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Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to by Roman Numerals I–III.

I. **Brūmele, B**., Mutso, M., Telanne, L., Õunap, K., Spunde, K., Abroi, A., & Kurg, R. (2021). Human TRMT112-Methyltransferase Network Consists of Seven Partners Interacting with a Common Co-Factor. International Journal of Molecular Sciences, 22(24), 13593. https://doi.org/10.3390/ijms222413593

II. **Brūmele, B**., Serova, E., Lupp, A., Suija, M., Mutso, M., & Kurg, R. (2024). Cross-Reactivity of N6AMT1 Antibodies with Aurora Kinase A: An Example of Antibody-Specific Non-Specificity. Antibodies (Basel, Switzerland), 13(2), 33. https://doi.org/10.3390/antib13020033

III. Mutso, M., **Brūmele, B**., Serova, E., Väärtnõu, F., Suija, M., & Kurg, R. (2024). The methyltransferase N6AMT1 participates in the cell cycle by regulating cyclin E levels. PloS one, 19(2), e0298884. https://doi.org/10.1371/journal.pone.0298884

The author's contributions to each article are as follows:

I. The author is the main author of this article, having done a large proportion of the experiments and contributed to data analysis and writing of the manuscript.

II. The author is the main author of this article and was responsible for the experimental design, most of the experiments, as well as data analysis and writing of the manuscript.

III. The author is the second author of this article, having done a large proportion of the experiments and contributed to data analysis and writing of the manuscript.

ABBREVIATIONS

(S)mchm^{5}U	–	5-methoxycarbonylmethyl-2′-O-methyluridine
acetyl-CoA	–	acetyl-coenzyme A
AMD	–	age-related macular degeneration
APC/CCDC20	–	anaphase-promoting complex/cyclosome and cell division cycle 20 complex
aRF1	–	archaeal release factor 1
ATM	–	checkpoint protein kinases ataxia telangiectasia mutated kinase
ATR	–	ataxia telangiectasia and Rad3-related protein kinase
BioID	–	proximity-dependant biotin identification
BRD4	–	chromodomain reader brominated domain protein 4
CDKs	–	cyclin-dependent kinases
CHD5	–	chromodomain-helicase-DNA-binding protein 5
CHK1/2	–	checkpoint kinase ½
cm^{5}U	–	5-carboxymethyluridine
DLAT	–	dihydrolipoamide transacetylase
DMA	–	dimethyl arsenic
DSB	–	double stranded break
ECM	–	extracellular matrix
eRF1	–	eukaryotic release factor 1
FN1	–	Fibronectin-1
GFP	–	green fluorescent protein
H4K12	–	lysine 12 of histone 4
IP	–	immunoprecipitation
lncRNA	–	long noncoding RNA
LS-MS/MS analysis	–	liquid chromatography-tandem mass spectrometry
m^{2}G	–	methylguanosine
m^{6}A	–	N6-adenenosine
m^{6}dA	–	N6-methyl-deoxyadenosine
m^{7}G	–	N7-methylguanosine
MCC	–	mitotic checkpoint complex
mcm^{5}s^{2}U	–	5-methoxycarbonylmethyl-2-thiouridine
mcm^{5}U	–	5-methylcarboxymethyluridine
mcm^{5}Um	–	5-methoxycarbonylmethyl-2′-O-methyluridine
MTase	–	methyltransferase
NUT	–	nuclear protein of the testis
RB	–	retinoblastoma protein

RF1	–	release factor 1
RF2	–	release factor 2
ROS	–	reactive oxygen species
SAC	–	spindle assembly checkpoint
SAM	–	S-adenosyl methionine
SILAC	–	stable isotope labelling by amino acids in cell culture
SNP	–	single nucleotide polymorphism
snRNA	–	small nuclear RNA
THUMPD2	–	THUMP domain-containing protein 2
THUMPD3	–	THUMP domain-containing protein 3
THUMPD3-AS1	–	THUMPD3 antisense RNA 1
TRMT112	–	TRM112-like protein
WBSCR22	–	Williams-Beuren syndrome chromosomal region 22 protein
WT	–	wild type

1. INTRODUCTION

Epigenetic modifications of DNA, post-transcriptional modifications of RNA, and post-translational modifications of proteins are important regulators of various cellular processes. In these modifications, a chemical tag is added to the substrate at a particular position, leading to specific changes in the activity of the substrate. The correct patterns of these modifications are vital for ensuring cellular functions in healthy cells. Alterations of these complex modification patterns often lead to abnormal cellular functions, which can cause various diseases. One such modification is methylation, in which a methyl group is transferred from a methyl donor, usually S-adenosylmethionine (SAM), to the substrate. This reaction is catalysed by a specific protein group called methyltransferases (MTases). Methylation plays an important role in gene regulation at both transcriptional and post-transcriptional levels. It affects various cellular processes, including metabolism, protein synthesis, and neural signalling.

Approximately 200 proteins function as methyltransferases in human cells. Dysregulation of these proteins and subsequent abnormal methylation patterns have been linked to multiple diseases, including cancer, diabetes, neurodegenerative disorders, and intellectual disabilities.

Many methyltransferases require a cofactor for activation and metabolic stability. One such cofactor is the small, evolutionarily conserved protein TRMT112. Prior studies in various organisms have revealed that TRMT112 interacts with and activates several MTases that act as post-transcriptional regulators of the protein synthesis machinery and are highly important for various essential cellular processes. However, to date, knowledge about TRMT112-network MTases, as well as many other human MTases and their functions in human cells, is still limited.

This dissertation focuses on the human TRMT112-network of MTases, with a particular focus on the interaction partner N6AMT1. N6AMT1 is an especially intriguing TRMT112-network member, as several studies have demonstrated contradictory results regarding N6AMT1 methylation substrates and effects. During this study, it was demonstrated that the human TRMT112 network consists of at least seven MTases as stable TRMT112 interaction partners: N6AMT1, WBSCR22, METTL5, ALKBH8, TRMT11, THUMPD2, and THUMPD3. A mutual stabilisation effect was observed between TRMT112 and MTases. In this study, by analysing the effect of point mutations on the TRMT112-MTase interaction surface, it was also shown that TRMT112 forms the interaction with all MTases similarly, but differences can be observed, especially in the case of THUMPD2 and THUMPD3. It was also shown that the TRMT112 interaction partner N6AMT1 participates in the cell cycle regulation, and N6AMT1 depletion leads to a reduction of cyclin E level throughout the cell cycle. Finally, several N6AMT1 commercial antibodies were found to strongly cross-react with the mitosis-associated protein Aurora kinase A, complicating research and data interpretation regarding N6AMT1 functions in mammalian cells. These findings provide more knowledge about the diverse functions of TRMT112 and its partner MTases.

2. LITERATURE REVIEW

2.1. Methyltransferases

The complex systems of epigenetic modifications that impact DNA transcription, post-transcriptional modifications of RNA, and post-translational modifications of proteins have recently emerged as important regulatory mechanisms in biological processes and the pathogenesis of various diseases [1,2]. These modifications provide the chemical diversity required to regulate and form the intricate structures necessary for DNA transcription regulation, and protein translational apparatus [2].

One such modification is methylation. The addition of a methyl group from a methyl donor to a wide range of substrates is mediated by specific enzymes called methyltransferases (MTases) [3]. Methylation plays an important role in epigenetic control, lipid biosynthesis, protein repair, hormone inactivation, and tissue differentiation in living organisms [4]. MTase dysfunctions are often associated with various diseases, including cancer and neurological disorders [4]. MTases are divided into classes based on their structure, as they typically do not share high sequence similarity but only short and conserved motifs [2,4,5]. In eukaryotic cells, most MTases use S-adenosyl methionine (SAM) as a methyl donor [2,5].

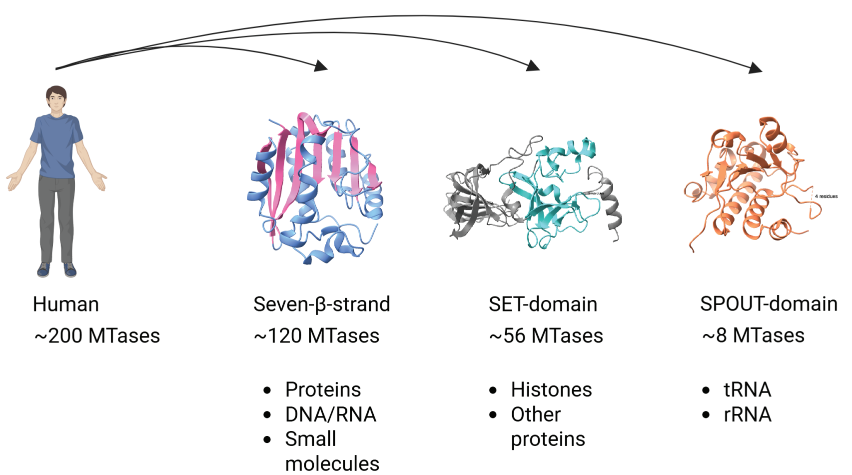

Figure 1. Schematic depiction of the three largest classes of human methyltransferases (MTases). Typical structures and substrates of the seven-β-strand superfamily (PDB accession code 6KMR), SET-domain MTases (PDB accession code 1N6C), and SPOUT domain MTases (PDB accession code 4FMW).

There are approximately 200 different MTases in human cells (Figure 1) [4]. The three largest classes in humans are the seven-β-strand superfamily, followed by the SET and SPOUT family MTases (Figure 1) [2]. The typical structures of the seven-β-strand superfamily MTases, SET family, and SPOUT family MTases are depicted in Figure 1. The SET domain MTases, comprising approximately 56 enzymes, were initially identified as methylating histone lysine residues. However, recent studies have demonstrated their ability to methylate non-histone lysine residues on molecules such as transcription factors and tumour suppressors [2,6]. The SPOUT protein family is the smallest, consisting of only eight proteins, which mainly mediate tRNA and rRNA methylation, with one reported case of protein methylation [2,7]. The seven-β-strand superfamily is described in more detail in the following section.

2.2. Seven-β-strand methyltransferases

The largest class of MTases in human cells is the seven-β-strand superfamily also referred to as "Class I" MTases, consisting of about 120 SAM-dependant MTases [2,4,8].

All of the seven-β-strand MTases adopt a Rossmann fold, consisting of a twisted central seven-stranded β-sheet, where all strands except the last one are parallel [8,9]. Typically, there are three α-helices on one side of the β-sheet, and at least two α-helices on the other side, thus creating a three-dimensional structure of an αβα sandwich. Within this fold, certain sequence motifs, such as those for SAM and substrate binding, are found at distinct locations [8,9].

Seven-β-strand methyltransferases (MTases) can be categorised into three distinct subgroups: protein MTases, DNA/RNA MTases, and small-molecule MTases, which include those acting on metabolites, neurotransmitters, and toxic compounds. These enzymes play a crucial role in regulating various cellular processes, such as cellular metabolism, protein synthesis, neural signalling, and gene regulation at both transcriptional and post-transcriptional levels [8].

Several of the seven-β-strand MTases require an activating subunit for their MTase activity, where the MTase forms a tight heterodimer with the partner, stabilising the metabolic activity of the MTase and enhancing SAM binding [10–17]. One such activating subunit is the eukaryotic multifunctional methyltransferase TRM112-like protein (TRMT112), which is discussed in more detail in the following section.

2.3. Methyltransferase activating subunit TRMT112

2.3.1. TRMT112 interactome and functions

TRMT112 is a small 15 kDa evolutionarily conserved protein. Its orthologues are found in all three domains of life. In eukaryotes and archaea, TRMT112 functions as an allosteric regulator of several seven-β-strand SAM-dependent MTases involved in rRNA, tRNA, snRNA, and protein methylation [11,16–22], whereas bacterial TRMT112 orthologue functions are unknown [23,24].

The archaeal orthologue of eukaryotic TRMT112, Trm112, has been confirmed to interact with three archaeal MTases: Trm9 (ALKBH8 in humans), Mtq2 (N6AMT1 in humans), and Trm11 (TRMT11 in humans) [20,25]. Although Trm112 is not present in all archaeal species [20], data indicate that in species where it is found, Trm112 may have more interaction partners than currently known and should be studied further [20,25].

In yeast, Trm112 interacts with and activates four MTases: Bud23 (WBSCR22 in humans), Mtq2 (HEMK2 or N6AMT1 in humans), Trm9 (ALBKH8 in humans), and Trm11 (likely TRMT11 in humans) [22,26–28]. Multiple studies have shown that TRM112 is not an essential protein in yeast; however, Trm112 knockout strains exhibit reduced growth rates [26–28].

Similar results have been shown with Trm112 functional homologue SMO2 studies in *Arabidopsis thaliana*, where SMO2 inactivation leads to multiple defects in plant development, such as impaired cell growth and slower proliferation rates. Additionally, SMO2 knockout plants have smaller aerial organs and shorter roots than wild-type plants, suggesting that SMO2 is required for optimal *Arabidopsis thaliana* development [29].

Studies from human cells have revealed that TRMT112 forms a complex with several different seven-β-strand SAM-dependant MTases – N6AMT1 [17], METTL5 [16], ALKBH8 [30], WBSCR22 [11], THUMPD2 [31] and THUMPD3 [32]. Another putative MTase identified as a potential TRMT112 interactor is TRMT9B (also known as TRM9L). Both TRMT9B and ALKBH8 share significant sequence similarity with yeast Trm9; ALKBH8 is a confirmed MTase, whereas the exact biological function of TRMT9B remains unclear [33,34]. It is suggested that TRMT112 plays an important role during mammalian embryogenesis, as the TRMT112 homologue in mice, Trmt112, is highly expressed during early brain and nervous system tissue development [35].

Recently, a novel study suggested that TRMT112 plays an important role in promoting inflammatory cytokine expression in post-traumatic osteoarthritis and the chromodomain reader brominated domain protein 4 (BRD4) has been identified as a novel TRMT112 interaction partner. Through this interaction expression of specific cytokine transcripts (IL-1β, TNF-α, and IL-6) are regulated to promote inflammatory responses during disease progression [36]. BRD4 and TRMT112 interaction was also identified in another study where proximity-dependent biotin identification (BioID) method was applied to identify the TRMT112-interactome. In this method, a biotin protein ligase was fused to TRMT112, facilitating the biotinylation of nearby endogenous proteins, which were then precipitated and identified through mass spectrometry analysis. BRD4 was identified as one of the biotinylated proteins, but this study did not further focus on this specific interaction [21]. Overall, the TRMT112 interactome might be more complex than current studies show and still needs further research.

2.3.2. Crystal structures of TRMT112, together with its partners

Crystal structures of eukaryotic TRMT112 have revealed that it consists of two domains: the Zn-knuckle domain and a helical domain (Figure 2). The Zn-knuckle domain is conserved in all three domains of life, and it consists of a short α-helic and an anti-parallel β-sheet composed of four β-strands as depicted in Figure 2 [16,20,22,23]. This domain was initially identified in yeast, where four cysteine residues chelate one zinc atom [22]. Although conserved in fungi, parasites, bacteria, and some archaeal species, the four cysteine residues and, therefore, the Zn-binding ability, are not conserved in metazoans [24]. The helical domain, observed only in eukaryotes, consists of three α-helices (Figure 2) and is inserted in the middle of the Zn-knuckle domain between strands β1 and β2 [23,24].

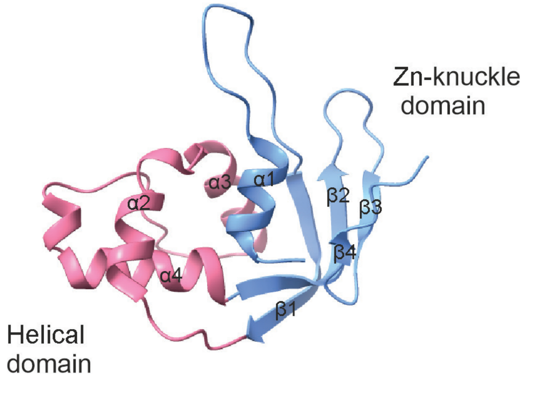

Figure 2. Eukaryotic TRMT112 secondary structure (PBD accession code 6H2U). TRMT112 consists of two domains: the Zn-knuckle domain (blue) and the helical domain (pink).

