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**Characterization of the TRMT112-methyltransferase network in
mammalian cells**

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Characterization of the TRMT112-methyltransferase network in mammalian cells

TRMT112 is a small evolutionary conserved protein that acts as a co-factor and activator for different methyltransferases involved in rRNA, tRNA and protein methylation. In mammalian cells TRMT112 interacts with WBSCR22, N6AMT1, METTL5, ALKBH8, TRMT11, THUMP2 and THUMP3. During this research subcellular localisation and mutual interactions of TRMT112 and its partners were studied. TRMT112 level and localization in the cells is tightly regulated and there is a positive feedback loop between TRMT112 and its partners. TRMT112 interacts with all its partners in a similar way, but single amino acid mutations on the surface of TRMT112 reveal several differences as well. Lastly, partial genomic knockout of TRMT112 using CRISPR/Cas9 system significantly reduces WBSCR22 protein level in mammalian cells. In summary, mammalian TRMT112 can be considered as a central “hub” protein that regulates the activity of several of the methyltransferases it interacts with.

Key words: TRMT112, WBSCR22, N6AMT1, METTL5, ALKBH8, TRMT11, THUMP2, THUMP3, CRISPR/Cas9, methyltransferases

CERCS: T490 Biotechnology; B200 cytology, oncology, cancerology

TRMT112 pooli aktiveeritavate metüültransferaaside võrgustiku iseloomustus imetajarakkudes

TRMT112 on väike evolutsiooniliselt konserveerunud valk, mis on vajalik rRNA ja tRNA metüültransferaaside ensümaatiliseks aktiivsuseks. Imetajarakkudes interakteerub TRMT112 vähemalt seitsme erineva metüültransferaasiga - WBSCR22, N6AMT1, METTL5, ALKBH8, TRMT11, THUMP2 ja THUMP3-ga. Projekti käigus uuriti TRMT112 ja tema partnerite subtsellulaarset lokalisatsiooni ja vastastikuseid koosmõjusid. TRMT112 tase ja asukoht rakkudes on rangelt reguleeritud ning TRMT112 ja tema partnerite vahel esineb positiivne tagasiside. TRMT112 suhtleb kõigi oma partneritega sarnasel viisil, kuid üksikud aminohappemutatsioonid TRMT112 pinnal näitavad ka mitmeid erinevusi. Lisaks vähendab TRMT112 osaline genoomne väljalülitamine CRISPR / Cas9 süsteemi abil märkimisväärselt WBSCR22 valgu taset imetajarakkudes. Kokkuvõtvalt võib imetaja TRMT112-d pidada keskseks regulaatorvalguks, mis reguleerib mitme RNA metüültransferaasi aktiivsust.

Märksõnad: TRMT112, WBSCR22, N6AMT1, METTL5, ALKBH8, TRMT11, THUMP2, THUMP3, CRISPR/Cas9, metüültransferaasid

CERCS: T490 Biotehnoloogia; B200 tsütoloogia, onkoloogia, vähiteadus

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Abbreviations

5mC - 5-Methylcytosine

6mA - N⁶-methyladenine

ALKBH8 - alkylation repair homolog 8

AS3MT - arsenic (+3 oxidation state) methyltransferase

ATF4- Activating transcription factor 4

BSA – bovine serum albumine

Cas9 - CRISPR-associated protein 9

Cbh- chicken β -actin hybrid promoter

CDK1 - cyclin-dependant kinase 1

cm5s2U - -carboxymethyl-2-thiouridine

cm5U - 5-carboxymethyluridine

CRISPR - clustered regularly interspaced short palindromic repeats

Cas9- CRISPR associated protein 9

DAPI - 4',6-diamidino-2-phenylindole

DMA - dimethylarsonic acid

EDTA - Ethylenediaminetetraacetic acid

eRF1 - eukaryotic translation termination factor 1

FBS – fetal bovine serum

GRIK2 - Glutamate receptor ionotropic, kainate 2

gRNA – guide ribonucleic acid

HDR - homology-directed repair

HRP - horseradish peroxidase

IMDM - Iscove's Modified Dulbecco's Media

IP – immunoprecipitation

KMT9 - lysine methyltransferase 9

LB media- Luria Bertani media

m6A - N⁶-methyadenosine

mcm5U - 5-methoxycarbonylmethyluridine

METTL5 - methyltransferase Like 5 protein

MMA^{III} - monomethylarsonous acid

MTase – methyltransferase

N6AMT1- N6-adenine-specific DNA methyltransferase

NFDM – non-fat dry milk

NHEJ - non-homologous end joining
PBS - phosphate-buffered saline
PVDF - polyvinylidene fluoride
ROS – reactive oxygen species
RT – room temperature
SAM - S-adenosylmethionine
SDS-PAGE - sodium dodecyl sulphate–polyacrylamide gel electrophoresis
siRNA – small interfering ribonucleic acid
SNP – single nucleotide polymorphism
STIL - centriolar assembly protein
THUMPD2 - THUMP domain-containing protein 2
THUMPD3 - THUMP domain-containing protein 3
TRMT11 - tRNA (guanine(10)-N2)-methyltransferase homolog
TRMT112 - Multifunctional methyltransferase TRM112-like protein
WBSCR22 - Williams Beuren syndrome chromosome region 22 protein
ZBD – zinc binding domain

Introduction

Methylation is an essential epigenetic modification mainly catalysed by S-Adenosyl methionine dependent methyltransferases. Several methyltransferases require a cofactor for their metabolic stability and enzymatic activity. One of such co-factors is the small evolutionary conserved protein TRMT112 that interacts and forms a complex with at least seven different rRNA, tRNA and protein methyltransferases. Several members of the TRMT112-methyltransferase network are involved in vital biological processes in the cell, such as gene expression regulation, cell growth and division. Disruption of their normal activity can lead to development of different malignancies such as cancer.

The crystal structures of human TRMT112 and its orthologues in yeast have revealed that TRMT112 interacts with its partners in a similar way, by covering a large hydrophobic cluster of the partner protein and therefore protecting it from external environment, hence increasing solubility and stability. All methyltransferases studied so far use the same surface of their MTase domain to bind the same region of TRMT112 raising the question whether they compete to interact with TRMT112.

The aim of this project is firstly, to describe subcellular localisation of TRMT112 and its partner methyltransferases, secondly, to study what impact single amino acid mutations have on TRMT112 protein stability and interactions with its partners and lastly to adopt the CRISPR/Cas9 system for isolating a human cell line containing a genomic TRMT112 knockout that could be used in future studies to further elucidate the functions of TRMT112-methyltransferase network.

This study was carried out in Tartu University Institute of Technology.

1 LITERATURE REVIEW

1.1 Methylation and methyltransferases

Methylation is a biochemical process during which a methyl group ($-CH_3$) is added to a wide range of molecular substrates such as DNA, RNA, proteins, carbohydrates, lipids, neurotransmitters, and other small molecules. Methylation has an important role in epigenetic regulation of all living organisms (Menezo *et al.*, 2020). Most of the methylation reactions in eukaryotic cells are catalysed by S-adenosyl methionine dependant methyltransferases (MTases), which use the S-adenosyl methionine as a methyl donor (Martin and McMillan, 2002).

In recent years it has been shown that methylation has a significant role in the translational machinery as it is commonly found in ribosomal, transfer and messenger RNAs as well as ribosomal proteins, translation and transcription factors (Clarke, 2018). The most commonly found eukaryotic RNA modification is N⁶-methyladenosine (m6A) and studies reveal that RNA methylation pattern has a significant impact on the overall gene expression in cells as it affects ribosome biogenesis, assembly and structure stability as well as protein translation efficiency (Sloan *et al.*, 2017; Shi, Wei and He, 2019). Abnormal RNA methyltransferase expression and m6A methylation pattern can lead to development of certain diseases, for example both have been detected in many different cancers, such as glioblastoma, acute myeloid leukaemia, breast cancer, prostate cancer and others (Sun, Wu and Ming, 2019). Furthermore, certain RNA methyltransferases and therefore certain modifications for molecules of the translational machinery are essential for cell survival in various organisms as knockout cells and organisms of certain methyltransferases are not viable (Sloan *et al.*, 2017).

