glycine-rich repeats (FG-repeats) that constitute a permeability barrier (Frey *et al.*, 2006; Frey and Görlich, 2007). In the cytoplasm, where

the concentration of RanGTP is low, cargo proteins form complexes with import receptors (importins), which bridge interactions between cargo proteins and the FG-repeat meshwork to facilitate the complex to be translocated across the NPC (reviewed in Schmidt and Görlich, 2016). In the nucleus, the high levels of RanGTP disassemble the nuclear import complex and release the cargo proteins (reviewed in Görlich, 1998; Görlich and Kutay, 1999). We found that the nuclear import of EMG1 can be mediated by Importin (IMP) $\alpha/\beta$  or by the IMP $\beta/7$  heterodimer. Besides the primary function of import receptors in mediating nuclear import of proteins, they can also chaperone basic proteins, such as RNA binding proteins or histones, by shielding their basic regions and preventing their multivalent interactions with polyanions in the cytoplasm that could otherwise lead to aggregation (Jäkel *et al.*, 2002; Bäuerle *et al.*, 2002). The presence of a basic patch on the surface of Emg1, within which the BCS mutation lies, lead us to hypothesize that IMP $\alpha/\beta$  and/or the IMP $\beta/7$  heterodimer might chaperone EMG1 and EMG1<sub>D86G</sub> in the cytoplasm (Leulliot *et al.*, 2008; Taylor *et al.*, 2008; Thomas *et al.*, 2010). Indeed, we found that the IMP $\beta/7$  heterodimer chaperones EMG1 and EMG1<sub>D86G</sub> efficiently, suggesting that it represents the physiological nuclear import receptor that chaperones EMG1 and EMG1<sub>D86G</sub> in the cytoplasm. This is consistent with previous reports showing that IMP $\beta/7$  heterodimer can chaperone highly basic proteins, such as the histone H1 and the ribosomal proteins RPL4 (uL4) and RPL6 (eL6) (Jäkel *et al.*, 2002; Bäuerle *et al.*, 2002). Interestingly, it has been suggested that the nuclear disassembly of the cargo-IMP $\beta/7$  complex takes place in two successive steps: (i) binding of RanGTP to IMP $\beta$  and release of the cargo-IMP7 complex (ii) transfer of the cargo from the cargo-IMP7 complex to its binding site (Jäkel *et al.*, 1998, 1999). This suggests that IMP7 remains associated with the basic proteins in the nucleus, chaperoning them until they form specific interactions with their binding partners.

In addition to importins, a group of proteins (called “dedicated chaperones”) were recently shown to prevent ribosomal proteins from aggregation and degradation (reviewed in Pillet *et al.*, 2016). Since ribosomal proteins and ribosome biogenesis factors possess highly basic regions that make them prone to aggregation, it is likely that they are shielded already during their synthesis. Indeed, several ribosomal proteins, Rps3, Rpl3, Rpl4, Rpl5 and Rpl10 were shown to be bound by their dedicated chaperones co-translationally (Pausch *et al.*, 2015; Pillet *et al.*, 2015). Although the co-translational binding of importins to their substrates remains to be documented, it is tempting to speculate that EMG1 is likely chaperoned by the IMP $\beta/7$  heterodimer while it is being translated. Alternatively, it is possible that an additional cytoplasmic chaperone protects EMG1 from aggregation on the ribosome before it is captured by the Imp $\beta/7$  heterodimer. While importins chaperone basic proteins in the cytoplasm and dissociate from the import complex in the nucleus,