Various crystal structures of TRMT112 in complex with its partner MTases from archaea, yeast and human cells have revealed that MTases form the interaction with TRMT112 in a similar manner in all organisms. In all the crystal structures revealed so far, the same region of TRMT112 interacts with the same region of the MTase partner [16,18,20,25,37–41]. Specifically, the binding forms when β-strand 3 of the MTase interacts with the β-strand 4 of TRMT112 through a parallel β-zipper that creates an extended β-sheet made of the central β-sheets from both proteins. This can be observed from the crystal structures of METTL5-TRMT112 (Figure 3A) and N6AMT1-TRMT112 (Figure 3B) in human cells [16,39]. TRMTT112 binding is crucial for MTase functionality due to several factors. First, the interaction between TRMT112 and its MTase partners covers a large hydrophobic area of MTase and a smaller one on TRMT112. This might be one of the main explanations for the observation from multiple studies that TRMT112 and MTase interactions stabilise many of its partners [11,16,19,26,42]. For example, it has been suggested that if the hydrophobic region of WBSCR22 is exposed to a solvent, it becomes unstable, leading to the degradation of WBSCR22 by proteosomes [11]. TRMT112 interaction also appears to be important for SAM binding to MTase partners. Even though crystal structures of TRMT112 in a complex with MTase and SAM reveal that no TRMT112 residue is in direct contact with SAM, it is thought that the loop between β-strand 3 and β-strand 4 of the MTases is stabilised by TRMT112 binding, which in turn stimulates SAM binding [9,16,18,23,24,37,39,40,43]. Intriguingly, studies in yeast involving Mtq2-Trm112 [18] and Trm11-TRMT112 [43] have revealed that Trm112 mutations at positions that do not directly interact with the MTase affect the enzymatic activity of MTase, even though they do not directly influence SAM binding or Trm112-MTase complex formation.

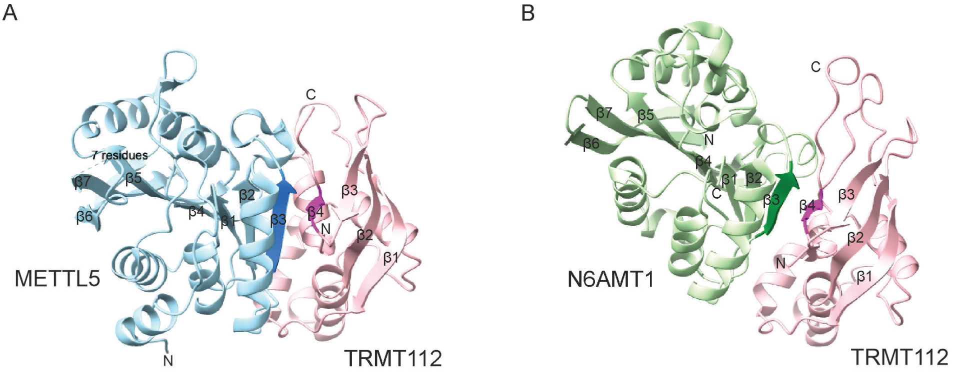

Figure 3. A. **Crystal structure of the human TRMT112-METTL5 complex.** TRMT112 (light pink) and METTL5 (light blue) binding forms between β-strand 3 of METTL5 (blue) and β-strand 4 of TRMT112 (pink) through a parallel β-zipper that creates an extended β-sheet composed of the central β-sheets from both proteins (PBD accession code 6H2U). B. **Crystal structure of the human TRMT112-N6AMT1 complex.** TRMT112 (light pink) and N6AMT1 (light green) binding forms between β-strand 3 of N6AMT1 (green) and β-strand 4 of TRMT112 (pink) through a parallel β-zipper that creates an extended β-sheet composed of the central β-sheets from both proteins (PBD accession code 6KMR).

2.4. TRMT112-network methyltransferases

In eukaryotic cells TRMT112-network MTases are important post-transcriptional regulators of protein synthesis. These complexes catalyse methylation of substrates that ensure biogenesis of both ribosomal subunits, stability of tRNAs, correct translation termination and efficient pre-mRNA splicing [16,17,19,21, 31,32,44,45]. In this literature review, the functions of the TRMT112-interacting MTases, which are all members of the seven-β-strand methyltransferase family: METTL5, WBSCR22, THUMPD2, THUMPD3, TRMT112, ALKBH8, and N6AMT1, are discussed in more detail. A simplified summary of TRMT112 network methyltransferases and their confirmed modifications, substrates, main bioprocesses, and their most important associated human diseases are described in Table 1.

Table 1. Summary of TRMT112-network methyltransferases

Human methyl-transferase	*Modification and substrate*	*Bioprocesses involved*	*Associated human diseases*	*Organisms*
METTL5	m^6A on 18s RNA	Protein translation regulation	Intellectual disability, microcephaly, carcinogenesis	Archea, higher eukaryotes
WBSCR22	m^7G on 18s rRNA	40s ribosome maturation	Carcinogenesis, deletion causes embryonic lethality	Eukaryotes
THUMPD2	m^2G on snRNA	Spliceosome regulation, splicing efficiency	Carcinogenesis, age-related macular degradation	Higher eukaryotes
THUMPD3	m^2G on tRNA	Protein translation, alternative splicing regulation	Carcinogenesis	Higher eukaryotes
TRMT11	m^2G on tRNA	tRNA function optimization, tRNA tertiary-structure regulation	Carcinogenesis	Archaea, eukaryotes
ALKBH8	mcm^5U on tRNA	Translation regulation, oxygenase activity towards $tRNA_{Gly}$, DNA damage response	Neurodevelopmental disorders, intellectual disability, carcinogenesis	Archaea, eukaryotes
N6AMT1	N^5-methyl-glutamine on eRF1, CHD5 and NUT	Translation termination regulation	Deletion causes embryonic lethality, carcinogenesis	Prokaryotes, archaea, eukaryotes.

2.4.1. N^6-adenenosine methyltransferase METTL5

Human METTL5 is a 24 kDa protein that is a member of the METTL family of over 30 different S-adenosyl-methionine-dependent MTases. Homologues of human METTL5 are conserved in archaea species, plants and metazoans, but are not found in yeast and prokaryotes [16,46,47]. METTL5 has been shown to catalyse the formation of N^6-adenenosine (m^{6}A) on 18s rRNA at position 1832 in human cells [16,48] and mice [49–51], but this modification is not required for correct 18s rRNA biogenesis [16,46]. The METTL5 homologue activity as m^{6}A 18s rRNA MTase has also been described in *C. elegans* [48,52] and Drosophila [46].

In mice, humans, and Drosophila cells, METTL5 requires TRMT112 interaction to gain metabolic stability and increased enzymatic activity [16,46,47]. Furthermore, it has been shown that in human cells, a siRNA knockdown of TRMT112, in turn, downregulates METTL5 protein level [16].

Several studies have shown that METTL5 plays an important role in the regulation of protein translation [47,48]. The METTL5 mediated 18s rRNA m^{6}A1832 modification is located in the middle of the ribosome decoding centre and may regulate the conformation of the decoding centre by altering the binding affinity for mRNAs [48]. However, there are conflicting data on whether METTL5 regulates global translation or whether this regulation is transcript-specific [47,48]. A recent study demonstrated intriguing data arguing that METTL5 indeed regulates global mRNA translation, and that m^{6}A1832 modification strengthens the connection between 60S ribosomal protein L24 (RPL24) and 18S rRNA for correct 80S ribosome assembly [53]. Additionally, it has been demonstrated that loss of the METTL5 homologue in mouse leads to impaired early differentiation of mouse embryonic stem cells, potentially due to a decrease in the global translation rate [50,51].

METTL5 and its homologues are suggested to play an important regulatory role in mammalian neuronal cells, particularly in brain cells [54]. Biallelic mutation variants that cause a frameshift mutation in the METLL5 protein have been linked to intellectual disability and microcephaly in human patients [54], while Mettl5 knockout mice exhibit distinct developmental abnormalities, including cranial deformities and behavioural alterations [50], as well as have metabolic defects, such as lipid biosynthesis and storage defects [47]. Drosophila strains with Mettl5 knockout also display behavioural pathologies and locomotion and orientation defects [46]. Intriguingly, METTL5 mutations associated with microcephaly and intellectual disability disturb METTL5 interaction with TRMT112 [47].

Additionally, METTL5 has been implicated as a contributor to cancer development in several studies. It is upregulated and correlated with poor prognosis in multiple cancer types, such as breast cancer [48], hepatocellular cancer [55], and lung adenocarcinoma [56], but not in gastric cancer, where higher METTL5 expression is correlated with better patient outcomes [57]. METTL5 knockouts from various cancer and non-cancer cell lines have shown both decreased [48] or no change [16] in cell growth compared to the wild-type. METLL5 has also been suggested to modulate immune responses in cancerous tissues [55,58]. Taken together, these data indicate that METTL5 is a highly important protein in various cell types.

2.4.2. N^7-guanosine methyltransferase WBSCR22

Another confirmed rRNA MTase that interacts with TRMT112 in mammalian cells is WBSCR22 [11]. The WBSCR22 gene was originally detected in human cells as one of 18 genes in the deletion area of Williams-Beuren syndrome. Williams-Beuren syndrome (WBS) is a rare, complex neurodevelopmental disorder due to an approximately 1.5 Mb large deletion at 7q11.23 [59].

WBSCR22 and its homologues play an important role during the biogenesis of the 40s ribosomes in eukaryotic cells [60]. Specifically, WBSCR22 catalyses the formation of N^7-methylguanosine (m^7G) at position 1575 on 18s rRNA in yeast cells [26,37,61] and position 1639 on 18s rRNA in human cells [19,62]. It has been shown that WBSCR22 mediates the m^7G modification at a late stage of 40s ribosome subunit maturation [37]. The deletion of the WBSCR22 homologue in yeast Bud23 causes reduced cell growth rate and a significant loss of 40s subunits and polysomes, indicating that Bud23 is highly important for ribosome biogenesis [61]. It has been shown that WBSCR22 depletion results in reduced cell growtn and the nuclear accumulation of 18s pre-rRNA intermediates, resulting in impaired 18s rRNA maturation [60]. Intriguingly, catalytically inactive WBSCR22 mutant restored normal cytoplasmic levels of 18SE pre-rRNA [19,62], suggesting that WBSCR22/Bud23 function during early ribosome biogenesis is independent of its MTase activity.

Studies have shown that WBSCR22 forms a heterodimer with its co-factor TRMT112 to gain MTase activity and metabolic stability [11,19,26]. Down-regulation of TRMT112 using siRNA significantly reduced WBSCR22 levels, indicating that the interaction with TRMT112 is essential for WBSCR22 stability and protein level regulation [11].

Potentially due to its role in 40s ribosome maturation, WBSCR22 can be considered an essential protein during embryogenesis of higher eukaryotes, as homozygous deletion of the WBSCR22 homologue Bud23 in mice results in embryo lethality [63]. It has been shown that even targeted deletion of Bud23 only in murine cardiac tissue shortens the lifespan of animals and leads to sudden death of knockout mice between the age of 28–25 days. Furthermore, the loss of Bud23 in mice was linked to mitochondrial function and mitochondrial protein dysregulation in cardiomyocytes, possibly due to impaired translation of mitochondrial regulatory proteins resulting from impaired ribosome biogenesis [63].

Additionally, WBSCR22 has been shown to be important not only in healthy tissues but also to have diverse functions in disease progression, particularly cancer development. WBSCR22 displays enhanced expression levels in breast cancer cells, and it has been suggested to promote metastasis by enhancing tumour cell survival in lung and melanoma cells [64]. The expression of WBSCR22 is also increased in multiple myeloma [65], colorectal [66], and glioma cancer cells, where loss of WBSCR22 inhibits the proliferation, migration, and invasive properties of glioma cancer cells, whereas its overexpression has the opposite effect [67]. WBSCR22, on the other hand, is downregulated in pancreatic cancer, and high WBSCR22 expression in pancreatic cells correlates with a better 5-year survival rate in patients with pancreatic cancer [68]. WBSCR22 is also downregulated in inflammatory and neoplastic pulmonary pathologies [69].

Finally, it has also been reported that the knockdown of WBSCR22 increases cell sensitivity to several chemotherapy drugs, such as the antineoplastic agents SN-38 and 5-Fluorouracil in the small lung cancer cell line H460 harbouring wild-type (WT) p53, while having no effect on p53-null H1299 cells [70]. It has also been shown that WBSCR22 knockdown increases sensitivity to cancer drug oxaliplatin in the colorectal cancer cell line Caco-2 [66].

2.4.3. N^2-guanosine methyltransferases THUMPD2, THUMPD3 and TRMT11

Human THUMP domain-containing protein 2 (THUMPD2), THUMP domain-containing protein 3 (THUMPD3), and TRMT11 are overall poorly characterised proteins that have been recently identified as TRMT112 interaction partners in mammalian cells [21,31,32]. THUMPD2, THUMPD3, and TRMT11 share a THUMP domain along with a MTase domain (UniProt database, accessed on 05.02.2025). The THUMP domain is an ancient RNA-binding domain found in various RNA-modifying enzymes, such as thiouridine synthases, RNA methylases, and pseudouridine synthases, adopting an alpha/beta fold structure to facilitate RNA modification activities [71]. Human THUMPD2 and THUMPD3 are paralogs that share 26% identity at the amino acid level and do not have yeast or archaeal orthologues (UniProt database, accessed on 03.02.2025), while TRMT11 is a likely homologue of Trm11 because of high sequence similarity, but this has not been confirmed experimentally.

2.4.3.1. THUMPD2 and THUMPD3

During Study I it was revealed that THUMPD2 and THUMPD3 form a complex with TRMT112. This was recently confirmed in another study, which also demonstrated that THUMPD2-TRMT112 acts as an N^2-methylguanosine (m^{2}G) MTase that catalyzes the methylation of U6 snRNA at position G72, which is a core component of the catalytic spliceosome in human cells and may affect splicing efficiency [21,31]. This modification, located at the core of the spliceosome catalytic centre, is not present in lower eukaryotes but only in higher vertebrates. Loss of THUMPD2 in human cells results in impaired endogenous pre-mRNA splicing activity [21,31] and upregulation of nonsense-mediated mRNA decay pathway-associated genes [31]. The nonsense-mediated mRNA decay pathway eliminates transcripts with premature stop codons [21,31].

THUMPD2 has also been linked to cancer development; for example, it has been shown that THUMPD2 has different regulatory roles at various tumour stages of epithelial ovarian cancer, where it is expressed at low levels during early ovarian cancer but becomes upregulated in late stages [72]. It has been suggested that lower levels of THUMPD2 promote cell proliferation in early ovarian cancer stages and inhibit metastasis formation, while higher THUMPD2 expression has the opposite effect in later cancer stages, which also correlates with poor prognosis in patients [72]. THUMPD2 may also play a role in the development of cancer cell resistance to two common drugs, cisplatin and 5-fluorouracil, in human oesophageal squamous cell carcinoma cells, where its downregulation increases cancer cell resistance to both drugs [73].

Recently, THUMPD2 has been associated with another common disease, age-related macular degeneration (AMD), which is one of the leading causes of blindness in the elderly. Through a genome-wide association study, a genetic variant of THUMPD2 was identified as being associated with the development of AMD [74]. Moreover, a recent study revealed that many AMD-associated genes and genes with retina-specific functions are direct targets of THUMPD2-mediated regulation of splicing, suggesting that THUMPD2-mediated U6 m^2G_{72} modification plays a role in age-related macular degeneration and retinal function [31].

Human THUMPD3 has recently been shown to act as a tRNA MTase in human cells. THUMPD3 forms a complex with TRMT112, which then catalyzes the m^2G at position 6/7 on the tRNAs in mammalian cells [21,32]. THUMPD3 is not an essential protein, as it can be successfully knocked out from mammalian cells, but its knockout decreases the cell proliferation rate and suppresses the global translation rate of the cell [32,75]. In contrast, its overexpression increases cell proliferation in both normal lung and lung cancer cells [75]. In lung cancer cells, THUMPD3 has been implicated as a regulator of alternative splicing of several extracellular matrix (ECM) transcripts, particularly affecting Fibronectin-1 (FN1). When THUMPD3 is depleted, it specifically promotes an alternative splicing event that reduces the pro-tumour isoform of FN1, leading to reduced cellular proliferation and migration in lung cancer cells [75].