Protein methylation also has an important role in gene expression regulation, as it can have a significant impact on protein-protein interactions (Bedford and Clarke, 2009; Huang, 2019; Husmann and Gozani, 2019). For example, lysine methylation pattern in histones is an important mechanism for regulating chromatin and therefore gene expression (Husmann and Gozani, 2019). Arginine methylation also plays an important role in gene expression regulation and is also commonly found on histones as well as many other substrates, such as molecules involved in DNA damage signalling, mRNA translation, cell signalling and others (Blanc and Richard, 2017). Altered gene expression of protein methyltransferases are commonly found in various pathologies, including cancers, neurodegenerative diseases and developmental disorders (Blanc and Richard, 2017; Husmann and Gozani, 2019).

It has also been demonstrated that DNA methylation has an important role in the epigenic regulation as it also controls gene expression, imprinting, X chromosome inactivation

and others (Moore, Le and Fan, 2013). So far two modifications have been observed in mammals- the extensively studied 5-Methylcytosine (5mC) and recently detected N⁶-methyladenine (6mA) (Wu *et al.*, 2016; Luo, Hajkova and Ecker, 2018). 5mC modification pattern is highly specific in differentiated tissues, mainly found in CpG islands, furthermore it is typically associated with transcription repression as actively transcribed sites usually lack this modification (Luo, Hajkova and Ecker, 2018). 6mA function in the DNA is not as well studied as 5mC, nonetheless it has been shown that it frequently occurs in exon regions in actively coding sites (Xiao *et al.*, 2018).

Several studies have revealed that several methyltransferases require a cofactor for their metabolic stability and enzymatic activity (Öunap *et al.*, 2015; Metzger *et al.*, 2019; van Tran *et al.*, 2019).

1.2 Mammalian TRMT112-methyltransferase network

1.2.1 TRMT112

Multifunctional methyltransferase TRMT112-like protein (TRMT112) is a small evolutionary conserved protein that functions as a co-factor for several methyltransferases that are involved in rRNA, tRNA and protein methylation (Liger *et al.*, 2011; Öunap *et al.*, 2015; Zorbas *et al.*, 2015; Bourgeois, Létoquart, *et al.*, 2017; van Tran *et al.*, 2018, 2019; Metzger *et al.*, 2019).

The most characterised member of the TRMT112 family is the human TRMT112 ortholog Trm112 from yeast *Saccharomyces cerevisiae*. It has been demonstrated in several studies that Trm112 is not an essential protein in yeast and Trm112 knockout yeast strains are viable, nevertheless these strains have severely impaired growth speed (Purushothaman *et al.*, 2005; Mazauric *et al.*, 2010; Figaro *et al.*, 2012). Similar results have been obtained from studies with Trm112 functional homologue SMO2 in *Arabidopsis thaliana*. Inactivation of SMO2 also leads to impaired cell growth, proliferation and overall plant development, for example SMO2 mutants have smaller aerial organs and shorter roots than wild type plants (Hu *et al.*, 2010).

TRMT112 homologue in mice, Trmt112, is highly expressed during mouse embryonic development in various tissues, therefore authors of this study suggest that Trmt112 might play an important role in the development of brain, nervous system and other tissues (Gu *et al.*, 2012). Currently it is unknown whether TRMT112 is an essential protein in mammalian cells.

Studies have revealed that in human cells TRMT112 forms a complex and interacts with four methyltransferases – N6AMT1 (Metzger *et al.*, 2019), METTL5 (van Tran *et al.*, 2019), ALKBH8 (Songe-Møller *et al.*, 2010) and WBSCR22 (Öunap *et al.*, 2015). Recent SILAC study from the Tartu University Institute of Technology has demonstrated that in addition to

these four proteins, TRMT112 also interacts with TRMT11, THUMP2 and THUMP3 (manuscript in preparation).

1.2.2 WBSCR22

The first methyltransferase known to interact with TRMT112 is WBSCR22 (Öunap *et al.*, 2015). WBSCR22 was first identified as one of the genes deleted in the Williams-Beuren Syndrome (WBS). WBS is a complex neurodevelopmental disorder associated with deletion of several genes on chromosome 7q11.23 (Doll and Grzeschik, 2001). WBSCR22 has an S-adenosyl methionine binding domain (Doll and Grzeschik, 2001) and it participates in pre-RNA processing and the 40S ribosomal subunit biosynthesis in mammalian cells. (Öunap *et al.*, 2013). Studies have revealed that WBSCR22 mediates methylation of N7 at nucleotide G₁₆₃₉ in 18S rRNA (Haag, Kretschmer and Bohnsack, 2015), nonetheless its presence, but not methyltransferase activity is required in the cells during ribosome biogenesis (Zorbas *et al.*, 2015). WBSCR22 protein amount in the cells is tightly controlled and its expression level control takes place through the ubiquitin-proteasome pathway (Öunap *et al.*, 2015).

WBSCR22 requires to form a heterodimer with TRMT112 in order to gain metabolic stability as it has been shown that downregulation of TRMT112 with siRNA significantly reduces WBSCR22 expression (Öunap *et al.*, 2015; Zorbas *et al.*, 2015). WBSCR22-TRMT112 heterodimer localises in the cell nucleus, but while WBSCR22 protein alone accumulates in nucleolus, TRMT112-WBSCR22 heterodimer does not (Öunap *et al.*, 2015).

Studies have revealed that targeted deletion of the mouse WBSCR22 homologue *Bud23* in the embryo heart tissue causes early post-natal death, but homozygous deletion of *Bud23* causes embryo-lethality, therefore *Bud23* can be considered an essential protein in mammals (Baxter *et al.*, 2020). However, WBSCR22 homologue in yeast *Bud23* is not an essential protein, but instead causes growth defects in the cells, in addition, WBSCR22 has the ability to partially compensate the growth defects of *bud23* deletion mutant yeast strain (Öunap *et al.*, 2013).

Recently several studies have illustrated that WBSCR22 has an important role in cancer progression. It is upregulated in multiple myeloma (Tiedemann *et al.*, 2012), colorectal (Yan *et al.*, 2017), glioma (Chi *et al.*, 2020) as well as breast cancer cells (Nakazawa, Arai and Fujita, 2011). WBSCR22 is also involved in repressing the p53 coactivator Zac1 by methylating lysine 9 of the core histone H3 in the promoter region, which enhances tumour cell survival and promotes metastasis formation (Nakazawa, Arai and Fujita, 2011). Moreover, inhibition of WBSCR22 expression in liver cancer cells slows cell growth and reduces invasive properties (Stefanska *et al.*, 2014). There is also evidence that WBSCR22 affects chemosensitivity of

cancer cells as it has been reported that knockdown of WBSCR22 protein increases chemosensitivity to antineoplastic agents SN-38 and 5-Fluorouracil in small lung cancer cells (Yan *et al.*, 2015). Furthermore, Yan *et al.* also report that WBSCR22 knockdown increases colorectal cancer cell sensitivity to oxaliplatin (Yan *et al.*, 2017). However, WBSCR22 appears to be highly downregulated in inflammatory and neoplastic pulmonary pathologies (Jangani *et al.*, 2014). The data suggest that WBSCR22 has several diverse functions in various tissues and it could potentially be implicated in numerous diseases. As it has been shown to be stabilised through the interaction with TRMT112, it is crucial to study this interaction further as it could present a possibility for a new drug target.