most dedicated chaperones accompany ribosomal proteins to their assembly sites on nucleolar pre-ribosomes (reviewed in Pillet *et al.*, 2016). It is likely that these basic proteins require to be shielded until they reach their final incorporation sites. Interestingly, importins can hand over the substrate proteins to dedicated chaperons in the nucleus. For example, Rps26 is chaperoned in the cytoplasm by its transport receptors (Kap123, Kap121/Pse1 and Kap104) and after it is imported to the nucleus, Rps26 is bound by the dedicated chaperone Tsr2, which displaces the protein from its transport receptors and facilitates its nucleolar recruitment and integration into pre-ribosomal complexes (Schütz *et al.*, 2014). It is possible that EMG1 is chaperoned in the nucleoplasm by its interaction partners (NOP14, NOC4L and UTP14A), which may form a complex with EMG1 that is then incorporated into the nucleolus. Alternatively, EMG1 can be chaperoned in the nucleus by IMP7, which may remain associated with EMG1 after the release of IMP $\beta$  by RanGTP. In BCS, it is possible that the residence of EMG1<sub>D86G</sub> with its import receptors in the nucleus is not sufficient to enable the protein to bind its specific interaction partners, which is in line with our observation that EMG<sub>D86G</sub> binds less than wildtype EMG1 to its interactions partners and is not fully recruited to the nucleolus. Collectively, our data suggest that EMG1<sub>D86G</sub> is chaperoned in the cytoplasm by the IMP $\beta$ /7 heterodimer, but after its import to the nucleus, the disassembly of the EMG1<sub>D86G</sub>-IMP $\beta$ /7 complex and the release of EMG1<sub>D86G</sub> leads to its aggregation and degradation. The reduced levels of EMG1<sub>D86G</sub> lead to defects in maturation of the small ribosomal subunit.

Impaired ribosome biogenesis results in the inhibition of the E3 ubiquitin ligase HDM2 by the 5S RNP, causing increased levels of the tumor suppressor p53 (Sloan *et al.*, 2013). Together with the observation that the knockdown of EMG1 in U2OS cells leads to increased expression of p53 (unpublished data, the Bohnsack lab), it is possible that p53 levels are elevated in BCS patients, leading to cell cycle arrest and apoptosis. This is supported by the previous finding that BCS patient fibroblasts accumulate in the G2/M transition of the cell cycle, resulting in reduced proliferation rates (Armistead *et al.*, 2014). Similar to other ribosomopathies (reviewed in Narla and Ebert, 2010), symptoms of BCS include bone marrow failure and bone abnormalities. These common symptoms may result from p53-mediated apoptosis of rapidly growing cells, such as erythroblasts and bone-forming cells, which are sensitive to impaired ribosome biogenesis. However, each ribosomopathy is clinically distinct. For example, some ribosomopathies such as X-linked Dyskeratosis Congenita (X-DC) lead to increased incidence of cancer, which is surprising considering the elevated p53 levels due to impaired ribosome biogenesis. One possible explanation is that defects in ribosome biogenesis may lead to the production of impaired ribosomes that have differential translation activities that promote tumorigenesis. For example, in X-DC, the produced ribosomes have defects in translating a subset of

mRNAs, including the mRNA encoding the tumor suppressor p27, leading to malignant transformation (Yoon *et al.*, 2006).

To date, bone marrow transplantation is the only definitive treatment for the severe haematological dysfunction in most ribosomopathies (Vlachos *et al.*, 2001). Based on our data, screening for a drug that prevents the degradation of EMG1<sub>D86G</sub>, for example, by targeting one of the ubiquitin ligases that specifically target EMG1<sub>D86G</sub> for proteasome-dependent degradation, may be one step towards developing a treatment for BCS. However, our data showed that EMG1<sub>D86G</sub> is prone to aggregation, therefore such a drug might lead to secondary pathogenic effects due to increased aggregated protein levels. Alternatively, developing a drug that prevents the misfolding and aggregation of EMG1<sub>D86G</sub> may be a better approach. This could be achieved, for example, by fluorescence microscopy-based screening for a compound that prevents the accumulation of EMG1<sub>D86G</sub> in nuclear foci. Taken together, our data provide insights into the molecular basis of BCS and raise the possibility of developing therapeutics for treatment of the syndrome.