THUMPD3 has been implicated in cancer development, not only in its protein form but also in the form of the long noncoding RNA (lncRNA) THUMPD3-AS1 (THUMPD3 antisense RNA 1), which partially overlaps with the THUMPD3 gene. Studies show that THUMPD3-AS1 is involved in the occurrence and development of non-small cell lung cancer [76], colorectal cancer [77], and gastric cancer [78] by enhancing the proliferation and invasiveness of cancer cells. THUMPD3-AS1 has been linked not only to cancer but also to other diseases such as osteoarthritis, where the knockdown of THUMPD3-AS1 increased apoptosis and facilitated inflammatory responses in chondrocytes [79].

Overall, THUMPD2 and THUMPD3 are intriguing proteins and deserve further investigation to fully uncover their cellular effects.

2.4.3.2. TRMT11

TRMT11 was identified as a novel TRMT112 interaction partner during Study I of this PhD thesis. This interaction was subsequently confirmed in another study, which reported that the TRMT11-TRMT112 complex catalyzes the methylation of m^2G at position 10 or tRNAs [21]. This corresponds to data obtained from the likely TRMT11-TRMT112 complex homologue in archaea and yeast, Trm11-Trm112, which catalyses the formation of m^2G at position 10 of some tRNAs in both archaea [20,80] and yeast [28,43], but is absent in bacteria [23]. Interestingly, in some archaeal species, Trm11 functions independently without requiring a binding partner. Nevertheless, in species such as *Archaeoglobus fulgidus*, which possesses Trm112, although Trm11 is active alone, its enzymatic activity is significantly enhanced when it is complexed with TRM112 [20]. It has been suggested that the m^2G10 modifications play a role in the tRNA tertiary structure [28] and are therefore highly important for tRNA function optimisation during translation [21].

Nonetheless, TRMT11 is not an essential gene in eukaryotes, as Trm11 deletion in yeast cells [28] and deletion of TRMT11 in the human colon cancer cell line HCT116 did not cause detectable changes in growth rate [21]. However, in HCT116 cells, the deletion of TRMT11 in combination with the other tRNA m^2G MTase, THUMPD3, led to a near-complete loss of m^2G modification of tRNAs. This did not affect tRNA structure and stability but did lead to a significant reduction in the cell proliferation rate. Additionally, TRMT11 and THUMPD3 deficient cells exhibited reduced polysome levels, 80s ribosome subunits, and newly synthesised proteins, indicating that the loss of both $m^2G_{6/7}$ and m^2G_{10} may lead to less efficient translation [21]. This could be either due to the direct effect of $m^2G_{6/7}$ and m^2G_{10} modifications on tRNA functions, or these modifications might affect the deposition of another tRNA modification important for translation efficiency [24].

Similar to other MTases, TRMT11 has been linked to carcinogenesis, although not in its protein form, but as part of a fusion gene in multiple cancer types. In all cases, the TRMT11 gene was fused with the ionotropic glutamate receptor GRIK2 (glutamate receptor ionotropic, kainite 2) [81–83]. The fusion of both genes leads to the loss of function of TRMT11, which may contribute to cancer development due to less efficient and stable mRNA translation in cancer cells because of tRNA defects or protein translation repression [82]. Nonetheless, further studies are required to investigate TRMT11 role in carcinogenesis.

2.4.4. Uridine methyltransferase ALKBH8

One more confirmed TRMT112 interactor is the tRNA MTase ALKBH8 [30]. Orthologues of ALKBH8 have been described in multiple organisms, ranging from archaea and yeast to mammals [25,27,30,84–86].

The archaeal homologue of ALKBH8, HvoTrm9, in a complex with Trm112 catalyzes the modification of 5-carboxymethyluridine (cm^5U) into 5-methylcarboxymethyl uridine (mcm^{5}U) at position 34 of tRNAs [25]. Similar data have been obtained from yeast, where Trm9 specifically methylates cm^{5}U to mcm^{5}U at the wobble positions of tRNA$^{Arg}_{UCU}$, tRNA$^{Gly}_{UCC}$, tRNA$^{Lys}_{UUU}$, tRNA$^{Gln}_{UUG}$, and tRNA$^{Glu}_{UUC}$ [23]. The modification mcm^{5}U is further converted to 5-methoxy-carbonylmethyl-2-thiouridine (mcm^{5}s^{2}U) through a multi-step process involving at least 15 proteins [23,27,84]. The tRNA anticodon loop modifications, such as at the wobble positions, are known to influence translation rate and accuracy [85,87].

Mammalian ALKBH8 is a member of the ALKBH protein family, which consists of nine homologous enzymes [88]. ALKBH8 contains a DNA and RNA recognition motif (RRM), SAM-dependent MTase domain, and a conserved AlkB domain [89]. Similar to its yeast and archaeal homologues, the MTase domain of the ALKBH8-TRMT112 complex catalyzes the formation of mcm^{5}U from cm^{5}U at the wobble position of certain tRNAs [30,90]. Additionally, through its AlkB domain, ALKBH8 functions as an oxygenase that hydroxylates mcm^{5}U into (S)-mchm^{5}U, specifically in tRNA$_{Gly}$ (UCC) [44,91]. Similar functions have also been recorded in *Arabidopsis thaliana*, where the functional Trm9-Trm112 homologue AtTRM9-AtTRM112a/b, is required for mcm^{5}U formation of tRNAs. Intriguingly *Arabidopsis thaliana* possesses another enzyme, that appears to be a functional ALKBH8 homologue, AtALKBH8, which catalyses the hydroxylation of mcm^{5}U to 5-methoxycarbonylmethyl-2′-O-methyluridine ((S)mchm^{5}U) in tRNA$^{Gly}_{UCC}$ [92].

It has also been demonstrated that ALKBH8 plays a role in the translation regulation of DNA damage response proteins in both yeast and human cells [85,90]. For example, ALKBH8 knockout in mouse embryonic fibroblasts leads to slower cell growth, increased DNA damage response, increased sensitivity to DNA-damaging agents, and increased levels of reactive oxygen species (ROS) [30,91,93]. It is suggested that the increased ROS level in ALKBH8 deficient cells might be due to ALKBH8 involvement in the regulation of essential enzymes that protect cells against oxidative stress, called selenocysteine-containing enzymes [30,93]. Selenocysteine-containing proteins contain a selenocysteine (Sec) amino acid residue. Sec insertion is mediated by a specific tRNA^{Sec} via a specialised mechanism involving stop codon recoding and tRNA modifications. tRNA^{Sec} contains two subpopulations: those containing mcm^{5}U and those containing mcm^{5}U with a further methylated ribose, 5-methoxycarbonylmethyl-2′-O-methyluridine (mcm^{5}Um). It has been suggested that during oxidative stress, ALKBH8 mediates mcm^{5}Um modification of tRNA^{Sec} to enhance selenoprotein translation, such as glutathione peroxidases and thioredoxin reductases, which possess antioxidant activity. In the absence of ALKBH8, this stop codon recoding process is disrupted, leading to decreased levels of mcm^{5}Um and reduced selenoprotein expression [93].

It has been suggested that the loss of ALKBH8 mediated selenocysteine based ROS detoxification processes might lead to an increased sensitivity to ROS-inducing agents, which increases the likelihood of diseases linked to abnormal ROS production, such as neurodegenerative diseases or cancer [93,94]. Indeed, high ALKBH8 expression has been linked to poor prognosis in patients with glioblastoma [95] and bladder cancer [96].

Several studies have also linked ALKBH8 with neurodevelopmental disorders; in particular, it has been reported that a loss-of-function homozygous missense mutation of ALKBH8 leads to a neurodevelopmental disorder and intellectual disability in patients [97–99]. Furthermore, similar data have been obtained from studies of the ALKBH8 deficient mice, which display behavioural abnormalities, abnormal brain phenotypes, dysregulated oxidative stress responses, and abnormal mitochondrial function [100]. This is contradictory to two other studies, which reported that ALKBH8 knockout mouse strains do not exhibit any noticeable phenotypic changes [30,91]. However, contradictory results could be explained by the difference in methods: in a study reported by Honda et al. [100] mice were subjected to specific behavioural tests, which were not reported in the studies by Songe-Moller et al. [30] and van den Born et al. [91]. Interestingly, it has been suggested that ALKBH8 is involved in neuronal development in *C. Elegans* as it transitions from larvae to adult worm, and ALKBH8 is widely expressed throughout larvae but becomes restricted to only a small number of neurones in adult worms [101]. Overall, ALKBH8 is an intriguing protein and deserves further research to decipher its precise role in living organisms.

2.4.5. Glutamine methyltransferase N6AMT1

The only confirmed protein MTase that interacts with TRMT112 is N6AMT1 [17]. N6AMT1, similar to TRMT112, is an evolutionarily conserved protein whose homologues have been described in multiple organisms [17,25,40,102–105].

2.4.5.1. N6AMT1 methylates glutamine of the GGQ motif

N6AMT1 catalyzes the methylation of the nitrogen atom of the glutamine side chain at the universally conserved GGQ motif of class 1 release factors in organisms ranging from prokaryotes to humans [17,102,104–106]. In bacteria, there are two class 1 release factors, RF1 and RF2, which recognise UAA and UAG, or UAA and UGA stop codons, respectively. In eukaryotes and archaea, there is a single class 1 release factor, called eukaryotic release factor 1 (eRF1) and archaeal release factor 1 (aRF1), both of which share high sequence similarity and recognise all stop codons [107]. In all eukaryotes, archaeal, and bacterial species, release factors are essential for optimal translation termination, as they promote the release of newly synthesised peptides from ribosomes [107].

Translation termination typically occurs when the mRNA stop codon reaches the ribosome A site. After the eRF1 N-terminal domain recognises the stop codon, eRF1 rearranges itself, resulting in the insertion of the GGQ motif into the peptidyl transferase centre to release the nascent protein. N6AMT1 bacterial homologue PrmC functions without a binding partner and catalyses the methylation of the GGQ motif in bacterial RF1 and RF2 [102,103], while in eukaryotes and archaea GGQ is methylated by a complex of N6AMT1-TRMT112 and their homologues [17,18,22,25]. Methylation of the glutamine side chain is thought to stabilise the GGQ position inside the peptidyl release centre in the large subunit of the ribosome by increasing the van der Waals interactions with the adjoining nucleotides [108–110]. Recently, it was shown that the enzymatic activity of the N6AMT1-TRMT112 homologue in yeast, Mtq2-Trm112, is essential for ribosomal pre-60S maturation and nuclear export. Mtq2-Trm112 potentially functions as a quality control mechanism to proof-test the eRF1 binding site on maturing subunits or by methylating another, yet unidentified, substrate [45].

Studies have identified additional glutamine target residues and substrate proteins that are methylated by N6AMT1 in human cells. Kusevic et al. identified glutamine residues on chromodomain-helicase-DNA-binding protein 5 (CHD5) and nuclear protein of the testis (NUT) as N6AMT1 methylation targets [111]. CHD5 is a poorly researched chromatin remodelling enzyme that has been suggested to function as a tumour suppressor [112], whereas NUT plays a role in germ cell maturation and has been implicated in the development an aggressive form of squamous cell carcinoma [113].

Similar to all previously described TRMT112-network MTases, N6AMT1 must form a heterodimer with TRMT112 to gain MTase activity in eukaryotic cells [17,22]. It has been suggested that N6AMT1 requires the TRMT112 interaction to bind SAM, as neither N6AMT1 nor TRMT112 can bind SAM alone [40]. Intriguingly, in human cells, N6AMT1 encodes two alternatively spliced isoforms that are equally expressed at the mRNA level. However, only isoform 1 interacts with TRMT112, suggesting that TRMT112 may play a role in regulating the levels of N6AMT1 isoforms in cells [42]. Unlike WBSCR22 and METTL5, downregulation of TRMT112 had no effect on N6AMT1 protein levels, suggesting that N6AMT1 is also stable alone and potentially has other functions independent of TRMT112 and its MTase activity [42].

2.4.5.2. N6AMT1 might have methyltransferase activity towards lysine and DNA

In addition to functioning as a glutamine MTase, the N6AMT1-TRMT112 complex has been suggested to function as a lysine MTase. It has been shown that N6AMT1 monomethylates lysine 12 of histone H4 (H4K12) *in vitro* and *in vivo* [40] and lysine 596 of the pyruvate dehydrogenase subunit dihydrolipoamide transacetylase (DLAT) in prostate cancer cells, but not in other cancer cells [114]. In contrast, another *in vitro* study recently showed that N6AMT1 has a much higher affinity for glutamine than lysine at the peptide and protein levels [115]. Furthermore, N6AMT1 strongly favoured eRF1 sequence as a substrate over the H4K12 sequence and although N6AMT1 displayed H4K12 methylation activity *in vitro*, this methylation was notably weak. In contrast, the MTase belonging to the SET-domain class, SETD6, exhibited significantly higher activity towards H4K12 than N6AMT1 [115]. Additionally, another study demonstrated that knockdown of the N6AMT1 homologue HemK2 in fruit fly germ cells results in loss of methylated eRF1, but no change in H2K12 levels [116]. Nonetheless, the N6AMT1 status on whether it is a lysine MTase needs further investigation.

Apart from being a glutamine and lysine MTase, N6AMT1 has also been proposed to act as a DNA N^6-methyl-deoxyadenosine (m^6dA) MTase in human cells [117–120]. Initially, N6AMT1 was thought to be a SAM-dependent DNA MTase because it possesses the NPPy domain that is characteristic of bacterial N6-adenine and N4-cytosine DNA MTases [121,122]. However, several studies have argued that N6AMT1 is an unlikely m^6dA MTase because of the negatively charged substrate-binding pocket surrounding the N6AMT1 active site, which is not suitable for DNA binding. These studies have also failed to detect any biochemical activity of N6AMT1 towards DNA or any change in m^6dA levels after N6AMT1 depletion in human cells [39,123,124] or fruit fly germ cells [116]. Moreover, there is an ongoing debate on how widespread m^6dA is and whether it is a true epigenetic mark in the human genome, as ultrasensitive antibody-free methods have detected very low levels of m^6dA in mammalian cells [125–127]. It has been suggested that instead of being a heritable epigenetic DNA marker in mammalian cells, its source could be the nucleotide salvage pathway, where free ribo-N6-methyladenosine is processed to the respective triphosphate and then misincorporated into DNA by DNA polymerases [126].

2.4.5.3. Diverse functions of N6AMT1 in cellular processes and disease

Although the targets of N6AMT1 MTase activity are under debate, N6AMT1 is an intriguing protein with important functions in mammalian cells. N6AMT1 has recently been implicated in the regulation of cellular energy processes, with an unexpected role in mitochondrial function. N6AMT1 has been shown to regulate mitochondrial gene expression in the cytosol by facilitating the translation of key components of the mitochondrial RNA processing machinery, specifically TRMT10C (MRPP1) and PRORP (MRPP3), which are two subunits of the mitochondrial RNAse P enzyme. Loss of N6AMT1 leads to failure of mitochondrial RNA processing and accumulation of unprocessed and double-stranded RNA, which ultimately disrupts mitochondrial protein synthesis and oxidative phosphorylation [128]. Another study has recently shown that N6AMT1 is involved in the regulation of the pyruvate dehydrogenase complex. The pyruvate dehydrogenase complex is involved in the regulation of mitochondrial metabolism by catalysing the oxidative decarboxylation of pyruvate to acetyl-coenzyme A (acetyl-CoA). N6AMT1 knockdown in prostate cancer cells leads to increased levels of pyruvate, NAD+, and lactate, whereas the level of acetyl-CoA is reduced [114].

N6AMT1 is an essential protein during mammalian embryonic development, as loss of N6AMT1 leads to early embryonic lethality in mice [129]. Furthermore, data from the N6AMT1 homologue in *Drosophila* HemK2 have revealed that the loss of HemK2 in germline cells leads to arrested oogenesis and female fruit fly sterility, likely due to the loss of eRF1 methylation [116]. Similarly, it was recently shown that N6AMT1 function is required during meiosis of mouse spermatocytes; in particular, N6AMT1 knockout induces meiotic arrest during Prophase I and an increase in spermatocyte cell death [130]. The data also show that the loss of N6AMT1 affects the expression of various genes involved in DNA double-strand break (DSB) repair and results in delayed DSB repair during mouse spermatogenesis [130]. Controlled double-stranded break repair is crucial for genetic material exchange during meiotic chromosome recombination between homologous chromosomes, which occurs during Prophase I [131]. It remains to be elucidated whether N6AMT1 affects DSB repair only in spermatocytes or in other cell types.