1.2.3 METTL5

Secondly, TRMT112 interacts with rRNA methyltransferase METTL5 (van Tran *et al.*, 2019; Chen *et al.*, 2020). METTL5 is a poorly characterised protein from the METTL family that also has an S-adenosyl methionine binding domain as well as N6 adenine-specific conserved site (Richard *et al.*, 2019). METTL5 has been shown to catalyse m6A formation in 18s rRNA at position A1832 (Chen *et al.*, 2020; Ignatova *et al.*, 2020) on the rRNA substrate that contains UAACA motif (Rong *et al.*, 2020).

METTL5 similarly to WBSCR22 and TRMT112, is evolutionary conserved in multiple species, for example m6A activity on 18s rRNA has also been observed with METTL5 drosophila homologue Mettl5 (Leismann *et al.*, 2020).

Studies reveal that METTL5 catalytic activity *in vivo* requires interaction with TRMT112, and it has been recently demonstrated that METTL5 must form a heterodimer with TRMT112 to gain metabolic stability. Similarly to WBSCR22, knockdown of TRMT112 with siRNA significantly reduces METTL5 protein level, however it does not influence the cell growth (van Tran *et al.*, 2019).

Several studies report that METTL5 is involved in stress response regulations in multiple organisms. For example, in B16 mouse melanoma cells, mRNAs typically translated in cells as a stress response are downregulated when METTL5 homologue Mettl5 is knocked out. Furthermore, key mediator of integrated stress response, the translation factor ATF4, is also reduced in the Mettl5 knockout cells, therefore suggesting that Mettl5 has a significant role in mediating integrated stress response in cells via regulating ATF4 translation (Chen *et al.*, 2020). In addition, similar results have been obtained from two studies with *C.elengans*, where the knockout of METTL5 homologue metl-5 appeared to result in enhanced stress tolerance, such as heat resistance and longer lifespan (Liberian *et al.*, 2020; Rong *et al.*, 2020).

It has been demonstrated that Mettl5 knockout mice are viable, however they have clear developmental pathologies, such as cranial abnormalities, reduced fertility together with several developmental changes like reduced activity and reduced urge to explore their surroundings (Ignatova *et al.*, 2020). Human METTL5 bi-allelic frameshift mutations resulting in truncated METTL5 with missing C-terminus have been linked with autosomal recessive intellectual disability and microcephaly in a study of two families of Pakistani and Yemeni origin. Moreover, METTL5 homologue in zebrafish mettl5 knock out mutant embryos have reduced head size similarly to human microcephaly (Richard *et al.*, 2019). Richard *et al.* suggest, that as METTL5 is highly expressed in the brain, it potentially has an important regulatory role, in particular the epigenetic regulation of RNA and possibly even DNA in the neurons, even though METTL5 activity towards DNA has so far not been reported (Richard *et al.*, 2019).

METTL5 is also overexpressed in breast cancer tissues, while downregulation of it reduces the growth of four cancer cell lines, which indicates that METTL5 might play a part in breast cancer tumorigenesis (Rong *et al.*, 2020). However, more research is needed to elucidate on METTL5 role in these pathologies as well as to understand whether TRMT112 plays a part. It is highly important to study the METTL5-TRMT112 interaction, as it has been shown that METTL5 gains metabolic stability through the interaction with TRMT112 and therefore could also be utilised as a new drug target (van Tran *et al.*, 2019).

1.2.4 N6AMT1

Another MTase that forms a complex with TRMT112 is N6-adenine-specific DNA methyltransferase 1 (N6AMT1, HEMK2). N6AMT1 has two isoforms, nevertheless only isoform 1 interacts with TRMT112, suggesting that TRMT112 might be involved in N6AMT1 isoform expression regulation. However, contrary to WBSCR22 and METTL5, downregulation of TRMT112 does not impact the N6AMT1 level in HeLa and U2OS cells, suggesting that N6AMT1 does not need TRMT112 interaction to gain stability in cells and potentially functions independently as well (Leetsi *et al.*, 2019).

Several studies have identified N6AMT1-TRMT112 complex as a protein MTase. TRMT112-N6AMT1 complex, but not N6AMT1 alone, is able to catalyse the N5-glutamine methylation of eukaryotic translation termination factor 1 (eRF1) (Figaro *et al.*, 2008), specifically glutamine 185, which has been observed *in vivo* and *in vitro*, moreover loss of N6AMT1 leads to early embryonic lethality in mice (Liu *et al.*, 2010). Metzger *et al.* have reported, that N6AMT1 must form a complex with TRMT112 in order to bind to S-adenosyl methionine and neither N6AMT1 nor TRMT112 are able to bind it alone. Furthermore,

TRMT112-N6AMT1 complex acts as a lysine methyltransferase (also named as KMT9) and methylates histone 4 lysine 12 *in vitro* and *in vivo*. KMT9 is also involved in transcriptional regulation of molecules that regulate the cell cycle, such as CDK1 (cyclin-dependant kinase 1), STIL (centriolar assembly protein) expression and others. (Metzger *et al.*, 2019). TRMT112-N6AMT1 complex is also involved in prostate cancer cell proliferation regulation, as siRNA mediated knock down of N6AMT in PC3-M cell line resulted in reduced cell proliferation (Metzger *et al.*, 2019).

N6AMT1 was recently proposed to be the N6-adenine methyltransferase in DNA in human cells. Xio *et al.* demonstrated that overexpression of N6AMT1 leads to an increased level of 6mA in DNA, however N6AMT1 knockdown reduces it (Xiao *et al.*, 2018). Moreover, *in vitro* overexpression of N6AMT1 in primary cortical neurons also leads to an increased level of 6mA, whereas downregulation decreases it (X. Li *et al.*, 2019).

However, there are contradicting reports that suggest that N6AMT1 does not act as a DNA N6-adenine methyltransferase, for example Xie *et al.* have revealed that in human glioblastoma cells knockout of N6AMT1 does not influence the level of DNA 6mA (Xie *et al.*, 2018). Moreover, Li *et al.* have argued that the N6AMT1 protein structure is not suitable for DNA binding, as the potential substrate binding domain of N6AMT is negatively charged. During their studies, Li *et al.* were unable to provide evidence that N6AMT1-TRMT112 complex possesses any biochemical activity towards DNA, instead they confirmed that it has a binding affinity towards the GGQ peptide of eRF1 (W. Li *et al.*, 2019) which has also been shown in two separate studies previously (Figaro *et al.*, 2008; Kusevic, Kudithipudi and Jeltsch, 2016). Woodcock *et al.* have also recently demonstrated that N6AMT1-TRMT112 heterodimer is only active on protein glutamine and lysine, but not on DNA (Woodcock *et al.*, 2019). So far data remain inconclusive whether N6AMT1 is or certainly is not involved in DNA methylation and more studies are needed to resolve this.

Several studies have suggested that N6AMT1 is involved in the arsenic metabolism in human cells, for example one study suggests that N6AMT1 can convert monomethylarsonous acid (MMA^{III}) to the less toxic dimethylarsonic (DMA) acid when overexpressed in human urothelial cells (Ren *et al.*, 2011), however its role is relatively minor compared to arsenic (+3 oxidation state) methyltransferase AS3MT (Zhang *et al.*, 2015). A study of N6AMT1 polymorphisms in Andian women concluded, that certain N6AMT1 SNPs and N6AMT1 haplotypes can be associated with MMA^{III} metabolism, as the percentage of MMA^{III} in the urine increased according to the number of alleles for each SNP (Harari *et al.*, 2013). Another study in Chilean population was not able to obtain concrete link between MMA^{III} metabolism and N6AMT1 polymorphisms as their results were too inconsistent (Rosa *et al.*, 2017). However, a

recent study in China provided evidence, that N6AMT1 SNPs and certain haplotypes can be associated with lower arsenic metabolism (Chen *et al.*, 2017). As the data remains conflicting, more studies are needed to elucidate on the N6AMT1 role in arsenic metabolism.