#### **4.4 RNA modifications and RNA modification enzymes**

Interestingly, some modifications are widely spread in cellular RNAs while others are rare. For example, m<sup>6</sup>A and m<sup>5</sup>C modifications are found in different classes of RNA, whereas so far f<sup>5</sup>C has been only detected in the wobble position (f<sup>5</sup>C34) of mt-tRNA<sup>Met</sup> (Dominissini *et al.*, 2012; Squires *et al.*, 2012; Moriya *et al.*, 1994). This is likely due to the diverse roles that individual modifications have on RNAs. For example, m<sup>6</sup>A modifications influence RNA secondary structure by destabilizing RNA duplexes or by promoting base stacking and the stability of single-stranded RNAs (Roost *et al.*, 2015). These RNA-stabilizing effects are likely required in different contexts for the functions of RNAs and RNA-protein complexes. Similarly, the topological effects of m<sup>5</sup>C modifications, such as base stacking and stability of RNA structures, are likely required in multiple cellular RNAs (Motorin *et al.*, 2010). In contrast, f<sup>5</sup>C was shown to allow base pairing of cytosine to adenine and guanine, which is a specific feature that enables mt-tRNA<sup>Met</sup> to decode AUG and AUA codons, therefore f<sup>5</sup>C appears to be a rare modification in cellular RNAs (Bilbille *et al.*, 2011). Similarly, although the exact function of the m<sup>1</sup>acp<sup>3</sup>Ψ modification is not known, it is likely that the particular chemical effect from this chemically complex modification is only required in limited contexts.

In a similar way, some RNA modification enzymes modify only a limited number of targets while other enzymes have multiple substrates. For example, only one site (U1248 of the 18S rRNA) is suggested to be modified by EMG1 (Meyer *et al.*, 2011), whereas our data indicated that METTL16 introduces m<sup>6</sup>A modifications in numerous RNA substrates. Furthermore, the METTL3-METTL14 complex was shown to install m<sup>6</sup>A modifications in

even more RNA substrates than METTL16 (Liu *et al.*, 2014). This may reflect co-regulation between m<sup>6</sup>A modifications on different substrates. Grouping of certain mRNA transcripts, for example, for a certain process such as translation or decay can be important for regulation of cellular events including the cell cycle, the circadian rhythm, differentiation and development (reviewed in Zhao *et al.*, 2016). Furthermore, some RNA modification enzymes can act on more than one type of modification substrate. Interestingly, although the f<sup>5</sup>C modification generated on mt-tRNA<sup>Met</sup> by ABH1 is rare, ABH1 was also reported to demethylate m<sup>1</sup>A modifications in several cytoplasmic tRNAs and to demethylate m<sup>3</sup>C modifications in single-stranded RNA and DNA, and to act as a DNA lyase at abasic sites (Liu *et al.*, 2016; Westbye *et al.*, 2008; Müller *et al.*, 2010; Ougland *et al.*, 2012). Moreover, our data indicate that NSUN6 methylates cytoplasmic tRNAs and recently, NSUN6 was shown to be involved in methylation of Hippo/ MST (Li *et al.*, 2017). The ability of RNA modification enzymes to modify more than one type of substrate may represent a mechanism for cross-regulation of these substrates and their action in certain cellular pathways.

Besides their modification functions, some enzymes can also perform additional functions in the cell. For example, Emg1 was shown to have an essential assembly role in the biogenesis of the small ribosomal subunit, in addition to its function in the hypermodification of the 18S rRNA (Meyer *et al.*, 2016). Interestingly, METTL16 was shown to promote splicing of MAT2A pre-mRNA, but in a methylation independent manner (Pendleton *et al.*, 2017). Together with our model that the METTL16-mediated m<sup>6</sup>A43 in the U6 snRNA has a role in splicing, this suggests that METTL16 may regulate pre-mRNA splicing in methylation dependent and independent ways. The ability of RNA modification enzymes to perform additional functions may represent a means to coordinate RNA modification with other aspects of RNA/RNP biogenesis or function. In the case where RNA modifications are essential for the function of RNAs, this could be a means of quality control to ensure that only correctly modified, mature RNAs are produced.