Moreover, N6AMT1 has been implicated as having an important role in cancer development. N6AMT1 has been shown to be highly expressed in various cancers [40,132,133]. Several studies have reported that N6AMT1 is involved in the regulation of cell proliferation, migration, invasion, and cell cycle gene transcription in multiple cancer cell lines [40,118,133–136]. Inhibition of N6AMT1 has been suggested as a potential cancer therapeutic strategy. In particular, a study has shown that N6AMT1 is a promising target in bladder cancer and conventional therapy-resistant prostate cancer therapeutics, as N6AMT1 silencing reduced cancer cell proliferation and migration, and even restricted xenograft growth in mice [135,136]. Potentially, N6AMT1 could also be used as a drug target in other forms of cancer, as multiple studies have shown that depletion of N6AMT1 leads to decreased cell proliferation, migration, and invasive capabilities in various cancer cell lines, apart from breast cancer, where knockdown of N6AMT1 enhances the proliferation and migration of cancer cells [118]. Knockdown of N6AMT1 has also been associated with the induction of conventional apoptosis pathways in prostate cancer cells [40], as well as increased non-apoptotic cell death in lung cancer cells [134]. It has been suggested that N6AMT1 participates in maintaining cancer stem cell properties in colorectal cancer, while its depletion drastically reduces colorectal tumorigenesis in mice and prevents the growth of both murine and human patient-derived tumour organoids [133].

However, studies have indicated that N6AMT1 depletion may not have a beneficial therapeutic effect in all cancer types, as a recent study demonstrated that N6AMT1 is normally highly expressed in oestrogen receptor-positive breast cancer cells but is downregulated in tamoxifen-resistant cells. In experimental models, N6AMT1 overexpression enhanced tamoxifen sensitivity, whereas knockdown reduced sensitivity, suggesting its potential as a biomarker for oestrogen-receptor-positive breast cancer and a therapeutic target for overcoming tamoxifen resistance [137].

Finally, several studies have suggested that N6AMT1 may be involved in arsenic metabolism in human cells, as it has been shown that N6AMT1 converts monomethylarsonous acid (MMA^{III}) to the less toxic dimethylarsinic acid (DMA) in human cells [138]. Despite this, its activity is minor compared to that of the arsenic (+3 oxidation state) MTase AS3MT [139]. Furthermore, studies that looked at certain single nucleotide polymorphisms (SNP) have found contradictory results; N6AMT1 genetic polymorphisms were associated with arsenic methylation in a study in Northern Argentina [140] and China [141], but not in Chile [142] or Spain [143].

Overall, N6AMT1 is an important protein with potentially diverse functions and future studies are necessary to address the contradictory results about its methylation substrates and cellular effects.

2.5. Eukaryotic cell division

MTases can be considered key regulators of various biological processes inside the cells, including regulation of cell division, also called the cell cycle, through various direct and indirect effects. For example, MTases can directly regulate cellular signalling and division by methylating one of the four core histones, which are subjected to multiple post-translational modifications [144]. Histone methylation mostly occurs at Lys, Arg, or His residues [145], but a methylated glutamine residue has also been detected in yeast and mammalian cells [146]. Histone methylation directly affects the physical properties and organisation of chromatin, which influences gene expression by either activating or suppressing cell-cycle-related gene transcription, thus playing a direct role in cell cycle regulation [145].

MTases can also indirectly affect the cell cycle, for example by regulating the enzymatic activity of numerous other proteins that are key players in cellular signalling cascades that determine biological processes inside the cell. Furthermore, various kinases and proteins involved in mediating phosphorylation modifications are known substrates of MTases [147]. Methylation of residues may influence protein-binding properties, structure, enzymatic activity, subcellular localisation, and interactions with other proteins, all of which may play a role in the regulation of the cellular signalling process that determines cell division [147].

To provide more context of cellular division, the key regulatory proteins and processes of the eukaryotic cell cycle will be described in more detail in the following sections.

2.5.1. Eukaryotic cell cycle

The cell cycle can be divided into two parts: interphase, which consists of G1, S, and G2 phases, where the cell duplicates its cellular contents, and the M phase, where the cell segregates its cellular contents and divides into two daughter cells (Figure 4). The cell can also exit the cell cycle and enter the non-proliferative G0 cell cycle (Figure 4), which is called quiescence. Quiescence is typically reversible, and in favourable conditions, cells can return to the G1 phase and subsequently initiate DNA replication again [148–150].

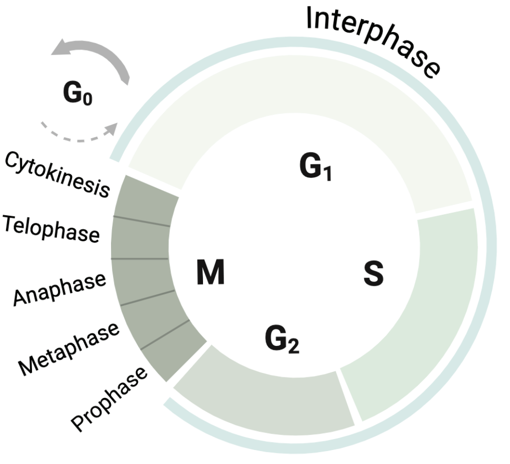

Figure 4. Schematic representation of the cell cycle. Figure was created using BioRender.com.

The main regulators of G1, S, G2, and M phase transitions are a family of serine-threonine kinases called cyclin-dependent kinases (CDKs) and their associated cyclin subunits. The main CDK family regulators of the cell cycle are CDK1, CDK2, CDK4, and CDK6 (Figure 5). As schematically depicted in Figure 5, CDK4/6 binds with cyclin D, CDK2 pairs with Cyclins E and A during G1-S phases, while CDK1 associates with cyclins A and B for mitotic entry initiation [151]. CDK activity throughout the cell cycle is mostly regulated by phosphorylation, where some phosphorylation modifications activate CDKs, while others inhibit their activity. CDK phosphorylation is dynamic, and CDKs are phosphorylated and dephosphorylated at specific sites throughout the cell cycle [152]. CDK activity is also regulated by two families of specific CDK inhibitors: the INK4 and Cip/Kip families. INK4 inhibitors consists of 4 members, p15INK4A (p15), p16INK4A (p16), p18INK4C (p18), p19INK4D (p19), while the Cip/Kip family has three members – p21Waf/Cip1 (p21), p27Kip1 (p27) and p57Kip2 (p57) [153,154]. INK4 inhibitors specifically inactivate CDK4/6 through competitive binding, preventing them from forming a complex with cyclin D. Cip/Kip inhibitors bind to and inactivate G1 phase CDK-cyclin complexes [155–157].

CDK levels remain consistent during the cell cycle; in contrast, cyclin protein levels rise and fall at specific times during the cell cycle (Figure 5). Cyclin levels are regulated by transcriptional regulation and ubiquitin-mediated degradation at specific times and through distinct mechanisms in each cell cycle phase [149,158]. Cyclin-CDK complexes are responsible for expression regulation of various cell cycle genes as well as epigenetic regulation of various cell cycle-related proteins [150,157,158].

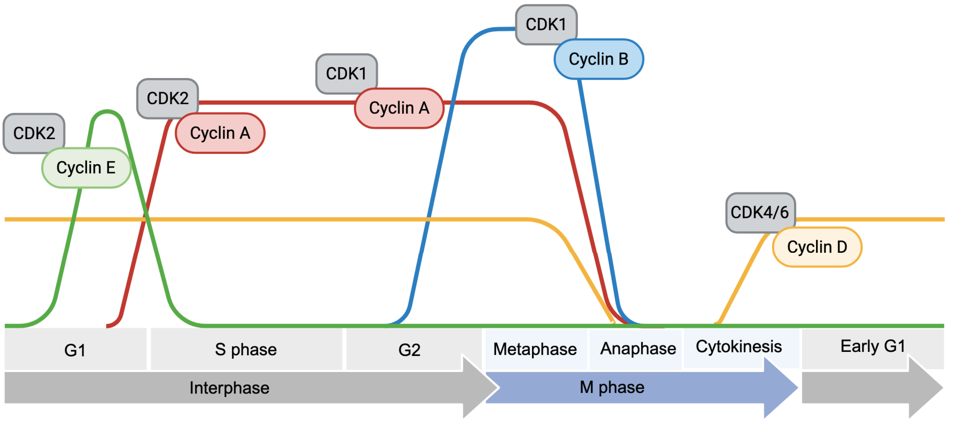

Figure 5. CDK and cyclin activity during the cell cycle. Figure adapted from Matthews, Bertoli and De Bruin, 2022 and created with BioRender.com.

A new cell division cycle starts immediately after cytokinesis, and the first part of the cell cycle is regulated mainly by cyclin D-CDK4/6, cyclin E-CDK2 and cyclin A-CDK2 (Figure 5). Cyclin D in a complex with CDK4/6 accumulates first to prevent cell cycle exit to quiescence. After cyclin CDK4/6, accumulation follows cyclin E-CDK2 accumulation and then cyclin A-CDK2 accumulation (Figure 5). More specifically, the build-up of the Cyclin E-CDK2 complex creates a positive feedback loop during the late G1-S phase transition, allowing for S phase entry and accumulation of Cyclin A-CDK2 complexes [148,150,159,160].

During the G1-S transition, a crucial part of the commitment to enter the S phase is the activation of the E2F transcription factor network. The E2F network is a family of transcription factors and their dimerisation partner proteins, called pocket proteins (most importantly retinoblastoma protein (RB), p107, and p130), which are typically associated with transcription activation or repression. E2F network proteins bind to E2F-regulated gene promoters at different stages of the cell cycle and are therefore involved in cell cycle control, DNA replication, and regulation of growth mechanisms [159,161]. Interaction with the RB pocket protein arguably plays the most important regulatory role for E2F transcription factors. During the pre-replicative stage of the G1 phase, RB in its active unphosphorylated or mono-phosphorylated by cyclin D-CDK4/6 form suppresses E2F-dependent transcription initiation. Afterwards, RB is inactivated from hyper-phosphorylation by gradually increasing cyclin E-CDK2, which creates the positive feedback loop that further increases the cyclin E-CDK2 as well as cyclin A-CDK2 activity and drives the S phase entry [150,159,162,163].

After the S phase, the cell enters the G2 phase, where the inactivation of CDK2 and activation of CDK1 and subsequent accumulation of Cyclin A-CDK1 and Cyclin B-CDK1 complexes drive mitotic (M phase) entry (Figure 5). CDK1 is kept inactive at the beginning of the cell cycle through inhibitory phosphorylation by WEE1 and MYT1 kinases. CDK1 activation occurs when the CDC25 phosphatase removes these inhibitory phosphorylation modifications. The decision to enter mitosis is tightly regulated by the balance between WEE1, MYT1, and CDC25 activities, as well as additional layers of regulation that together ensure that a high level of CDK1 activity is switched on at the right time at mitotic entry [152,164–166].

At the mitotic entry trigger point, CDK1 phosphorylates more than a thousand of its substrates and, most importantly, activates the mitotic kinases PLK1, Aurora A, and Aurora B, which then phosphorylate their substrates [167,168]. This sudden increase in phosphorylation is crucial for mitotic progression, as it triggers widespread structural changes inside the cell. It prepares the cell for DNA separation and division, for example, cell rounding, centrosome reorganisation, loss of the nuclear envelope, and mitotic spindle formation. CDK1 activity remains high during mitosis through cyclin B interaction until the APC/C^{CDC20} (anaphase-promoting complex/cyclosome and cell division cycle 20) complex triggers cyclin destruction to enable mitotic exit, effectively resetting the cell cycle in daughter cells to the beginning of the G1 phase [150].

2.5.2. Cell cycle checkpoints

Errors during the cell cycle can lead to the accumulation of cells with genetic errors, leading to the development of various diseases, such as cancer. To prevent this, there are several important decision-making windows during the cell cycle, where the cell can either commit to continue or block further cell cycle progression. This decision largely depends on cell cycle checkpoints, which monitor cells for defects and, if needed, can trigger either temporary cell cycle arrest or permanent exit through quiescence (G0), senescence, or apoptosis, depending on the stage of the cell cycle and damage severity [150,169,170]. As schematically depicted in Figure 6, there are three main cell cycle checkpoints that function based on evolutionarily conserved signalling pathways: the DNA damage checkpoint, DNA replication stress checkpoint, and spindle assembly checkpoint [150,169].

The DNA damage checkpoint functions throughout the interphase to prevent genetic errors during cell division, with separate actions during the early (G1) and late (S/G2) interphase (Figure 6). It is activated by double-stranded DNA breaks and relies on checkpoint protein kinases ataxia telangiectasia mutated (ATM) and checkpoint kinase 2 (CHK2) to stop cell cycle progression. In early G1, ATM-CHK2 activates p53 via phosphorylation. Phosphorylated p53 activates the CDK inhibitor protein p21, which blocks Cyclin E/A-CDK2 activity and prevents S phase entry. In late interphase (S/G2), the response involves ATM-CHK2 with CHK2-dependant phosphorylation and subsequent degradation of tyrosine phosphatase CDC25 and nuclear kinase WEE1-dependent phosphorylation and subsequent inhibition of CDK1 to block mitotic entry [171,172].

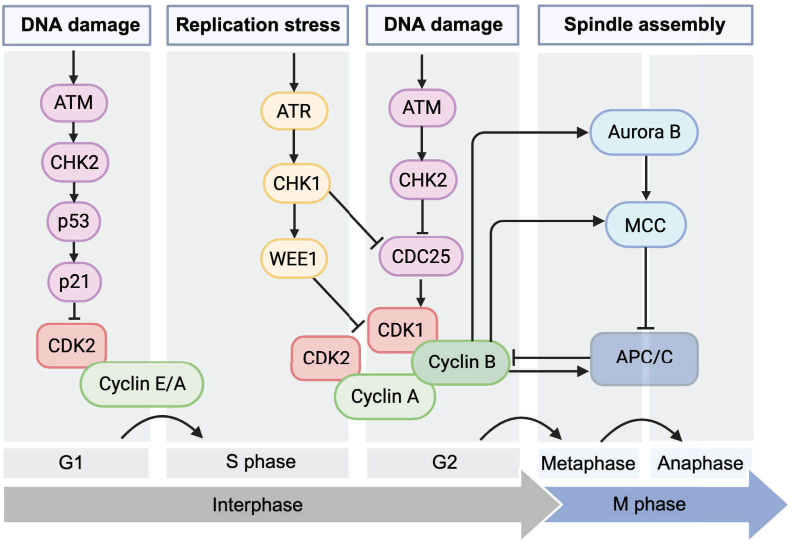

Figure 6. Cell cycle checkpoints. Figure adapted from Matthews, Bertoli and De Bruin, 2022 and created with BioRender.com.

The DNA replication stress checkpoint operates during the S phase to ensure error-free genomic DNA duplication and prevent DNA damage caused by replication stress (Figure 6). When DNA replication forks encounter obstacles and stall, single-stranded DNA becomes exposed, which activates checkpoint protein kinases, including ataxia telangiectasia and Rad3-related protein (ATR) kinase and its downstream effector CHK1. Similar to the DNA damage checkpoint, the activated checkpoint prevents mitotic entry by restricting CDK activity through two main mechanisms. The first one is CDC25 phosphorylation by CHK1 (CHK2 in the DNA damage checkpoint) and subsequent degradation, and the second one is WEE1 activation, which then mediates the phosphorylation and inactivation of CDK1/2 [149,150,165,173].

The final checkpoint is the spindle assembly checkpoint (SAC) (Figure 6). SAC is a multiprotein complex that helps ensure that DNA is distributed equally between daughter cells during the M phase by acting as a surveillance mechanism that looks for errors in chromosome attachment to kinetochores. When an unattached chromosome is detected, SAC is recruited to the site because of the phosphorylation of its component proteins by Aurora B and CDK1. The SAC then catalyzes the formation of the mitotic checkpoint complex (MCC), consisting of MAD2, BUB3, BUBR1, and CDC20, which inhibits APC the C^{CDC20} complex that subsequently preventing anaphase initiation. This cell cycle arrest is maintained until all chromosomes are correctly attached to the spindle microtubules. Once all kinetochores are properly attached and in the correct orientation, the SAC is deactivated, allowing APC/ C^{CDC20} activation, cyclin B degradation, and anaphase initiation [170].