Lastly, a recent genome wide association study has revealed that N6AMT1 might be involved in clopidogrel pharmacokinetics. Clopidogrel is a widely used treatment for coronary artery disease (Zhong *et al.*, 2017). Another study conducted in China has demonstrated a link between certain N6AMT1 polymorphisms with clopidogrel resistance in patients with coronary artery disease (Li *et al.*, 2018), however it is not clear whether N6AMT1 is directly or indirectly involved in clopidogrel metabolism and more studies are needed to elucidate this.

1.2.5 ALKBH8

Another protein that forms a heterodimer with TRMT112 is the alkylation repair homolog 8 (ALKBH8). ALKBH8 is a tRNA hypermodification enzyme, that has a S-adenosyl methionine dependant methyltransferase domain as well as an oxygenase domain. It has been shown that the methyltransferase domain of ALKBH8 is active only if ALKBH8 has formed a complex with TRMT112 (Fu *et al.*, 2010; Songe-Møller *et al.*, 2010; van den Born *et al.*, 2011). The methyltransferase domain catalyses the 5-carboxymethyluridine (cm5u) and 5-carboxymethyl-2-thiouridine (cm5s2U) methylation at the wobble position of several tRNA species, while the AlkB oxygenase domain is responsible for hydroxylation of 5-methoxycarbonylmethyluridine (mcm5U) at the wobble position of several tRNA species including the 21st amino acid selenocystein tRNA (tRNA^{Sec}). Studies have revealed that both functions act independently of each other, but in sequence starting with the MTase and following with the oxygenase action (Fu *et al.*, 2010; Songe-Møller *et al.*, 2010; van den Born *et al.*, 2011; Endres *et al.*, 2015).

It has been reported that ALKBH8 is involved in DNA damage repair as its reduction leads to increased cell sensitivity to the DNA damaging agent methyl methane sulfonate (Fu *et al.*, 2010). Similar results have been obtained from both human and murine cells. Endres *et al.* have demonstrated, that fibroblasts derived from ALKBH8 deficient knockout mice exhibit slower growth, increased DNA strand breaks, activated DNA damage response, increased sensitivity to DNA damaging agents and increased levels of reactive oxygen species (ROS) within the cells. They have also shown that ALKBH8 has a roll in detoxification of ROS through the regulation of selenocysteine-containing ROS detoxification enzymes. Loss of ALKBH8 and therefore loss of an important anti-oxidant defence system might be highly relevant in development of various pathologies, such as cancer, as it is known that cancer cells have an enhanced ability to tolerate high ROS levels (Endres *et al.*, 2015). Recently another

study has confirmed that loss of ALKBH8 leads to a reduction of selenoprotein level in mammalian cells (Lee *et al.*, 2020).

However, it has been reported that ALKBH8 is upregulated in bladder cancer cells and silencing of ALKBH8 leads to increased apoptosis in human urothelial carcinomas *in vitro*. Furthermore, silencing of ALKBH8 *in vivo* reduces cancer cell invasion, angiogenesis and bladder tumour growth in an orthotopic mouse model using human bladder carcinoma cells (Shimada *et al.*, 2009). Ohshio *et al.* have confirmed that knockdown of ALKBH8 induces apoptosis as well as reduces cell proliferation in human bladder cancer cells. They have also shown that overexpression of human ALKBH8 in transgenic mice leads to accelerated rate of tumour growth and cancer invasiveness in an N-butyl-N-(4-hydroxybutyl)-nitrosamine-induced bladder cancer model (Ohshio *et al.*, 2016). It is very likely that ALKBH8 has different roles in different cancer types and stages of cancer development, nonetheless, more studies are needed to elucidate on ALKBH8 role in carcinogenesis as well as DNA damage response and whether TRMT112 also plays a part, because ALKBH8-TRMT112 interactions could potentially present a new target for drug development.

1.2.6 TRMT11

In a recent SILAC study from Reet Kurg lab, Tartu University, Institute of Technology, TRMT11 has been identified as TRMT112 partner (manuscript in preparation). According to Uniprot, TRMT11 is tRNA (guanine(10)-N2)-methyltransferase Trm11 homolog due to sequence similarity, however so far there is no experimental data to confirm this.

TRMT11 homologue Trm11 in *Saccharomyces cerevisiae* is a S-adenosyl methionine dependent tRNA methyltransferase and it forms a complex together with Trm112 and this complex methylate the guanosine nucleotide at position 10 in yeast tRNA. It has also been shown that deletion of Trm11 in yeast causes no detectable phenotype changes (Purushothaman *et al.*, 2005).

Recently, TRMT11 has been identified as part of a fusion gene recurrent in several cancer types, such as prostate cancer, breast cancer, colon cancer, non-small cell lung cancer, oesophageal adenocarcinoma, glioblastoma multiforme, ovarian cancer, liver cancer and hepatocellular carcinoma. In all those cancers TRMT11 is fused together with GRIK2 (Glutamate receptor ionotropic, kainate 2) which according to Uniprot is an ionotropic glutamate receptor (Yu *et al.*, 2014; Yu, Liu, *et al.*, 2019; Yu, Tsung, *et al.*, 2019). Fusion of these two genes leads to loss of function of both TRMT11 and GRIK2, and it is suggested that loss of TRMT11 leads to tRNA defects in cells which causes unstable mRNA translation into proteins which in turn contributes to tumorigenesis (Yu, Liu, *et al.*, 2019). However more

research is needed to elucidate the functions of TRMT11 alone and functioning as a complex with TRMT112 which would help understand its functions and role in cancer development.

1.2.7 THUMPD2 and THUMPD3

THUMP domain-containing protein 2 (THUMPD2) and THUMP domain-containing protein 3 (THUMPD3) are two uncharacterized proteins that, in a recent SILAC study from Reet Kurg lab, Institute of Technology, have recently been identified as TRMT112 partners (manuscript in preparation). According to Uniprot database, both proteins are involved in RNA binding and have a tRNA methyltransferase activity, however detailed functions are currently unknown. THUMPD2 and THUMPD3 are paralogues, contain both the THUMP and methyltransferase domains and share 26% of identity on amino acid level (Uniprot database).

The THUMP domain is a module that has 100-110 amino acid residues, and it has RNA binding and modifying activity. It is shared by enzymes that are predicted to catalyse three types of RNA-modifications - methylation, pseudouridylation and thiouridylation. The THUMP domain can be found in single form or together with other catalytic domains, for example methyltransferase domain NDPPY (Aravind and Koonin, 2001).

THUMPD2 has been recently identified as cisplatin and 5-fluorouracil resistance gene candidate in human oesophageal squamous cell carcinoma cells (Hayashi *et al.*, 2019), while THUMPD3 has been previously predicted to interact with TRMT112 in two interactome studies (de Crécy-Lagard *et al.*, 2019), nonetheless more research is needed to describe the THUMPD2 and THUMPD3 functions and interaction with TRMT112.

1.3 Crystal structures of TRMT112 together with its partners

Crystal structures of TRMT112 in complex with N6AMT1 and METTL5 have revealed that TRMT112 interacts with both of them and potentially other partners in a similar way (Metzger *et al.*, 2019; van Tran *et al.*, 2019; W. Li *et al.*, 2019; Gao *et al.*, 2020).

As can be seen in Figure 1, in a complex with a partner MTase, TRMT112 consists of two domains: a central domain and a zinc binding domain (ZBD). ZBD consists of three α -helices and an anti-parallel β -sheet from four β -strands, but the central domain consists of three α -helices (van Tran *et al.*, 2019; W. Li *et al.*, 2019). The complex TRMT112- METTL5 assembles when main chain atoms between β -strand 3 of METTL5 and β -strand 4 of TRMT112 interact and form a parallel β -zipper which results in a β -sheet consisting of eleven strands (Figure 1). It has also been shown that after the heterodimer has formed, a large hydrophobic area on METTL5 is covered by TRMT112, which increases the stability and solubility of the protein while protecting it from outside factors (van Tran *et al.*, 2019). This same phenomena

has also been observed with TRMT112 and METTL5 homologues in *Drosophila* (Leismann *et al.*, 2020).