An emerging concept in the RNA modifications field is the presence of residues that are partially/ substoichiometrically modified (reviewed in Sloan *et al.*, 2017; Roundtree *et al.*, 2017). In the context of rRNA, partially modified residues were proposed to fine-tune translation, whereas constitutively modified residues, which are present in functionally important regions, are essential for rRNA folding and the assembly of ribosomes (Krogh *et al.*, 2016). The m<sup>1</sup>acp<sup>3</sup>Ψ modification, where EMG1 functions, was found to be fully modified in yeast (Taoka *et al.*, 2016), suggesting that the modification has an important function on the ribosome. This is in line with the finding that depletion of snR35, which installs the first step of the modification, leads to ribosome biogenesis defects (Liang *et al.*,



2009). Similarly, our data indicated that C34 of mt-tRNA<sup>Met</sup> is almost fully modified by NSUN3 and ABH1 *in vivo*. This is supported by our finding that NSUN3 and ABH1 are required for mitochondrial translation by expanding codon recognition by mt-tRNA<sup>Met</sup>. Similarly, and consistent with our model that METTL16 installs m<sup>6</sup>A43 in an essential conserved sequence in the U6 snRNA, it is likely that m<sup>6</sup>A43 is not dynamically regulated. In contrast, it is possible that the intronic m<sup>6</sup>A modifications installed by METTL16 on pre-mRNA introns are dynamically regulated, in line with previous findings that many modifications, such as m<sup>6</sup>A, m<sup>1</sup>A and  $\psi$ , occur at substoichiometric levels on mRNAs (Zheng *et al.*, 2013; Dominissini *et al.*, 2016; Carlile *et al.*, 2014).

RNA modifications can be regulated according to environmental signals such as stress signals and the metabolic status of the cell. For example, in yeast, oxidative stress was shown to cause a change in the levels of m<sup>5</sup>C, 2'-O-methylcytosine, N<sup>2</sup>,N<sup>2</sup>-dimethylguanosine and t<sup>6</sup>A modifications in tRNAs (Chan *et al.*, 2010). Consistent with this, m<sup>5</sup>C methylation of tRNA<sup>Leu(CAA)</sup> was later shown to facilitate the translation of a TTG-rich mRNA that encodes RPL22A, which is suggested to be involved in oxidative stress response (Chan *et al.*, 2012). Recently, mRNA modifications were suggested to be dynamically regulated by demethylases in response to cellular stresses. For example, UV-induced DNA damage was shown to regulate m<sup>6</sup>A levels on mRNAs by the METTL3-METTL14 methyltransferase complex and the demethylase ALKBH5 (Xiang *et al.*, 2017). However, response to environmental stresses may be only one side of RNA modifications dynamics. Many RNA modification enzymes require cofactors or utilise chemical moieties that are interconnected with, or produced during metabolic pathways, suggesting that RNA modifications may be adjusted according to the availability of metabolites and play roles in maintaining cell homeostasis (reviewed in Helm and Alfonzo, 2004). For example, the biosynthesis of i<sup>6</sup>A, which is present at position 37 of several tRNAs, requires DMAPP (dimethylallylpyrophosphate). DMAPP is derived from acetyl-CoA, which may be derived for example from glycolysis via pyruvate, suggesting a link between the metabolic status and the function of t<sup>6</sup>A in promoting translation efficiency (Benko *et al.*, 2000; Thiaville *et al.*, 2016). We have shown that the oxidation of m<sup>5</sup>C34 to f<sup>5</sup>C34 in mt-tRNA<sup>Met</sup> is catalysed by ABH1, which requires alpha ( $\alpha$ )-ketoglutarate as a cofactor, that itself is an intermediate of the citric acid cycle (CAC; reviewed in Akram *et al.*, 2014).  $\alpha$ -ketoglutarate may signal that the CAC is active and that enough NADH and succinate are produced and can be utilized in oxidative phosphorylation (OXPHOS). The  $\alpha$ -ketoglutarate-mediated activation of ABH1 is required for efficient translation of mitochondrial proteins, which form the OXPHOS complexes that utilize NADH and succinate for the production of the majority of cellular energy (reviewed in Dennerlein *et al.*, 2017).

Taken together, RNA modifications are integral for the functionality of core aspects of the gene expression machinery, as they can be specifically regulated in different cellular conditions and can play major roles in regulation of gene expression for the maintenance of cell homeostasis.

## Chapter Five: Bibliography

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