2.5.3. Role of TRMT112-network methyltransferases in cell cycle regulation

Dysregulation of cell cycle is one of the hallmarks of cancer, as the components of cell cycle signalling pathways and checkpoints are often altered in cancer cells [154]. The common factor among the TRMT112-network MTases is that their deregulation has been linked to carcinogenesis (Table 1). Furthermore, as discussed above, loss of various TRMT112-network MTases have been linked to cell proliferation rate changes in healthy cells and/or different cancer cell lines [40,48,67,72,118]. Slower or faster proliferation rate in various TRMT112-network MTase-deficient cells indicates that the cell cycle is either prolonged or faster, respectively. However, currently, as discussed above, it is known that TRMT112-network MTases are important post-transcriptional regulators of protein synthesis, but there is limited knowledge about how exactly different TRMT112-network methyltransferases impact cell proliferation. The TRMT11-network member that has been the most directly implicated in cell cycle regulation is N6AMT1, where it has been demonstrated from several studies that loss of N6AMT1 leads to changes in cell proliferation rate and differentially expressed cell cycle genes in various cancer cell lines [40,118,133–136]. As discussed above, it has been proposed that N6AMT1 acts as a lysine 12 of histone H4 methyltransferase, thus directly mediating gene expression of cell cycle genes [40], but recently contradictory results have been published suggesting that N6AMT1 possesses minimal, if any, MTase activity towards H4K12 [115,116]. Therefore, the exact role of how N6AMT1 impacts cell cycle remains unclear and deserves further research.

3. AIMS OF THE STUDY

Dysfunction of methyltransferases can lead to numerous diseases, ranging from neurodegenerative disorders to cancer. Therefore, it is necessary to fully understand the complex functions and interaction networks of these proteins in human cells. In eukaryotic cells, the methyltransferase cofactor TRMT112 and methyltransferases that bind to TRMT112 are important post-transcriptional regulators of the protein synthesis machinery. Nonetheless, knowledge about the TRMT112-network MTases and their functions in human cells is still limited. When the work on this thesis research started, TRMT112 was confirmed to interact with four MTases in human cells: WBSCR22 [11], N6AMT1 [17], ALKBH8 [30], and METTL5 [16]. The crystal structures of TRMT112 and its homologues in yeast have also revealed that TRMT112 interacts with all the partners in a similar way, by covering a large hydrophobic area of the partner protein, thus increasing its solubility and stability [16,18,25,37–40,43]. As the knowledge about TRMT112 interactome in human cells at the time was rather limited, we hypothesised that there are potentially undetected TRMT112-interacting methyltransferases in human cells and subsequently set out to investigate the TRMT112 and methyltransferase interactions in more detail.

In the second part of this study, we focused on the TRMT112 interaction partner N6AMT1, which is characterised by contradictory data regarding its methylation substrates and cellular effects. Prior studies had suggested that N6AMT1 might be involved in cell cycle regulation, as depletion of N6AMT1 leads to differentially expressed cell cycle genes and a reduction in cell proliferation rate [40,133,134]. This data coupled with our observation of centrosomal localisation signal in immunofluorescence analysis after staining with N6AMT1 primary antibodies in cells, led us to hypothesise that N6AMT1 indeed affects human cell cycle regulation and might have a particular role during mitosis.

Therefore, the aims of this thesis were as follows:

1. Identify MTases that form a stable complex with the cofactor TRMT112 in human cells.
2. Investigate the TRMT112 and MTase interactions in more detail by analysing point mutations on the TRMT112 surface.
3. Investigate if and how TRMT112 interacting MTase N6AMT1 is associated with cell cycle regulation in human cells.
4. Assess the specificity of commercial N6AMT1 antibodies.

4. MATERIALS AND METHODS

All materials and methods used in this dissertation are described in greater detail in the corresponding publications. This section provides a brief overview of the materials and methods used in this study.

Open reading frames for TRMT112-network MTases were amplified from the cDNA obtained from U2OS cells and cloned into pQM (Icosagen Ltd., Tartu, Estonia) and pEGFP-C1 plasmid vectors. In all cases, EGFP and E2Tag were located at the N-terminus of the MTase. TRMT112-EGFP and pQM-TRMT112 plasmids were used to introduce mutations into the TRMT112 sequence. Mutagenesis was performed using the polymerase incomplete primer extension method (Study I). The open reading frame for Aurora kinase A (Uniprot accession Q5QPD1) was amplified from cDNA obtained from U2OS cells and cloned into pEGFP-C1 and pEGFP-N1 plasmids (Study II).

Human osteosarcoma cells line U2OS were grown in complete culture media Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% foetal calf serum (FCS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were incubated at 37 °C in a 5% CO2 environment (Studies I, II, III). N6AMT1 knockout cell lines were generated from U2OS cell line using the pSpCas9(BB)-2A-Puro (PX459) V2.0 plasmid vector (Cat no 62988, Addgene, USA), and the absence of N6AMT1 protein expression was confirmed by immunoblotting, qPCR, and Sanger sequencing (Study II).

Cell proliferation was determined using the xCELLigence Real-Time Cell Analyser (RTCA SP Instrument, Version November 2009, ROCHE, USA) according to the manufacturer's protocol (Study III). Cell cycle phase distribution in a cell population was studied by flow cytometry using propidium iodide staining (Study III). Analysis was performed using the FlowJo software (Beckton Dickinson, Franklin Lakes, NJ, USA). The cell cycle was studied by synchronising cells in different cell cycle phases. Unsynchronized cell populations were collected 18 h after seeding. The starved cell population (G0/G1 arrested cells) was first seeded in complete growth media to allow the cells to attach. After cell attachment, the medium was replaced with serum-depleted medium, and the samples were collected 24 h later. Cells were arrested in the S phase with double thymidine treatment. Cells were treated with 2 mM thymidine for 16 h and then released for 6 h in complete culture medium, followed by another 16 h of treatment with 2 mM thymidine, after which the cells were collected. The mitotic cell population was obtained after incubation with 50 ng/ml nocodazole for 10 h, followed by mitotic shaking. Only mitotic cells were collected (Study III).

Plasmid vectors were transfected into U2OS cells using electroporation (Studies I, II, and III). Immunoprecipitation was used to study MTase-TRMT112 interactions 48h after transfection with protein expression plasmids. Cells were lysed with IP lysis buffer, and proteins were immunoprecipitated using GFP-Trap magnetic beads (ChromoTek GmbH, Munich, Germany) according to the manufacturer's protocol (Study I).

Endogenous and overexpressed proteins were detected and quantified using western blotting (Studies I, II, III) and flow cytometry (Study I), while protein localisation was studied using immunofluorescence assays (Studies I, III). Western blot images were quantified using the ImageJ software. The expression of all proteins of interest was normalised against the expression of GAPDH by calculating the protein of interest and GAPDH expression ratio. Graphs were prepared, and statistical analysis was performed using multiple unpaired t-test and Holm-Šidák method with GraphPad Prism 8.4.3 software (Study III). Proteomic analysis was performed at the Proteomics Core Facility of the Institute of Technology, University of Tartu (Study I and II).

MRNA levels in U2OS and N6AMT1 knockout cells were studied using quantitative PCR analysis. RNA was isolated from cells using the Zymo Quick-RNATM MiniPrep kit (Zymo Research, R1055), cDNA was synthesised using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, K1621) using random hexamer primers, and quantitative PCR (qPCR) was performed using a LightCycler 480 (Roche) with HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, 08-24-0000S) using the following conditions: 95 °C for 12 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 20 s (Study III).

Protein crystal structures were visualised using ChimeraX-1.9 software. This dissertation was spell-checked using the English language writing assistant software Grammarly (https://www.grammarly.com/) and the academic language style was edited using the academic writing software Paperpal (https://paperpal.com/).

5. RESULTS AND DISCUSSION

5.1. Human TRMT112 network consists of at least seven methyltransferases (Study I)

5.1.1. TRMT112 network consists of at least seven partners

At the time Study I was conducted, TRMT112 was confirmed to interact with four MTases in yeast cells: Bud23, Mtq2, Trm9, and Trm11 [26–28,106], as well as four MTases in human cells: WBSCR22, the homologue of Bud23 [11], N6AMT1, the homologue of Mtq2 [17], ALKBH8, the homologue of Trm9 [30], and METTL5 [16], which lacks a yeast homologue. Therefore, the initial goal of Study I was to determine whether additional stable TRMT112 interaction partners exist in human cells. From prior studies, it is known that TRMT112 forms a stable interaction with the partner MTase [16,18,25,37,38]; hence, it was decided to apply the immunoprecipitation method using recombinant TRMT112 with the small E2 tag of 15 amino acids as a bait. For this, a stable TRMT112-E2tag expressing cell line was developed from U2OS cells and used to perform stable isotope labelling by amino acids in cell culture (SILAC) assay coupled with co-immunoprecipitation (co-IP) assay and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Figure 1A, C; Study I).

LC-MS/MS analysis identified seven MTases as the most abundant TRMT112 interaction partners: N6AMT1, WBSCR22, METTL5, ALKBH8, TRMT11, THUMPD2, and THUMPD3, as schematically depicted in Figure 7. All TRMT112-MTase interactions were further confirmed by expressing GFP-tagged MTases in U2OS cells and verifying through IP that the GFP-tagged MTase interacted with endogenous TRMT112 (Fig 1E; Study I)

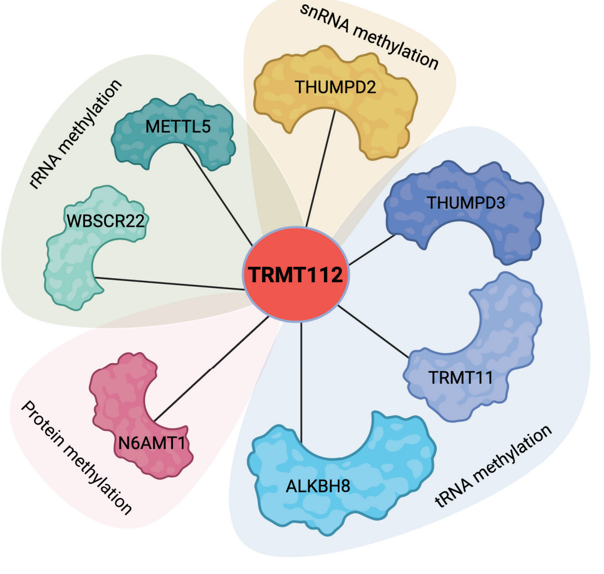

Figure 7. Schematic representation of the TRMT112 interactome in human cells. Methyltransferases that interact directly with TRMT112 are grouped based on their enzymatic activity.

Four of the identified MTases, WBSCR22, ALKBH8, N6AMT1, METTL5 (Figure 1D, E, F; Study I) have been previously described as TRMT112 partners not just in human cells, but also in other organisms as well [11,16,17,19,30,40, 46,47,92]. Three identified MTases were confirmed as novel interaction partners of TRMT112 in mammalian cells: THUMPD2, THUMPD3, and TRMT11 (Figure 1D, E, F; Study I). At the time of initial manuscript preparation, THUMPD3 was classified as a novel TRMT112 partner. During later stages of manuscript preparation, a study by Yang et al. also showed that THUMPD3 is a TRMT112-interacting MTase [32]. TRMT11 homologues in yeast [28,43] and archaea [20] have been shown to interact with TRMT112 in previous studies; however, this is the first time mammalian TRMT11 has been confirmed as a TRMT112 interaction partner. Another study recently confirmed that the TRMT112-MTase network consists of at least seven stable interaction partners, including TRMT11, THUMPD2, and THUMPD3 [21].

Despite this, it cannot be excluded that there are more unidentified TRMT112 interaction partners. Affinity-based techniques, such as SILAC coupled with co-IP, may not capture transient or weak interactions or interaction partners with low expression levels. A novel study recently applied the proximity-dependent biotin identification (BioID) method for more complex TRMT112 interactome identification. This approach allows the identification of transient and weak interactions, but does not distinguish between direct interaction or whether proteins are only closely localised in the same area [21]. Using this method, the authors were able to identify a more complex list of potential TRMT112 interactors beyond MTases, such as various kinases, protein MTases, and components of the ubiquitination machinery, which might include potential TRMT112 interactors and components of the TRMT112-MTase complexes [21]. In our SILAC data, we similarly identified low levels of various kinases, components of the ubiquitination machinery, components of the cytoskeleton, and various ribosomal proteins. A more complex TRMT112-interactome investigation beyond MTases certainly deserves further attention.

Nonetheless, the study by Wang et al. [21] failed to identify two well-studied TRMT112 interactors, WBSCR22 and N6AMT1, confirmed in multiple prior studies [11,17,25,26,37,40,45], including the present Study I. In Study I, as well as in the study by Wang et al. [21], TRMT112 was tagged at the C-terminus. In the case of TRMT112, the N-terminus is not suitable for a tag, as the crystal structures of TRMT112 together with METTL5 [16] and N6AMT1 [39,40] reveal that the N-terminus of TRMT112 is directly engaged in forming an interaction with its partner MTase. In our study, the plasmid vector of choice for recombinant TRMT112 expression was pQM with a small E2 tag (46 base pairs), adding 15 amino acids to TRMT112, which is a minor addition relative to the endogenous TRMT112 size (125 amino acids). The plasmid vector of choice in the study by Wang et al. [21] was pcDNA3.1 MCS-BirA(R118G)-HA (Addgene), which adds an approximately 1000 base pair tag to TRMT112, which is more than two times longer than TRMT112 itself. We were able to identify WBSCR22 and N6AMT1 interactions using the TRMT112-EGFP recombinant protein, which similarly adds a large, approximately 700 bp tag to TRMT112 (Figure 1F; Study I), suggesting that a large tag does not interrupt the TRMT112 interaction with N6AMT1 and WBSCR22. The authors suggested that WBSCR22 might be resistant to biotinylation for some unknown reason [21]. Nevertheless, it can be argued that to determine and confirm the entire TRMT112 interactome, a multimethod approach should be used, as it is difficult to capture all of the various TRMT112 interactions with a single method.

5.1.2. TRMT112 stabilises interacting methyltransferases

Next, while co-transfecting EGFP-fused MTases with E2tagged TRMT112, we noticed a prominent mutual stabilising effect in U2OS cells (Figure 3A, B; Study I). The stabilising effect of recombinant TRMT112 was visible for all recombinant MTase partners, with the most prominent effect observed for N6AMT1 and METTL5 (Figure 3A, lines 2–3,9–10; Figure 3B; Study I). This is consistent with prior studies showing stabilisation by TRMT112 for WBSCR22 [11,19,26] and METTL5 [16] proteins. Various crystal structures have revealed that the interaction between TRMT112 and its MTase partners covers a large hydrophobic area of the MTase and a smaller area on TRMT112 (Figure 2). If this hydrophobic area is exposed, it leads to protein instability and subsequent degradation [11,16,19,26,42]. This is consistent with prior studies that revealed that METTL5 and WBSCR22 levels are negatively affected by endogenous TRMT112 downregulation [11,16]. Intriguingly, this is not the case for N6AMT1 [42], even though a significant stabilisation effect can be observed between recombinant N6AMT1 and TRMT112.

Subsequently, it was also observed that all recombinant MTases, apart from THUMPD2, stabilised recombinant TRMT112 expression in U2OS cells (Figure 3C, lanes 2–8; Study I). Furthermore, the expression of recombinant TRMT112 reduced the endogenous TRMT112 level in the cells, suggesting that E2tagged TRMT112 is functional and that the overall TRMT112 level in the cells is tightly controlled (Figure 3C; D; Study I). However, we did not detect an increase in endogenous MTase protein levels, while observing a decrease in endogenous TRMT112 after recombinant TRMT112 overexpression (Figure 3D; Study I). These data suggest that the TRMT112 level in the cells is tightly controlled and that the cellular MTase level is not increased simply by increasing the TRMT112 level.

In summary, we observed a strong mutual stabilisation effect of MTases and the TRMT112 complex in U2OS cells by transiently co-expressing both proteins. Additionally, we detected a reduction in endogenous TRMT112 levels after overexpressing recombinant TRMT112. It is currently unknown how much TRMT112 protein is present in cells as a monomer and how much is in a complex with other proteins. It is also unknown how stable TRMT112-MTase heterodimers are in cells and whether they disassociate once formed in favour of a partner with a stronger binding affinity. Overall, these data suggest a dynamic network in which MTases might compete for interaction with TRMT112, while the internal levels of TRMT112 and potentially the MTases are tightly controlled.