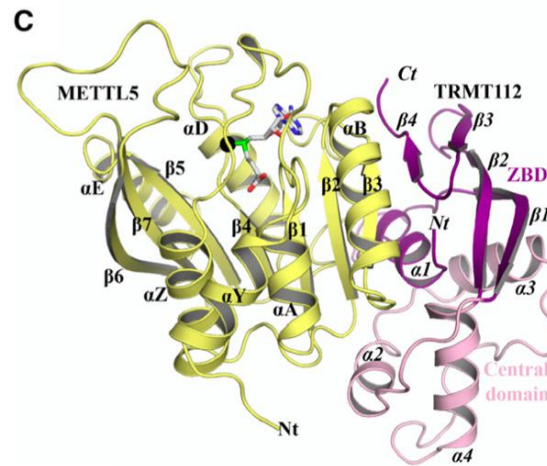


Figure 1. Crystal structure of the human TRMT112-METTL5 complex. TRMT112 consists of two domains: a central domain (pink) and a zinc binding domain (purple). The complex TRMT112- METTL5 assembles when main chain atoms between β -strand 3 of METTL5 (yellow) and β -strand 4 of TRMT112 interact and form a parallel β -zipper (van Tran *et al.*, 2019).

Crystal structure from the N6AMT1-TRMT11 complex has revealed that TRMT112 forms a complex with N6AMT1 similarly to METTL5. In Figure 2 it can be seen TRMT112 also forms a complex with N6AMT1 through the interaction of β -strand 3 of N6AMT1 and β -strand 4 of TRMT112 which therefore creates a continuous β -sheet with eleven strands (Figure 2). In this case TRMT112 similarly covers a large hydrophobic area of N6AMT (W. Li *et al.*, 2019).

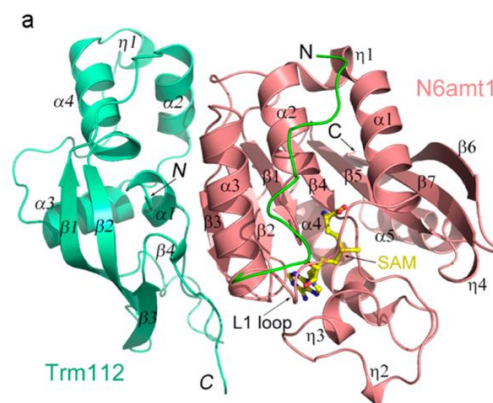


Figure 2. Crystal structure of the human TRMT112-N6AMT1 complex. The complex TRMT112- N6AMT1 assembles when main chain atoms between β -strand 3 of N6AMT1 (pink) and β -strand 4 of TRMT112 (green) interact and form a parallel β -zipper (W. Li *et al.*, 2019).

Crystal structures of TRMT112 yeast homologue Trm112 in a complex with Bud23, Trm11 and Mtq2 have also shown that Trm112 interacts with these partners in the same way as TRMT112 interacts with N6AMT1 and METTL5 in human cells (Liger *et al.*, 2011; L  toquart *et al.*, 2015; Bourgeois, Marcoux, *et al.*, 2017). Moreover, the crystal structure of Trm112 in a single form from yeast cells has revealed that when Trm112 is not bound with a partner MTase, the fourth β -strand, located in the C-terminal region, folds back onto the hydrophobic region of the ZBD (Bourgeois, L  toquart, *et al.*, 2017).

1.4 CRISPR/Cas9-directed genome editing

The clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated (Cas) system is a naturally occurring defence mechanism that bacteria use against phage infection and plasmid transfer (Jiang and Doudna, 2017). Several bacterial CRISPR-Cas systems have been recently engineered and repurposed as a tool for precise genome editing in living cells as well as multicellular organisms (Adli, 2018). Currently the most popular is CRISPR-Cas9 system which was first used to edit human cell genome in 2013 (Jiang and Doudna, 2017) and since then it has become one of the most widely used genome editing tools.

CRISPR/Cas9 belongs to Class 2, type II system and originates from *Streptococcus pyogenes* (Jiang and Doudna, 2017). At the core of CRISPR/Cas9 system is protein Cas9 which is an endonuclease guided by RNA duplex consisting of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). The crRNA contains a part that acts as a guiding sequence while the other part pairs with the tracrRNA (Jinek *et al.*, 2012). Cas9 uses the guide sequence from the RNA duplex to form base pairs with target DNA sequence, therefore allowing Cas9 to make a site-specific double stranded break (Doudna and Charpentier, 2014). Jinek *et al.* were the first to reveal that Cas9 can also be guided by a single chimeric RNA, that can be designed by fusing crRNA and tracrRNA called single guide RNA (Jinek *et al.*, 2012), therefore paving the way of using CRISPR systems as a gene editing tool.

Another important element of the CRISPR/Cas9 is the protospacer adjacent motif (PAM) site, as Cas9 makes a blunt cut to the DNA 3 bp upstream of the PAM site (Anders *et al.*, 2014). In the case of Cas9 the PAM sequence is 5'-NGG-3' and it has been shown that Cas9 interactions with PAM sequence are crucial for recognition and stable binding of Cas9 to the DNA strand, DNA strand separation, guide-RNA and DNA heteroduplex formation, subsequent endonuclease domain activation and DNA cleavage (Anders *et al.*, 2014; Sternberg *et al.*, 2014). Cas9 utilises two nuclease domains for cleaving the DNA: HNH-like nuclease domain and RuvC-like nuclease domain. In Figure 3 a complete setup of an engineered CRISPR-Cas9 system can be seen (Pickar-Oliver and Gersbach, 2019). HNH-like domain snips

the DNA strand complementary to the guide RNA, but the RuvC-like domain cleaves the DNA strand opposite to the complementary strand (Jiang and Doudna, 2017).

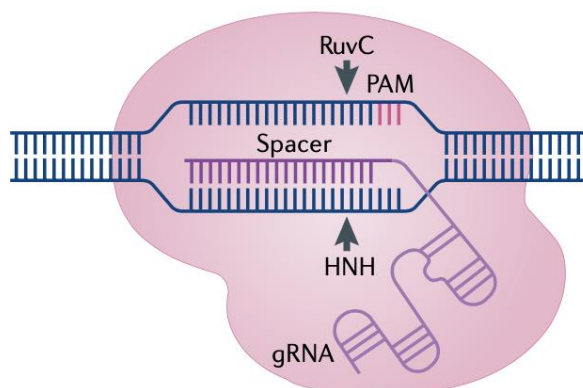


Figure 3. Engineered CRISPR-Cas9 system. Cas9 protein is guided by the sgRNA (purple). Cas9 interacts with the backbone of the sgRNA while part of it complementary pairs with a separated DNA strand (blue) adjacent to the PAM sequence (pink) which results in Cas9 nuclease domains RuvC and HNH-mediated double-strand break (Pickar-Oliver and Gersbach, 2019).

The Cas9-mediated cut activates DNA damage response pathways when the cell attempts to fix the broken DNA strand. One of the most common DNA repair pathways after a Cas9-induced double strand break is thought to be non-homologous end joining (NHEJ). This process is rather error prone, and in some cases results in small insertions or deletions which can cause a frameshift mutation and a subsequently a gene knockout. NHEJ is often used when attempting to create CRISPR/Cas9-induced gene knockouts in cultured cells (Sansbury, Hewes and Kmiec, 2019). Another mechanism that can be used for DNA repair is error-free homology-directed repair (HDR) where a repair template is introduced to the cell at the same time as Cas9 and therefore utilised as a template to repair the DNA. This technique can be used to generate precise gene knockouts as well as point mutations or longer insertions in the DNA sequence (Pickar-Oliver and Gersbach, 2019),

Cas9 can be delivered into the cells in the form of DNA plasmid encoding for Cas9 and the guide RNA, mRNA for Cas9 translation together with a separate guide RNA, or Cas9 protein in a complex with guide RNA (Lino *et al.*, 2018). The Cas9 delivery can be performed using a variety of methods, for example electroporation, microinjection, viral delivery, viral vectors, lipid nanoparticles, cell penetrating peptides, gold nanoparticles and others (Lino *et al.*, 2018). During this study CRISPR/Cas9 system was adapted to use for knocking out TRMT112 gene in human cells.

2 EXPERIMENTAL PART

2.1 Aims and objectives

As a part of the ongoing research in Tartu University Institute of Technology about the role of RNA methyltransferases in the cell growth and proliferation, a recent study has identified a network of methyltransferases, consisting of seven different proteins that interact simultaneously with the co-factor TRMT112. The interaction with TRMT112 in turn stabilises and regulates the methyltransferase activity in cells. The purpose of this research project was to study TRMT112-methyltransferase network in greater detail.

The aims of this study are:

1. Describe subcellular localisation on TRMT112 and its partner methyltransferases,
2. Study TRMT112-methyltransferase complex reciprocal stabilisation,
3. Analyse how single amino acid mutations on the surface of TRMT112 affect TRMT112 protein stability and interactions with its partners,
4. Adapt the CRISPR/Cas9 system for isolating a human TRMT112 knockout cell line, that can be used in future research,
5. Describe the change of TRMT112 partner methyltransferase protein levels in the TRMT112 genomic knockout cell line.

2.2 Materials and methods

2.2.1 Cell culture

Human osteosarcoma (U2OS) and human embryonic kidney cells (HEK293) (ATCC (American Type Culture Collection); Manassas, VA, USA) were cultured in IMDM (Iscove's Modified Dulbecco's Media) supplemented with 10% FBS (fetal bovine serum, Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Cells were incubated at 37 °C in 5% CO₂ environment (Panasonic, Kadoma, Osaka, Japan).

2.2.2 Plasmids

During this project plasmid vectors pQM (Icosagen Ltd., Tartu, Estonia) and pEGFP _C1 (Clontech, USA) (Tartu university, Institute of Technology) containing open reading frames for proteins of interest were used to express methyltransferases in eukaryotic cells. Once transfected to cells, plasmids were translated to methyltransferases tagged with EGFP in the N-terminus or E2Tag in the C-terminus. pQM and pEGFP vectors contain CMV (cytomegalovirus) promoter to drive gene expression. All the plasmids used in this research were obtained from Reet Kurg lab in Institute of Technology, Tartu University (Table 1).

Table 1. Plasmid vectors used for protein expression in eukaryotic cells.

Plasmid vector	Tag
pEGFP_TRMT112	EGFP
pEGFP_N6AMT1	EGFP
pEGFP_WBSCR22	EGFP
pEGFP_METTL5	EGFP
pEGFP_TRMT11	EGFP
pEGFP_ALKBH8	EGFP
pEGFP_THUMPD2	EGFP
pEGFP_THUMPD3	EGFP
pEGFP_C1	EGFP
pQM_TRMT112_WT	E2
pQM_TRMT112_T5A	E2
pQM_TRMT112_L8W	E2
pQM_TRMT112_S10F	E2
pQM_TRMT112_M45A	E2

pQM_TRMT112_K48A	E2
pQM_TRMT112_E50A	E2
pQM_TRMT112_E92A	E2
pQM_TRMT112_F107A	E2
pQM_TRMT112_I113F	E2
pQM_TRMT112_K48A+E92A	E2
pQM_empty	E2

2.2.3 Antibodies

For protein analysis primary antibodies specific to certain proteins were used. Antibodies that originated from mouse were monoclonal, whereas antibodies that originated from rabbit were polyclonal.

Table 2. Primary antibodies used for protein analysis.

Antibody	Origin	Manufacturer	Product code	Western blot	IF
Anti-TRMT112	mouse	Santa Cruz Biotechnology, Dallas, TX, USA	sc-398481	1:1000	1:50
Anti-WBSCR22	mouse		sc-135322	1:500	1:100
Anti-TRMT112	rabbit	Atlas antibodies, Bromma, Sweden	HPA030473	1:3000	1:100
Anti-N6AMT1	rabbit		HPA059242	1:500	1:100
Anti-ALKBH8	rabbit		HPA061514	1:500	1:100
Anti-TRMT11	rabbit		HPA030473	-	1:100
Anti-METTLL5	rabbit		HPA038223	1:1000	1:100
Anti-THUMPD2	rabbit	Invitrogen, Carlsbad, CA, USA	PA5-32045	-	1:100
Anti-THUMPD3	rabbit		PA5-57554	-	1:100
Anti- α -tubulin	mouse	Sigma Aldrich, St. Louis, MO, USA	T5168	1:20 000	-

Anti-5E11	mouse	Icosagen, Tartu, Estonia	-	1:10 000	-
Anti-EGFP	rabbit	Institute of technology; University of Tartu, Tartu, Estonia	-	1:10 000	-

Table 3. Secondary antibodies used for protein analysis.

Antibody	Manufacturer	Concentration	Western blot	IF
Anti-rabbit Alexa 568	Invitrogen, Carlsbad, CA, USA	1 mg/ml	-	1:1000
Anti-mouse Alexa 568		1 mg/ml	-	1:1000
Goat anti-rabbit	LabAS	1 mg/ml	1:10 000	-
Goat anti-mouse		1 mg/ml	1:10 000	-

2.2.4 Eukaryotic cell transfection

For protein expression analysis U2OS or HEK293 cells were transfected using the electroporation method. Cells were cultured in 100 mm plates until 70% confluency in previously described media. 1×10^6 cells were used per one transfection reaction. Cells were harvested using trypsin solution (0.05% trypsin, 0.02% EDTA PBS, PAN Biotech) and transferred to 15 ml centrifuge tubes. Next cells were centrifuged (5 min, 1000 rpm, 20 °C, Eppendorf Centrifuge 5810R), supernatant was then removed, and cells were resuspended in 250 µl of media. Cells were then mixed with 50 µg salmon sperm carrier DNA and 1 µg of EGFP or pQM plasmids encoding for proteins of interest. Cells, DNA, and carrier mix was transferred to 4 mm cuvettes and transfected using Bio-Rad GenePulser Xcell (settings 200 V, 975 µF) (Hercules, CA, USA). Afterwards electroporated cells were transferred to 15 ml tubes in 3 ml of media and centrifuged (5 min, 1000 rpm, +20 °C, Eppendorf Centrifuge 5810R). Next supernatant was removed, cells were resuspended in 10 ml of media, transferred to 100 mm cell culture plates, and incubated at 37 °C in 5% CO₂ environment. Cells were analysed 48 h post transfection by Western blot, immunofluorescence, or immunoprecipitation analysis.