5.1.3. TRMT112 interacts with methyltransferases similarly, but differences exist

Prior studies from various organisms have revealed that TRMT112 interacts with the same region of all of the MTase partners studied so far in a similar manner [16,18,20,23,25,37–41]. To analyse whether TRMT112 interacts with its partner MTases in a similar manner in mammalian cells, we mutated several amino acids on TRMT112 that were chosen based on an *in silico* analysis and crystallography data, as described in the Methods section of Study I. The locations of the mutations on the TRMT112-METTL5 interface are shown in Figure 8. In summary, hydrophobic amino acids shown to be involved in hydrophobic interaction formation with MTases in prior studies [16,23,39] were mutated accordingly, as depicted in Figure 8: leucine 8 was replaced with tryptophan (L8W), methionine 45 was replaced with alanine (M45A), and isoleucine 113 was replaced with phenylalanine (I113F). Polar amino acid threonine 5 was mutated to hydrophobic alanine (T5A), and serine 10 was mutated to hydrophobic phenylalanine (S10F). Charged amino acid lysine 48 was mutated to hydrophobic alanine (K48A) mutation. Additionally, we created mutants E50A, E92A, F107A, and a double mutant K48A + E92A.

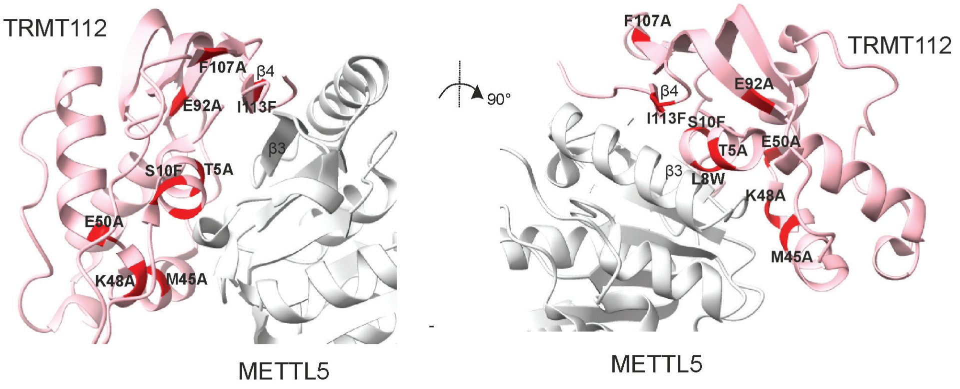

Figure 8. Localisation of the TRMT112 point mutations on the crystal structure of the TRMT112 (pink) and METTL5 (grey) complex (PBD accession code 6H2U). The mutated amino acids are shown in red.

We created E2Tag TRMT112 mutant protein plasmid expression vectors and expressed them in U2OS and Hek293 cells (Figure 4C,D; Study I). In U2OS, the mutation S10F increased the expression of recombinant TRMT112, whereas T5A, M45A, E92A, F107F, and K48A + E92A mutations reduced the expression of recombinant TRMT112. In contrast, the L8W, K48A, and E50A mutations did not have a noticeable effect on TRMT112 expression (Figure 4C; Study I). Similar results were obtained with Hek293 cell line as well (Figure 4D; Study I).

To study mutant TRMT112 interactions with MTases, EGFP-MTases were co-transfected with E2Tagged wild-type TRMT112 or its mutants, and immunoprecipitation assay was performed using EGFP-MTase as bait (Figure 4E; Study I). The results were analysed using western blotting (Figure 5; Study I). The results revealed that TRMT112 mutant-MTase interactions have several similarities, but individual characteristics could also be observed for each MTase. N6AMT1 interaction with TRMT112 appears to be one of the most stable among all MTases, as this interaction was only significantly disturbed by the loss of hydrogen-bond-forming T5 (Figure 5A; Study I). Crystal structure of the N6AMT1-TRMT112 complex has revealed that T5 of TRMT112 forms a hydrogen bond with Q98 of N6AMT1 and it can be concluded that this is a crucial bond for the protein complex stability [39]. In contrast, WBSCR22 was the only MTase that could interact with the T5A mutant in a stable manner, whereas the interaction was unexpectedly prevented by the F107A mutant (Figure 5B; Study I). Currently, there is no available crystal structure of TRMT112 and WBSCR22 in human cells, but, it is most likely that the benzyl group of TRMT112 F107 forms a bond with WBSCR22, which is highly important for TRMT112 and WBSCR22 protein stability.

TRMT112 interaction with METTL5 was disturbed the most by T5A, L8W, and M45A (Figure 5C; Study I). The crystal structure of METTL5 with TRMT112 revealed that T5 of TRMT112 forms a hydrogen bond with D103 of METTL5, whereas L8 and M45 are part of the hydrophobic core of TRMT112 [16]. These amino acids appear to form crucial bonds for TRMT112-METTL5 interaction stability. In the case of tRNA MTase ALKBH8 (Figure 5E; Study I), the TRMT112 interaction was most disturbed by T5A, M45A, and the double mutant K48A + E92A, whereas in the case of TRMT11 (Figure 5D; Study I), the greatest effect was observed for T5A, E92A, and the double mutant K48A + E92A. The MTases most affected by TRMT112 mutations were THUMPD2 and THUMPD3. Interaction with THUMPD3 (Figure 5F; Study I) was prevented by T5A, L8W, M45A, K48A, F107, I113F, and the double mutant. Meanwhile, THUMPD2 (Figure 5G; Study I) was most affected by T5A, S10F, K48A, F107, I113F, and the double mutant K48A + E92A.

Interestingly, we observed two types of effects of TRMT112 mutations. In the first case, the mutations resulted in the loss of the stabilising effect between TRMT112 and MTase. For example, this was the case for the T5A mutation, which in almost all cases displayed the loss of the stabilisation effect on the MTase (Figure 5, lines 3; Study I) and resulted in a completely reduced or lost interaction. In the other case, we observed a clear stabilisation effect, but the interaction was lost while using our immunoprecipitation method, suggesting that the MTase was interacting with the mutant, but the interaction was weak and easily disrupted. For example, this was the case with THUMPD3 and the mutants M45A, K48A, and I113F (Figure 5F, lines 6,7, 10; Study I) and THUMPD2 and mutants S10F and K48A (Figure 5G, lines 5 and 7; Study I), where the mutants exhibited a stabilisation effect, but the interaction was lost. In the case of ALKBH8 and the mutants T5A, M45A, and the double mutant K48A + E92A (Figure 5, lines 3,6,12; Study I), a stabilisation effect was observed; but, the interaction, although not completely lost, was significantly reduced.

In summary, the results obtained in Study I revealed that the TRMT112-network consists of at least seven MTases interacting with TRMT112, and MTases and TRMT112 mutually stabilise each other. Previous studies have suggested that TRMT112 binds to its partners in a similar manner. However, TRMT112 mutants revealed that specific differences can be observed in the binding of TRMT112 and MTases. N6AMT1, METTL5, WBSCR22, ALKBH8, and TRMT11 were the least affected by TRMT112 mutations, whereas THUMPD2 and THUMPD3 were affected the most. In the case of N6AMT1, METTL5, WBSCR22, and TRMT11, the effects of some of the TRMT112 mutations resulted in the loss of the stabilising effect on the partner MTase, which was already observed in the input. In the case of THUMPD2, THUMPD3, and to some extent ALKBH8, in addition to the mutations that disrupted the stabilisation effect, there were mutations that preserved the stabilisation effect while disrupting the stable interaction.

Currently, only the crystal structures of TRMT112-N6AMT1 [39] and TRMT112-METTL5 [16] complexes are available. The AlfaFold Protein Structure Database might be a useful tool to analyse the TRMT112-MTase interactions in more detail for the MTases lacking a crystal structure, especially with the release of the recent AlfaFold3 version which promises more accurate predictions of protein structures and protein-protein interactions [174]. Nonetheless, the data obtained through computational studies deserve experimental validation, and in future studies, crystal structures of TRMT112 and the other partner MTases could reveal more details about how TRMT112 and MTases interact. In particular, the crystal structures of THUMPD2 and THUMPD3 in a complex with TRMT112 might be especially intriguing, as these two MTases are the most affected by TRMT112 mutations.

5.2. TRMT112-network methyltransferase N6AMT1 participates in the cell cycle regulation (Study III)

Multiple studies have linked N6AMT1 to the regulation of cell proliferation and have shown that N6AMT1 downregulation leads to differentially expressed cell cycle genes [40,118,133–136]. During Study I and II, we observed through immunofluorescence staining with the N6AMT1 antibody HPA059242 (Atlas antibody) that in mitotic U2OS cells, the N6AMT1 antibody target localises at the centrosomes (Fig 2A; Study II). Owing to the prior data on N6AMT1 involvement in the cell cycle, coupled with the previously undescribed centrosome location of N6AMT1, we decided to focus on N6AMT1 as the main subject of our further research on the TRMT112 network MTases.

5.2.1. N6AMT1 disrupts the cell cycle in U2OS cells (Study III)

To study N6AMT1 functions in human cells, we used the CRISPR/Cas9 gene editing system to knockout N6AMT1 of U2OS cells targeting exon 1 of the N6AMT1 gene, as described in the methods section of Study III. Immediately, we observed a clear decrease in the proliferation rate of the N6AMT1 knockout cell line and an increase in the cell doubling time (Figure 1B,C; Study III). These initial results, coupled with prior studies, indicate that N6AMT1 may be connected to the regulation of the cell cycle. Therefore, next, we analysed whether N6AMT1 knockout affects a certain part of the cell cycle phase. For this, we compared the overall cell cycle distribution of U2OS cells and N6AMT1 knockout cells in an unsynchronized cell population, as well as cells that were synchronised with serum depletion, thymidine, and nocodazole treatments. Serum depletion arrests cells in the G1 phase, thymidine treatment arrests cells in the early S phase, and nocodazole treatment arrests cells in the early prophase (Figure 2A; Study III). Unsynchronized cell populations, serum depletion, and thymidine treatments did not reveal any noticeable differences between U2OS and N6AMT1 knockout cells (Figure 2B,D,E; Study III). In contrast, nocodazole treatment revealed a difference in the G1 population; approximately 20% of the cells remained in the G1 phase after nocodazole treatment, with only 7% in U2OS cells, suggesting that the lack of N6AMT1 may affect cell progression through the G1 phase of the cell cycle (Figure 2C; Study III).

As cyclin oscillations are at the core of cell cycle regulation, the protein levels of cyclins A, B, and E were analysed for U2OS and N6AMT1 depleted cells in unsynchronized cell populations, as well as for serum-depleted, thymidine-, or nocodazole-treated cells (Figure 3A; Study III). In the case of cyclin A and cyclin B levels, there were no noticeable differences apart from serum-depleted cells, where in both cases, cyclin A and B were increased in N6AMT1 knockout cells compared to U2OS cells (Figure 3A, C, and D; Study III). A considerable reduction in cyclin E protein levels was detected in N6AMT1 knockout cells and N6AMT1 siRNA-treated U2OS cells (Figure 3A, B, F; Study III). Interestingly, the cyclin E level was lower for all analysed cell cycle phases rather than being linked to a specific phase.

Next, we measured and compared the mRNA levels of cyclin A, B, and E in U2OS and N6AMT1 depleted cells to determine whether the lower cyclin E levels were due to mRNA level reduction or protein degradation. The results revealed that while cyclin A and B levels remained similar in both cell lines, cyclin E was downregulated in N6AMT1-depleted cells (Figure 3H; Study III), confirming that N6AMT1 regulates cyclin E expression at the mRNA level. Several RNA sequencing datasets have indicated that N6AMT1 depletion induces up and downregulation of various cell cycle-related genes, including genes that are directly related to cyclin E expression, for example RB, E2F1 and p53 [40,118,133,135]. Nevertheless, the exact mechanism by which N6AMT1 mediates the expression of these genes remains unclear.

It is suggested that N6AMT1 acts as a histone 4 lysine 12 methyltransferase which in turn might regulate the transcription of cell cycle-related genes [40]. In contrast, a recent study has shown that N6AMT1 methyltransferase activity towards H4K12 is very low [115], while another study failed to detect any change in H4K12 levels after N6AMT1 depletion in *Drosophila* cells [116]. N6AMT1 is also implicated as a DNA m6dA methyltransferase [119], which may lead to changes in the DNA methylation pattern in cyclin E and other cell cycle-associated genes, impacting gene transcription. Nonetheless, N6AMT1 status as a DNA m6dA methyltransferase has been disputed with several convincing studies demonstrating that it does not have DNA m6dA methyltransferase activity [39,116,123,124].

It is confirmed that N6AMT1 methylates the GGQ motif of the eukaryotic translation termination factor eRF1 [17], which may affect translation efficiency. Moreover, it has recently been shown in *Drosophila* germ cells that depletion of N6AMT1 results in ribosome stalling at stop codons of actively translated mRNA, thus activating the mRNA surveillance process, which targets mRNAs with stalled ribosomes for degradation [116]. This might explain the reduced cyclin E mRNA levels we observed. However, the authors did not observe any changes in cyclin E levels in their study. It cannot be excluded that the loss of cyclin E in our study is human-specific, as we used the human osteosarcoma cell line U2OS (Figure 3A, 2H; Study III) and human embryonic kidney cell line Hek293 (S1C, S1F; Study III). Another possibility to consider is that N6AMT1 might regulate cyclin E and other cell-cycle related genes through methylation of other protein targets, for example, it has been shown that N6AMT1 methylates glutamine residues or other substrates, such as the poorly characterised chromo-domain-helicase-DNA-binding protein 5 (CHD5), which is suggested to function as a chromatin remodelling enzyme [111,175].

Moreover, the loss of N6AMT1 has recently been linked to the dysregulation of DNA DSB repair mechanisms in mouse spermatocytes during meiosis [130]. Interestingly, in non-germ cells, DSB typically activate DNA damage check-points during interphase, which stalls further cell cycle progression by activating factors that inhibit cyclin and CDK complexes (Figure 6). It is possible that the downregulated cyclin E observed in our study is linked to an activated DNA damage checkpoint due to the N6AMT1 effect on DSB mechanisms and not due to a direct impact on cyclin E gene transcription. Nonetheless, the exact mechanism by which cyclin E transcription is regulated by N6AMT1 remains to be elucidated in further research.

5.2.2. N6AMT1-depleted cells are sensitive to serum removal

Our initial data revealed that N6AMT1 knockout cells exhibited elevated cyclin A and B levels after 24h of serum depletion compared to U2OS cells (Figure 3A; lines 3–4; Study III). Serum is typically added to cell cultures to provide a mixture of nutritional, hormonal, growth, and attachment factors. Serum depletion is often used to synchronise cells in the G1 population and to induce G0 arrest. However, it also dysregulates various cellular signalling pathways and has been shown to affect various processes, such as protein translation [176], as well as dysregulated cellular energetics, leading to excessive production of ROS and mitochondrial defects [177].

Therefore, we next analysed the ability of U2OS and N6AMT1 knockout cells to exit the G0/G1 block induced by 24 h of serum starvation (Figure 4B;

Study III). U2OS cells exhibited undetectable cyclin A, B, and E levels 24h post-starvation, typical for a G0 block [178], indicating that cells were successfully arrested in the G0/G1 phase (Figure 4C; Study III). 3h post-release, all cyclin levels gradually increased, indicating that U2OS cells re-entered the cell cycle (Figure 4C; Study III). In contrast, N6AMT1 knockout cells showed elevated cyclin A and B levels, which is inconsistent with a typical G0/G1 block (Figure 4D; Study III).

Prior study has shown that cyclin E-deficient cells can actively proliferate, but after G0 induction, they cannot re-enter the cell cycle [179]. As loss of N6AMT1 leads to cyclin E reduction (Figure 3A, lines 5–6; Study III), N6AMT1 may play a role in cell entry/exit from the G0 phase through its effect on cyclin E levels. The G0 phase, also called quiescence, has gathered attention as a mechanism how cancer cells evade traditional chemotherapy by becoming dormant for a period of time and then re-entering the cell cycle, thus causing cancer recurrence [178].

Recently, N6AMT1 has been linked to the regulation of mitochondrial activity through the translation regulation of two key components of the mitochondrial RNA processing machinery [128] and the regulation of the pyruvate dehydrogenase complex [114]. Our data indicate that after 24h of serum depletion, neither cell line proliferated, whereas cyclin levels remained elevated in N6AMT1 knockout cells. Therefore, the loss of N6AMT1 regulatory role in mitochondrial activity combined with serum removal may lead to a specific cellular response that results in atypical G0/G1 arrest (Figure 4A; Study III). One potential explanation for the phenotype observed in N6AMT1 knockout cells after 24 h of serum depletion could be the increased sensitivity of cells to the lack of nutritional, hormonal, growth, and attachment factors that are found in serum.