2.2.5 Immunofluorescence

For immunofluorescence analysis cells were electroporated and seeded on coverslips (VWR International, Radnor, PA, USA) in 24-well plates. 48 h after transfection cells were washed with PBS (phosphate buffered saline) (3x 5 min on a shaker) and fixed using 4% PFA-PBS solution (paraformaldehyde) for 10 minutes in RT. Cells were then washed with PBS (3x 5 min on a shaker) and permeabilised using 0,2% Triton X-100-PBS solution for 10 min while keeping the plate on ice. Next cells were washed with PBS (3x 5 min on a shaker) and blocked for 1 h in RT on a shaker in 3% BSA/PBS solution (3 µg BSA in 1 ml PBS). After blocking, cells were incubated overnight with protein specific primary antibodies (Table 2) diluted in 3% BSA/PBS solution. After incubation with primary antibody coverslips with cells were washed with PBS (3x 5 min on a shaker) and incubated with secondary anti-rabbit or anti-mouse antibodies conjugated to Alexa Fluor 488 or 568 (Table 3) (Invitrogen, Carlsbad, CA, USA) diluted in 3% BSA/PBS solution for 1h in RT. After incubation with secondary antibody coverslips were washed with PBS (3x 5 min on a shaker) and rinsed twice with dH₂O. Nuclei were counterstained with a DAPI (4',6-diamidino-2-phenylindole) containing reagent (SlowFade® Gold antifade reagent with DAPI, Life Technologies) and EGFP were detected using auto-fluorescence. The samples were analysed using 63x magnification with Zeiss LSM 710 confocal microscope and software (Carl Zeiss AG, Oberkochen, Germany).

2.2.6 Immunoprecipitation

For immunoprecipitation (IP) cells were electroporated and seeded on 100 mm cell culture dishes according to the protocol above. 48 h after electroporation cells were collected in 500 µl of immunoprecipitation buffer (150 mM NaCl; 20 mM Hepes (pH = 7.2) + 100 mM K-acetate; 2 mM MgCl₂; 0.1% Tween-20; 1% Triton X-100; H₂O and protease inhibitor (Complete ULTRA Tablets, Roche), then 50 µl were taken (input) for Western blot analysis, 450 µl were incubated with GFP-Trap_M magnetic beads (ChromoTek GmbH, Munich, Germany) for 1 hour on end-over-end shaker at +4 °C. Afterwards GFP-Trap_M magnetic beads were washed with 500 µl IP buffer 3 times. After the washing, 40 µl 2x Laemli buffer was added to the beads and 50 µl of 2x Laemli buffer was added to the input sample. Input and GFP-Trap_M magnetic bead protein samples were boiled for 10 min in +100 °C to denature the proteins.

2.2.7 SDS-electrophoresis and Western blot

Input and immunoprecipitated protein samples were separated using SDS (sodium dodecyl sulphate)-PAGE (polyacrylamide gel electrophoresis) method at 150 V using polyacrylamide gel and SDS buffer (125 mM Tris, 960 mM glycine, 0.5% SDS). For protein separation 12%

or 10% polyacrylamide gels were used according to the size of the protein in question. 10 µl of sample was loaded in each well. After separation, proteins were transferred to PVDF (polyvinylidene fluoride) membrane (Amersham™ Hybond™ P 0.45 PVDF Blotting Membrane, GE Healthcare Life Sciences, Marlborough, MA, USA) using semi-dry transfer method. Transfer was performed at 20 V 15 min for each membrane using TransBlot® SD Semi-Dry Transfer Cell (BioRad, Hercules, CA, USA) equipment. After transfer the membranes were blocked for 1 h RT in blocking buffer (100 mM Tris, pH 7.5; 170 mM NaCl; 0.05% Tween20; 5% NFDM (non-fat dry milk)) on a shaker. Afterwards primary antibodies were used to detect specific proteins. Antibodies used and appropriate dilutions are described in Table 2. All antibodies were diluted in 100 mM Tris, pH 7.5; 170 mM NaCl; 0.05% Tween20; 2.5% NFDM (non-fat dry milk). Membranes were incubated with primary antibodies at +4 °C over night or 1h in RT on a shaker. After incubation with primary antibodies, membranes were washed (3x10 min on a shaker) using Western blot washing buffer (100 mM Tris, pH 7.5; 170 mM NaCl; 0.05% Tween20). After washing membranes were incubated with HRP (horseradish peroxidase) conjugated secondary antibody based on the primary antibody origin organism (Table 3). Antibodies were diluted in 100 mM Tris, pH 7.5; 170 mM NaCl; 0.05% Tween20; 2.5% NFDM. All membranes were incubated with secondary antibodies for 1 h in RT. After incubation with secondary antibody membranes were washed (3x10 min on a shaker) with Western blot washing buffer. Protein signals were developed using ECL detection kit (Amersham™ ECL™ Western Blotting Detection Reagents, GE Healthcare Life Sciences, Marlborough, MA, USA) according to the manufacturers protocol. Protein signals were detected with autoradiography method using AGFA roentgen film (AGFA CP-BU new Medical X-ray Film Blue, Agfa HealthCare, Mortsel, Belgium). Images were scanned using EPSON EXPRESSION 1680 PRO scanner (Suva, Japan).

2.2.8 TRMT112 knockout cell line isolation using CRISPR/Cas9 system

CRISPR/Cas9 system was used to generate a genomic knockout of TRMT112 in U2OS cells. For this project it was chosen to use a plasmid delivery method using vector pSpCas9(BB)-2A-Puro (PX459) V2.0 (Ran *et al.*, 2013), as the Cas9 and guide RNA containing plasmids can be prepared and delivered to the cells in a standard laboratory set-up. Plasmid has its benefits, for example it offers a longer Cas9 expression in the cells, nonetheless it also takes the most time as Cas9 needs to go thorough transcription and translation. Furthermore, longer Cas9 expression in the cells also increases the possibility of off-target cuts, moreover plasmid DNA has the potential to cause mutagenesis through insertion (Yip, 2020).

It was decided to deliver the Cas9-containing plasmid through the commercially available lipid nanoparticle delivery system Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Lipofectamine 2000 contains cationic liposomes that form complexes with negatively charged nucleic acids, which can fuse with negatively-charged cell membrane or can be delivered into the cell through endocytosis (Lino *et al.*, 2018). The main drawback of this system is that the particle has to escape lysosomal degradation after entering the cell and afterwards translocate to the nucleus, due to this the delivery efficiency can be rather low (Lino *et al.*, 2018). Nonetheless, lipid nanoparticle delivery system has been shown to be an effective way of delivering plasmid DNA for Cas9 expression, targeted gene knock-out and subsequent knock-out cell line isolation (Yang *et al.*, no date).

2.2.8.1 CRISPR/CAS9 gRNA design

gRNAs were designed (done by Margit Mutso, PhD) targeting TRMT112 exon 1 after two separate PAM sequences, also two in exon2 and one in exon 4 using <http://crispr.mit.edu/> , <http://www.rgenome.net/cas-offinder/> websites and primer sequences were ordered from Microsynth, Balgach, Switzerland (Table 4). pSpCas9(BB)-2A-Puro (PX459) V2.0 was a gift from Feng Zhang (Addgene plasmid # 62988; <http://n2t.net/addgene:62988>; RRID:Addgene 62988).

Table 4. Guide RNA primer sequences for CRISPR/Cas9 plasmids targeting TRMT112 gene in exon 1, 2 and 4.

Oligo name	DNA sequence 5'-3'	Size (bp)
Exon 1.1 F	CACCGATGCGAGCTCAGCAGATTGT	25
Exon 1.1 R	AAACACAATCTGCTGAGCTCGCATC	25
Exon 1.2 F	CACCGGCTGAGCTCGCATGTGCGGG	25
Exon 1.2 R	AAACCCCGCACATGCGAGCTCAGCC	25
Exon 2.1 F	CACCGTCATACGCGCCACGAAGTTG	25
Exon 2.1 R	AAACCAACTTCGTGGCGCGTATGAC	25
Exon 2.2 F	CACCGGGCGCGTATGATACCTAAAG	25
Exon 2.2 R	AAACCTTTAGGTATCATACGCGCCC	25
Exon 4 F	CACCGCGTATGTTCCCATCAGCCG	25
Exon 4 R	AAACCGGCTGATGGGGAACATACGC	25

2.2.8.2 CRISPR/CAS9 plasmid cloning

Step 1. Annealing of oligos

1 µl of 100 µM Forward oligo, 1 µl of 100 µM Reverse oligo (Table 4), 1 µl 10x T4 Ligation Buffer (NEB) (Thermo Fisher Scientific, Waltham. MA, USA), 0,5 µl T4 PNK (Thermo Fisher Scientific, Waltham. MA, USA), and 6,5 µl ddH₂O (total reaction volume 10 µl) was mixed and then incubated in thermal cycle (Biosan, Rīga, Latvia) for 30 min at 37°C, then 5 min at 95°C and then thermal cycler with samples inside was switched off and cooled down to RT. Afterwards annealed oligo duplex was diluted with ddH₂O (1:2).