In summary, our data indicate that N6AMT1 knockout cells are highly sensitive to serum starvation; however, at this stage of this research, the reason behind this cannot be conclusively determined and deserves further investigation.

5.2.3. Loss of N6AMT1 leads to delayed progression out of S phase and mitosis

Next, to analyse cell progression through the cell cycle in more detail, cells were synchronised with thymidine and nocodazole treatments, then released and collected at certain time points and analysed by western blotting (Figure 5A and 6A; Study III).

In the case of S-phase arrest with thymidine, after release in both cell lines, cyclin B gradually increased and peaked at 18h (Figure 5B, C, E; Study III), while cyclin A gradually increased and reached a plateau at 9h and then decreased in U2OS cells (Figure 5B, D; Study III) and displayed a 9h delay compared to N6AMT1 knockout cells (Figure 5C, D; Study III). In U2OS cells, cyclin E levels remained high at 0–6h post-release and then dropped, indicating a progression to the S and G2 phases (Figure 5B, F; Study III). In contrast, N6AMT1 knockout cells did not exhibit a decrease in cyclin E levels, and the levels remained steady at all time points (Figure 5C, F; Study III). This indicates that the loss of N6AMT1 disrupts the classical pattern of up-and downregulation of cyclin E. As the overall level of cyclin E is reduced in N6AMT1 knockout cells throughout the cell cycle (Figure 3A; Study III), it could potentially mean that cyclin E has reached a minimum threshold to continue the cell cycle, leaving no margin to reduce it even further.

The model organism used in this study was the human osteosarcoma cell line U2OS, and previous studies have demonstrated that cyclin E is overexpressed in patients with human osteosarcoma [180]. Furthermore, high levels of cyclin E have been observed in various cancer types and are correlated with cancer aggressiveness [160]. In particular, increased cyclin E levels are correlated with increased genomic instability due to replication stress and mitotic defects [181]. Interestingly, cyclin E silencing inhibits osteosarcoma cell proliferation and increases sensitivity to the chemotherapeutic drug cisplatin [180]. N6AMT1 inhibitors have been proposed as potential candidates for novel treatment options in prostate cancer [136], and our results suggest that N6AMT1 and subsequent reduction of cyclin E could be investigated as a potentially beneficial treatment route in osteosarcoma therapeutics as well.

Another intriguing study has shown that loss of cyclin E results in decreased male fertility and unrepaired DNA double-stranded breaks [182], which correlated with results obtained by Luo et al. (2025), where the loss of N6AMT1 lead to meiotic arrest or spermatocytes during prophase I and decreased male fertility [130]. As noted above, the authors did not observe a reduction in cyclin E [130], suggesting that N6AMT1 and cyclin E may have separate roles and regulatory mechanisms in different cell types. Whether the reduction in cyclin E levels observed in U2OS cells is due to N6AMT1 MTase activity towards eRF1, additional substrates, or a completely novel function remains to be investigated.

Interestingly, in yeast cells, N6AMT1 knockout results in increased resistance to two microtubule-inhibiting drugs [105], while loss of N6AMT1 in prostate cancer cells leads to downregulation of various tubulin isoforms, which are the structural subunits that form microtubules [40], suggesting that N6AMT1 may play a role in microtubule organisation in eukaryotic cells. In this study, the microtubule-disrupting chemical nocodazole was used to synchronise cells in early mitosis using a method called mitotic shake (Figure 6A; Study III). In this method, adherent cells were treated with nocodazole and briefly shaken to detach the nocodazole-blocked mitotic cells from the plate. The media containing mitotic cells were subsequently collected, and the cells were pelleted, washed, and released in complete culture medium. In the case of mitosis block with nocodazole treatment, U2OS cells exhibited a sharp drop in cyclin A and cyclin B after the 0h timepoint and then gradually increased again at 12–18h (Figure 6B, D, E; Study III). Cyclin E levels exhibited a similar accumulation pattern in both cell lines (Figure 6A,B,F; Study III). Meanwhile, in N6AMT1 knockout cells, cyclin A and, most prominently, cyclin B gradually decreased over 2h (Fig 6C, D, E; Study III). Interestingly, in U2OS cells, cyclin B was barely detectable at –1–15h timepoint, whereas it remained detectable at all time points in N6AMT1 knockout cells (Figure B,C; Study III).

One potential reason for the slower cyclin B degradation could be the prolonged activation of the spindle assembly checkpoint (SAC) (Figure 6). Cyclin B degradation and subsequent mitotic exit are typically triggered by the APC/C^{CDC20} complex, which ubiquitylates cyclin B and targets it for degradation by proteosomes. The APC/C^{CDC20} complex is inhibited by the SAC, which monitors kinetochore attachment; the SAC is typically active until all kinetochores are bound to microtubules [170]. Nocodazole disrupts microtubule assembly/ disassembly dynamics, preventing the formation of the mitotic spindle and acting as a strong SAC activator [183]. It is possible that N6AMT1 knockout cells take longer to assemble the microtubules of the mitotic spindle after the nocodazole block, leading to prolonged SAC activation and slower cyclin B degradation. Moreover, SAC can also be activated in response to DNA damage during mitosis [184], and recently, loss of N6AMT1 function has been linked to the dysregulation of DNA DSB repair mechanisms in mouse spermatocytes during meiosis [130]. Nonetheless, at this point in the research, we cannot conclusively determine the exact mechanism underlying the slower cyclin B degradation due to the loss of N6AMT1.

Overall, the data show that N6AMT1 participates in the regulation of the cell cycle, and N6AMT1 depletion leads to a reduction in cyclin E levels throughout the cell cycle. It remains to be elucidated whether this effect is due to N6AMT1 MTase activity or a novel function that is currently unknown.

5.3. N6AMT1 antibody cross-reactivity complicates data interpretation about the N6AMT1 interactome and functions (Study I, II, III)

During the study, we aimed to analyse whether the N6AMT1 level remained steady throughout the cell cycle or whether phase-specific fluctuations could be observed. During these experiments, we encountered an unexpected issue of N6AMT1 antibody cross-reactivity, which will be described in the following chapter. To study N6AMT1 protein levels during different cell cycle phases, we collected mitotic U2OS and N6AMT1 knockout cells and released them from the mitotic block at designated time points from 0 to 33 h (Figure 4A, B; Study II). Here, we observed no cell cycle-specific N6AMT1 level fluctuation; however, to our surprise, we observed a distinct band with a size of 45–50 kDa corresponding to mitotic cells (Figure 4A, B, lines 1–2; Study II). Additionally, we observed no difference in the N6AMT1 antibody signal between N6AMT1 knockout cells and wild-type U2OS cells in immunofluorescence staining (Figure 1A,B, Figure 2A,B; Study II). This raised questions regarding the specificity of the antibodies used. We obtained the same result with two N6AMT1 antibodies from different manufacturers, HPA059242 and CQA1550 (Table 1, nr I–II; Study II), indicating that both antibodies cross-react with a centrosome-specific protein. To study the extent of N6AMT1 antibody cross-reactivity, we selected four other commercial antibodies: 16211-1-AP, ARP45845_P050, PA5-121076, and sc-517120 (Table 1, nr III–VI; Study II). Specific antibodies were selected to cover various manufacturers and a variety of N6AMT1-based immunogens, as well as host organisms.

5.3.1. Several commercial N6AMT1 antibodies cross-react with mitosis-specific protein Aurora kinase A (Study II)

To test these antibodies, we initially applied the immunofluorescence method to analyse U2OS cells with N6AMT1 antibodies for interphase (Figure 1A; Study II) and mitotic cells (Figure 2A; Study II). In summary, for mitotic cells, antibodies I, II, and III exhibited a signal colocalizing with the centrosome marker pericentrin, while no centrosomal signal was detected for antibody IV (Figure 2A; Study II). Moreover, there was no noticeable difference in the signal intensity between U2OS cells and the N6AMT1 knockout cell line (Figures 1B and 2B, Study II). Antibody V and VI signals were below detection levels. These results suggest that several N6AMT1 antibodies cross-react with one or multiple other targets, including a target that is localised in the centrosomes during mitosis.

Next, N6AMT1 antibodies were further validated using the immunoblotting method. We tested N6AMT1 antibodies I–VI with U2OS (endogenous N6AMT1 23kDa), N6AMT1 knockout cell line (no band at 23 kDa), and compensatory N6AMT1 knockout cell line expressing a recombinant N6AMT1-EGFP fusion protein with a size of 55 kDa (Figure 3; Study II). Antibodies I–IV displayed a band in U2OS cells corresponding to the endogenous N6AMT1 size, while in N6AMT1 knockout cell line no signal was detected at 23 kDa. These antibodies also recognised the recombinant N6AMT1-EGFP protein at 55 kDa, confirming their recognition of the N6AMT1 protein (Figure 3 A–D; Study II). To our surprise, antibody V did not produce any band at the size corresponding to N6AMT1 (Figure 3E; Study II), while antibody VI produced a similarly strong band at a size of 23 kDa in both U2OS and N6AMT1 knockout cell lines (Figure 3F; Study II), suggesting that the visible signal is not just N6AMT1, but an unknown protein(s) of the same size. Furthermore, neither antibody recognised the recombinant N6AMT1-EGFP protein, suggesting that they do not recognise N6AMT1 protein in immunoblots.

To identify the 45–50 kDa (Figure 4, lines 1–2; Study II) mitosis-specific protein with a centrosomal location that N6AMT1 antibodies recognise, we performed IP using Antibody I coupled with SDS gel electrophoresis, protein retrieval from polyacrylamide gel, followed by mass spectrometry (Figure 4C; Study II). Mass spectrometry analyses revealed that Aurora kinase A was the best candidate for further cross-reactivity analysis (Supplementary Table S1; Study II) because it is highly expressed during mitosis and localises to the mitotic spindle poles [185].

To analyse whether N6AMT1 antibodies I–VI cross-react with Aurora kinase A, we constructed a plasmid expression vector for the Aurora kinase

A-GFP fusion protein, which would create an additional band on the western blot image. The EGFP-AURKA (Figure 5A, lane 1; Study II) and AURKA-EGFP (Figure 5A, lane 3; Study II) proteins were expressed in U2OS cells and were detected at 72 kDa and 85 kDa, respectively, using EGFP and Aurora kinase A specific antibodies (Figure 5A; Study II). EGFP expression plasmids pEGFP-C1 and pEGFP-N1 were used as controls (Figure 5A, lanes 2,4; Study II). The results indicated that antibodies I–III, in addition to N6AMT1, specifically recognised Aurora kinase A (Figure 5B–D, lanes 1,3; Study II). Antibody IV displayed multiple bands, including bands of a similar size as the Aurora kinase A EGFP fusion proteins, and we could not conclusively determine whether this antibody cross-reacted with Aurora kinase A. Antibodies V and VI, which did not recognise N6AMT1 recombinant EGFP protein, also did not recognise Aurora kinase A recombinant protein; therefore, we were unable to determine the target of these antibodies (Figure F,G; Study II).

5.3.2. N6AMT1 and Aurora kinase A share similar motifs (Study II)

Typically, antibody cross-reactivity occurs because of the high similarity in protein sequences. Therefore, we performed a BlastP sequence alignment, as described in the Methods section of Study II. We found that N6AMT1 (UniProt accession Q9Y5N5) and Aurora kinase A (UniProt accession O14965) share two similar motifs: ENNPEE and EKVDL (Figure 6A; Study II). Motif EKVDL is more common and is shared by 26 different proteins. However, the ENNPEE motif is rare and is shared only by Aurora Kinase A and N6AMT1. In *silico* analysis of AURKA and N6AMT1-TRMT112 structures revealed that the ENNPEE motif is freely accessible (Figure 6C, D; Study II), whereas EKVDL is hidden in the secondary structure of both proteins (Figure 6F, G; Study II). In N6AMT1, the ENNPEE motif is located in the N-terminal part of the protein, position 176–181, and is included in immunogens for antibodies I–III (Figure 6A; Study II), which recognise both N6AMT1 and Aurora kinase A, suggesting that this is a likely reason for this case of antibody cross-reactivity.

5.3.3. N6AMT1 antibody cross-reactivity with Aurora kinase A complicates the N6AMT1 investigation process (Study I, II, III)

Many standard molecular biology methods used to study endogenous protein localisation and interactions, such as immunoprecipitation and immunofluorescence, are based on antibodies and due to cross-reactivity with Aurora kinase A, cannot be reliably used to study endogenous N6AMT1. In our studies, we applied the use of recombinant proteins fused with tags and failed to detect centrosomal localisation for recombinant EGFP-tagged N6AMT1 or E2tagged TRMT112 (unpublished data obtained during Study I and III). It cannot be determined whether N6AMT1 localises to the centrosomes, but it is unlikely, as there is no noticeable difference in the immunofluorescence staining between U2OS and N6AMT1 knockout cells (Figure 2; Study II). Despite this, recombinant proteins also pose certain issues, such as tags interrupting endogenous functions and protein levels not matching the native level, thus causing dysfunction in the cells. During study I of TRMT112-network MTases, it was noticed that in some cases, the recombinant MTase was localised differently than the endogenous protein staining pattern using commercial antibodies (Figure 2, Fig S1; Study I). Most of the used antibodies against MTases were polyclonal and produced multiple bands in western blotting, raising doubts about their specificity.

Research groups have used N6AMT1 antibodies for various methods, including immunoblotting, immunohistochemistry, immunocytochemistry, and immunoprecipitation. During the initial stages of our study, we found information about twenty-two N6AMT1 antibodies. It cannot be excluded that some of the twenty-two antibodies might be duplicates of the same antibody, as biomedical reagent resellers often re-label them [186]. One of the antibodies was clearly labelled as discontinued on the manufacturer's website (Table 1, nr VIII; Study II). Another antibody, which was not included in Table 1 of Study II (ab173804 by Abcam), was used for western blot analysis in two studies [42,123] and was likely discontinued. However, there was no information about this antibody on the manufacturer's website and we were unable to determine the immunogen, clonality, and host. It is a well known issue that antibodies in scientific studies are frequently poorly reported [187,188], often due to intellectual property concerns [189]. Furthermore, even if the antibody is correctly reported, tracking the original manufacturer of antibodies can be difficult because resellers frequently relabel them [186].

The most extensively cited N6AMT1 antibody was made in-house and is not commercially available (Table 1, nr VII; Study II). To date, this antibody has been used in seven studies and applied for chromatin immunoprecipitation multiple times, western blot, and immunohistochemistry and published in various research journals [40,114,132–136]. In the most recent study, this antibody was used for protein immunoprecipitation from the total cell lysate of prostate cancer cells coupled with LC-MS/MS analysis [114]. As revealed by the original proteomics data from the Supplementary information and original LC-MS/MS data (ProteomeXchange Consortium via the PRIDE partner repository under accession code PXD053942; accessed 15.03.2025), in this study, the authors have used two different in-house-made antibodies against N6AMT1: antibody anti-KMTα (N6AMT1) #27630 (Table 1, nr VII; Study II) and #28445. To our surprise, the original proteomics data set [114] revealed that #27630 antibody co-immunoprecipitates Aurora kinase A, along with well-established Aurora kinase A interactors CEP192 [190] and TPX2 [191]. When looking at Supplementary Data 1 and original LC-MS/MS data (ProteomeXchange Consortium via the PRIDE partner repository under accession code PXD053942; accessed 15.03.2025) for antibody #27630, it can be seen that Aurora kinase A show higher LFQ values and unique and razor peptide count than N6AMT1. Moreover, intriguingly, the most well-described N6AMT1 interaction partner, TRMT112, was not detected.

In the case of antibody #28445, a low level of TRMT112 was detected in the dataset, but this antibody did not precipitate Aurora kinase A.

It cannot be excluded that N6AMT1 interacts with Aurora kinase A; nevertheless, we failed to detect any mutual interaction in our experiments (Figure 9A, B). In one case, we used a stable N6AMT1-EGFP expressing cell line based on U2OS cells, followed by GFP-trap using unsynchronised cells and nocodazole-synchronised cells (Figure 9A). We detected endogenous Aurora kinase A in the input sample but not in the IP sample (Figure 9A). We have also analysed these samples using LC-MS/MS analysis, similar as described in the Methods section of Study I, and failed to detect any Aurora kinase A. In the other case, we co-expressed EGFP-AURKA and AURKA-EGFP together with either E2-tagged N6AMT1 or recombinant N6AMT1 without a tag, followed by GFP-trap, similar to as described in the Methods section of Study I (Figure 9B) and did not detect any N6AMT1 in the immunoprecipitated samples. Meanwhile, when using EGFP-tagged N6AMT1 as bait for co-immunoprecipitation, we were always able to detect endogenous TRMT112 in western blot images (Figure 1E; Figure 3A,C; Figure 5A; Study I) and mass spectrometry. TRMT112-N6AMT1 interaction has been well-established and shown by multiple groups in various organisms [17,22, 25,40].

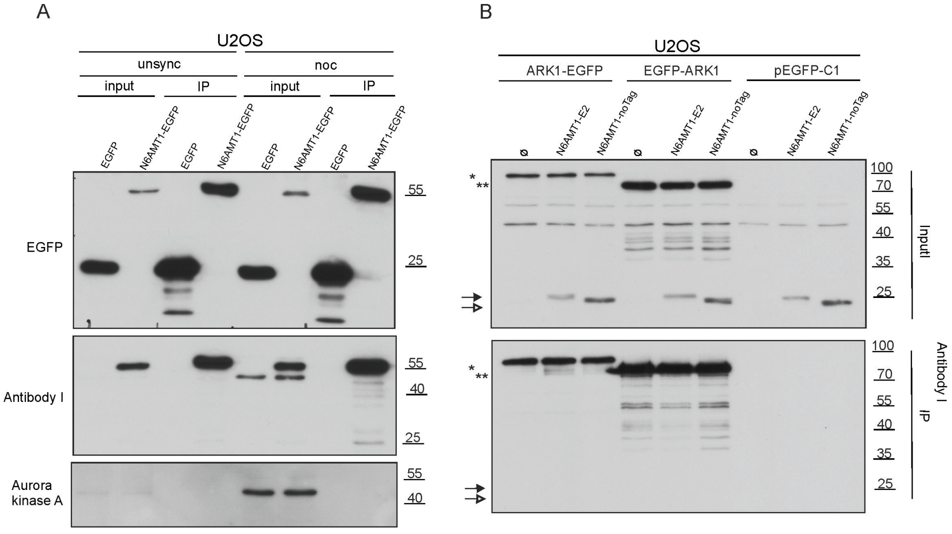

Figure 9. Recombinant N6AMT1 interaction with Aurora kinase A was undetectable by immunoprecipitation analysis. A. Unsynchronised stable U2OS cell lines expressing either EGFP or N6AMT1-EGFP, as well as cells synchronised with nocodazole treatment, were lysed, and immunoprecipitation was performed with GFP-trap beads using EGFP as bait. Samples were analysed by western blot using antibodies against EGFP (1:10000; Institute of Technology, University of Tartu), Aurora kinase A (1:500, sc-56881, Santa Cruz Biotechnology), and Antibody I against N6AMT1 (1:2000; HPA059242, Atlas Antibodies). Input represents the total cell lysate, and IP represents the immunoprecipitated fraction. B. U2OS cells were transfected with plasmids encoding for ARK1-EGFP, EGFP-ARK1 or pEGFP along with plasmids encoding for N6AMT1-E2tag or

N6AMT1-noTag, lysed and immunoprecipitation was performed with GFP-trap beads using EGFP as bait. Samples were analysed by western blot using Antibody I against N6AMT1 (1:2000; HPA059242, Atlas Antibodies). Input represents the total cell lysate, and IP represents the immunoprecipitated fraction. The band marked with a single asterisk represents ARK1-EGFP, the band marked with a double asterisk represents EGFP-ARK1, the band marked with a full arrow represents N6AMT1-E2tag, and the band marked with an empty arrow represents N6AMT1-noTag.

Intriguingly, the authors note that antibody #27630 is raised against the N-terminal part of N6AMT1, while antibody #28445 is raised against the C-terminal part of N6AMT1, but have not included the specific immunogen. The ENNPEE motif is located at the N-terminus of N6AMT1, and if the #27630 immunogen contains it, it is likely that #27630 cross-reacts with Aurora kinase A, which would explain its presence among the precipitated proteins. Similar to #28445, antibody V (Table 1, nr V; Study II) was generated against the C-terminal part of N6AMT1. However, we were unable to detect N6AMT1 using antibody V in western blot or immunofluorescence. This could mean that antibody V either does not recognise N6AMT1 at all or recognises it with too low affinity to be used for detection with western blotting or immunofluorescence (Figure 3E; Study II).

Antibody V was used by Sheng et al. to demonstrate a successful siRNA knockdown of N6AMT1 in western blotting [117]. Overall, immunoblotting can be considered the least sensitive method for issues arising from antibody cross-reactivity, as bands can be validated by knockout studies. Nonetheless, there have been documented cases where antibodies cross-react with proteins of the same size, producing false results. It cannot be excluded that the LOT or the specific antibody we purchased was faulty or damaged, as it has been demonstrated that antibody specificity varies between different LOT batches [192]. However, it is also possible that the first 50 amino acids of N6AMT1 are not suitable immunogens for antibody generation due to the N6AMT1 protein structure, which produces an unsuitable antibody for western blot detection.

The use of antibodies in biomedical research has led to many important discoveries; however, there are many issues associated with antibody use in biomedical research. Unfortunately, it is often impossible for both the industry and other scientific laboratories to reproduce previously published results [193]. Antibody cross-reactivity is a well-known issue and it has even been proposed that poorly validated antibodies are one of the main causes of the "reproducibility crisis" in biomedical research [192,194]. It has been reported that the data obtained using antibody-based methods frequently cannot be replicated using a different antibody, a different laboratory using the same antibody, or even by the same laboratory using a different batch of the same antibody [192,194–196]. Despite their widespread use in scientific research and clinical assays, a comprehensive framework for antibody specificity validation remains lacking [197,198]. A large study was conducted by the Human Protein Atlas, a Swedish consortium that attempted to generate antibodies for every protein in the human genome. During the study, approximately 20 000 commerical antibodies were tested, and it was found that more than half exhibited some cross-reactivity with other antigens, making them unsuitable for many applications [199]. Unsuspecting scientists often buy and use poorly performing and non-specific antibodies that give false signals, leading to wasted time and resources and incorrectly interpreted data [192,196,197]. During this study, resources and time were also wasted due to N6AMT1 antibody-cress reactivity with Aurora Kinase A. Antibodies will certainly remain an invaluable tool in biomedical research, but a more comprehensive reporting and validation framework is needed to improve data reproducibility with regard to antibody-based methods.

In conclusion, N6AMT1 is a multifunctional protein involved in the methylation of various substrates (eRF1, CHD5, and NUT), while also surrounded by controversial results regarding its methyltransferase activity towards lysine and DNA. The apparent difficulties in creating a specific well-performing antibody certainly complicate the effort to describe the total substrates, interactome, and cellular localisation of N6AMT1.

6. CONCLUSIONS

In eukaryotic cells, the methyltransferase cofactor TRMT112 and methyltransferases that bind to TRMT112 are important post-transcriptional regulators of protein synthesis. These protein complexes catalyse the methylation of substrates responsible for the biogenesis of both ribosomal subunits, stability of tRNAs, correct translation termination, and efficient pre-mRNA splicing. Nonetheless, knowledge of TRMT112-network methyltransferases and their functions in human cells is still limited. Furthermore, there are contradictory studies on methylation substrates and the cellular effects of the TRMT112-network member N6AMT1.

This thesis aimed to identify the methyltransferases that form a stable complex with the cofactor TRMT112 in human cells and to characterise whether TRMT112 binds to all its interaction partners in a similar manner through a TRMT112 point mutation study. This thesis also aimed to investigate the effect of TRMT112-network methyltransferase N6AMT1 on human cell cycle regulation and N6AMT1 antibody specificity in more detail.

Based on the results of the three studies included in this thesis, the following conclusions can be drawn:

- Methyltransferase co-factor TRMT112 interacts with at least seven methyltransferases: N6AMT1, WBSCR22, METTL5, ALKBH8, TRMT11, THUMPD2 and THUMPD3 in human cells.
- TRMT112 and its interacting methyltransferases have a mutual stabilisation effect on each other in human cells, and the TRMT112 cellular level is tightly controlled.
- Point mutations on the TRMT112 interaction surface revealed that TRMT112 forms the interaction with all methyltransferases similarly; however, subtle differences can be observed, especially in the case of THUMPD2 and THUMPD3.
- The TRMT112-network methyltransferase N6AMT1 participates in cell cycle regulation by regulating cyclin E levels, as N6AMT1 depletion in cells leads to the downregulation of cyclin E levels at the protein and mRNA levels.
- N6AMT1 depletion leads to decreased cell proliferation, delays cell progression through the G1 phase, S phase, and mitosis, and causes high sensitivity to serum depletion.
- Several commercial antibodies raised against N6AMT1 cross-react with the mitosis-associated protein Aurora kinase A, complicating the N6AMT1 investigation process and data interpretation.

SUMMARY IN ESTONIAN

TRMT112 poolt aktiveeritavate metüültransferaaside võrgustiku kirjeldamine ja TRMT112-võrgustiku liikme N6AMT1 raku funktsioonide iseloomustamine

DNA epigeneetilised modifikatsioonid, post-transkriptsioonilised RNA modifikatsioonid ja post-translatsioonilised modifikatsioonid on olulised erinevate rakuliste protsesside regulaatsioonil. Nende modifikatsioonide on olulised, et tagada rakuliste funktsioonide korrektne toimimine. Seetõttu viivad muutused modifikatsioonimustrites sageli häireid rakuprotsessides, mis võib omakorda põhjustada erinevaid haigusi. Üks selline modifikatsioon on metülatsioon, mille käigus toimub metüülrühma (-CH3) ülekanne ühelt substraadilt teisele. Rakkudes katalüüsivad seda reaktsiooni spetsiifilised valgud, mida nimetatakse metüültransferaasideks.

Metüülatsioon mängib olulist rolli geenide regulatsioonis nii transkriptsioonilisel kui ka translatsioonijärgsel tasandil. See mõjutab mitmesuguseid rakulisi protsesse, sealhulgas ainevahetust, valkude sünteesi ja raku signalisatsiooni. Suurim metüültransferaaside klass on inimrakkudes seitsme-β-ahela perekond, kuhu kuulub umbes 120 metüültransferaasi. Seitsme-β-ahela perekonna valgud katalüüsivad erinevate substraatide, sealhulgas valkude, DNA ja RNA, aga ka väikeste molekulide, nagu neurotransmitterite ja metaboliitide metülatsiooni.

Paljud metüültransferaasid vajavad enda katalüütiliseks aktiivuseks ja struktuurseks stabiilsuseks kompleksi moodustamist kofaktoritega. Üks selline kofaktor on 15 kDa suurune evolutsiooniliselt konserveeritud valk TRMT112. Selle ortolooge on leitud kõigist kolmest elu domeenist. Eukarüootides ja arhedes toimib TRMT112 paljude seitsme-β-ahela metüültransferaaside allosteerilise regulaatorina. Eukarüootides on TRMT112-ga interakteeruvad metüültransferaasid seotud rRNA, tRNA, snRNA ja valkude metülatsiooniga, samas kui bakterite TRMT112 ortoloogide funktsioonid on teadmata. TRMT112-võrgustiku metüültransferaasid toimivad post-transkriptsiooniliste reguleerijatena ja on väga olulised erinevate oluliste rakuliste protsesside jaoks. TRMT112-võrgustiku metüültransferaaside düsregulatsioon on seotud paljude haigustega, sealhulgas vähi ja neurodegeneratiivsete haigustega.

Esimese doktoritöö osana uuriti inimese rakkudes TRMT112-ga interakteeruvaid metüültransferaase. Uuringu käigus leiti, et inimese TRMT112 võrgustik koosneb vähemalt seitsmest metüültransferaasist: N6AMT1, WBSCR22, METTL5, ALKBH8, TRMT11, THUMPD2 ja THUMPD3. Seejuures leiti, et TRMT112 stabiliseerib temaga interakteeruvaid metüültransferaase. Lisaks näidati, et nii metsiktüüpi TRMT112 kui ka TRMT112 mutandid interakteeruvad metüültransferaasidega sarnaselt, kuid erinevusi on märgata eriti THUMPD2 ja THUMPD3 puhul. Täheldati kahte tüüpi efekte TRMT112 mutatsioonide mõjust metüültransferaasidega interaktsioonil. Esimesel juhul viisid mutatsioonid stabiliseeriva efekti kadumiseni TRMT112 ja metüültransferaaside vahel. Teisel juhul täheldati selget stabiliseerimisefekti, kuid kasutades ko-immunosadestamise meetodit täheldati nõrgemat interaktsiooni metüültransferaasiga. Kokkuvõttes viitavad need tulemused dünaamilisele võrgustikule, kus metüültransferaasid võivad omavahel konkureerida TRMT112-ga interaktsiooniks, mis omakorda on vajalik nii TRMT112 kui ka temaga interakteeruvate metüültransferaaside rakusisese taseme kontrolliks.

Töö teine osa keskendus TRMT112 ühele interaktsioonipartnerile N6AMT1. N6AMT1 on huvitav TRMT112-võrgustiku liige, kuna mitmed uuringud on näidanud vastuolulisi tulemusi N6AMT1 metülatsioonisubstraatide ja nende funktsiooni kohta. N6AMT1 funktsioonide uurimiseks tehti osteosarksoomi rakuliinis U2OS N6AMT1 *knockout*, kasutades selleks CRISPR-Cas9 süsteemi. Võrreldes normaalsete U2OS rakkudega, oli N6AMT1 rakkudega jagunemine aeglasem, mis viitas sellele et N6AMT1 osaleb rakutsükli regulatsioonis. Lisaks sellele leiti, et N6AMT1 vaigistamine põhjustas rakkudes rakutsükli G1/S faasi üleminekut reguleeriva valgu tsükliin E allaregulatsiooni nii valgu kui ka mRNA tasemel. Samuti põhjustas N6AMT1 vaigistamine rakkude suuremat tundlikust seerumnäljutamise suhtes ja taastusid aeglasemalt pärast S-faasi ja mitoosi arestist väljumisel. Töö tulemuste põhjal on tulevikus võimalik edasi uurida, kas kirjeldatud efektid on põhjustatud N6AMT1 metüültransferaasi aktiivsuse puudumisest või mitte.

Viimases uurimustöö osas uuriti erinevate kommertsiaalselt kättesaadavate N6AMT1 antikehade spetsiifilisust, kuna paljude molekulaarbioloogia meetodite eelduseks on antikehade seondumine spetsiifilise substraadiga. Töö käigus leiti, et mitmed N6AMT1 antikehad seonduvad lisaks N6AMT1 valgule tugevalt ka mitoosiga seotud valgu Aurora kinaas A-ga. Lisaks leiti bioinformaatilse analüüsi käigus, et mõlemad valgud sisaldavad motiivi ENNPEE, mis võib potentsiaalselt seletada antikehade ebaspetsiifilisust. Sellest tulenevalt tuleks tulevikus arvestada, et N6AMT1 antikehadel põhinevatel meetoditel saadud tulemused võivad olla mitteusaldusväärsed ja nende tõlgendamisel tuleb olla ettevaatlik.

Kokkuvõttes leiti, et inimeses interakteerub TRMT112 seitsme erineva metüültransferaasiga, mille puhul interaktsioon TRMT112-ga toimub sarnaselt. Töö tulemuste põhjal oleks tulevikus võimalik täpsemalt uurida TRMT112-võrgustiku metüültransferaaside dünaamilist interaktsiooni ja omavahelist regulatsiooni ning selle rolli erinevate haiguste arengus. Lisaks leiti, et TRMT112-võrgustiku liige N6AMT1 osaleb rakutsükli toimimises läbi tsükliin E taseme regulatsiooni. Edasised uurimistööd peaks aga täpsustama, kuidas N6AMT1 täpselt osaleb tsükliin E taseme regulatsioonis. Viimaks näidati, et antikehad N6AMT1 valgu vastu võivad ristreageerida mitoosi regulatsioonis olulise valgu Aurora kinaas A-ga, mida tuleks tulevikus N6AMT1 uurimisel arvestada.

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