Step 2. Plasmid vector digestion

2 µl (1 µg) of pSPCas9(BB)-2A-Puro vector was mixed with 1 µl FastDigest BbsI, 2 µl 10x Fast digest buffer and 15 µl of ddH₂O (total reaction volume 20 µl) then incubated in thermal cycle at 37°C (Biosan, Rīga, Latvia) for 30 min to digest the plasmid vector. Next the digested plasmid mix was treated with 1 µl Fast AP. BbsI restriction enzyme, FastAP (Thermosensitive alkaline Phosphatase) and the buffer were obtained from Thermo Fisher Scientific, Waltham. MA, USA (Catalogue number FD1014, EF651).

Step 3. Ligation

PX459 vector was ligated with oligo duplex by mixing 3 µl of oligo duplex, 0,05 µg of the digested PX459 vector, 2 µl of 10x T4 ligase buffer, 1 µl of T4 ligase and 13,5 µl of ddH₂O. Mix was then incubated at +16°C overnight in a PCR thermocycler (Tprofessional thermocycler, Biometra - Analytik Jena, Jena, Germany). T4 ligase and ligase buffer were obtained from Thermo Fisher Scientific, Waltham. MA, USA.

Step 4. Transformation

Plasmids were amplified using competent *E.coli* XL-10 gold cells. 10 µl of ligation mixture was mixed with thawed *E.coli* XL-10 gold competent cells and incubated on ice for 30 min followed by heat shock for 2 min at 42 °C. Samples were cooled down on ice for 2 min, then 500 µl of Luria Bertani (LB) liquid media was added and cells were incubated at 37 °C (Biosan, Rīga, Latvia) for 1,5 h. Afterwards cells were centrifuged at 4000 rpm for 5 min, supernatant was removed and cells were resuspended in 100 µl of LB liquid media. Cells were then transferred to LB-ampicilline plates as PX459 vector contains ampicilline resistance gene and incubated for 16 h at 37 °C using a Heraeus incubator (Heraeus Function Line, Hanau, Germany).

Step 5. Plasmid DNA extraction

Afterwards six colonies of each construct were selected from transformed colonies for plasmid extraction using FavorPrep plasmid extraction kit (Favorgen, Ping-Tun, Taiwan) according to the manufacturers protocol. DNA concentration was measured using NanoDrop™ 2000 (Thermo Fisher Scientific, Waltham. MA, USA) and control restriction was performed using NdeI and EcorI enzymes (Thermo Fisher Scientific, Waltham. MA, USA). Inserted oligo sequences in the plasmid were confirmed by Sanger sequencing in the University of Tartu, Institute of Genomics core facility laboratory (Tartu, Estonia). Afterwards plasmids with the correct sequence were selected and plasmid DNA was amplified using NucleoBond Xtra Midi EP (MACHERY-NAGEL, Düren, Germany) according to the manufacturers protocol. All plasmids obtained are listed in the Table 5. In pSPCas9(BB)-2A-Puro (PX459) V.2.0 vector Cas9 is expressed under chicken β -actin hybrid (Cbh) promoter, but the gRNA U6 promoter.

Table 5. CRISPR/Cas9 plasmids targeting TRMT112 gene.

Plasmid vector
pSPCas9(BB)-2A-Puro (PX459) _TRMT112_Exon_1.1
pSPCas9(BB)-2A-Puro (PX459) _TRMT112_Exon_1.2
pSPCas9(BB)-2A-Puro (PX459) _TRMT112_Exon_2.1
pSPCas9(BB)-2A-Puro (PX459) _TRMT112_Exon_2.2
pSPCas9(BB)-2A-Puro (PX459) _TRMT112_Exon_4

2.2.8.3 CRISPR/Cas9 knockout clonal cell line isolation

In the previous day, 1×10^6 of U2OS cells were seeded into 6-well plate. The following day, approximately 50% confluent cells were transfected using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham. MA, USA) according to the manufacturers protocol using 500 ng plasmid DNA and 1,5 μ l Lipofectamine 2000 per transfection reaction. Two days after transfection, selection media containing 5 μ g/ml of Puromycine (Cayman Chemicals, Ann Arbor, MI, USA) was added. After selection cells were allowed to recover and form colonies for 3 weeks. Visible colonies were picked and transferred to a 24 well plate. After cells had reached 80% confluency in a 24 well plate, cells were transferred to a 6 well plate and subsequently to a 100 mm plate. When cells reached confluency, 2×10^6 cells were lysed in 100 μ l 2x Laemli buffer and samples were boiled for 10 min in +100 °C to denature proteins. Next samples were separated using SDS (natrium dodecyl sulphate)-PAGE method and analysed with Western blot.

2.3 Results

2.3.1 TRMT112-interacting methyltransferases initiate endogenous TRMT112 re-localisation

First the subcellular localisation of TRMT112 and its partner methyltransferases was studied. Commercial antibodies were used to analyse localisation pattern of endogenous TRMT112 as well as its partner MTase expression (Figure 4A) and compared with the EGFP-MTase overexpression pattern (Figure 4B) in U2OS cells. Immunofluorescence assays were repeated two times. Cells overexpressed with EGFP-MTases were also counterstained with TRMT112 primary antibody to analyse TRMT112 localisation. Endogenous TRMT112 displayed a strong nuclear as well as cytoplasmic signal which also correlates with a previous study (Zorbas *et al.*, 2015). TRMT112-EGFP displayed a similar localisation pattern as the endogenous protein, however it is also similar to the free EGFP pattern and may also be caused by degradation products of TRMT112-EGFP fusion protein detected in previous experiments in Tartu University, Institute of Technology (manuscript in preparation). N6AMT1 endogenous protein was rather weakly detected all over the cell with a slightly stronger expression in the cytoplasm. Overexpressed N6AMT1 showed a similar pattern, but here the protein expression was more enhanced in the nucleus, furthermore, recombinant N6AMT1 appeared to slightly enhance the nuclear expression of TRMT112. Both endogenous and recombinant WBSCR22 displayed a strong signal in the cell nucleus with accumulation in the nucleoli, moreover recombinant WBSCR22 appeared to significantly enhance the expression of nuclear TRMT112 while reducing its cytoplasmic expression. At the same time WBSCR22 and TRMT112 co-expression was not detected in the nucleolus as it has already been demonstrated in previous study (Õunap *et al.*, 2015). Endogenous METTL5 displayed a stronger signal in the cytoplasm compared to nucleus, but at the same time accumulation in the nucleoli could be observed. Overexpressed METTL5 was detected all over the cell, while similarly to N6AMT1, TRMT112 expression was slightly enhanced in the nucleus compared to recombinant METTL5 negative cells. THUMPD2 protein showed a strong cytoplasmic and rather weak nuclear signal while the overexpressed protein could only be detected in the nucleus, raising the question whether the available antibody is sufficiently specific. Moreover, as with WBSCR22, recombinant THUMPD2 significantly enhanced TRMT112 expression in the nucleus while reducing TRMT112 amount in the cytoplasm. Both endogenous and recombinant THUMPD3 protein showed a similar localisation pattern with strong signal in the cytoplasm. Endogenous ALKBH8 displayed a cytoplasmic as well as nuclear signal with strong signal of nuclear bodies. However, recombinant ALKBH8 protein had a similar localisation pattern with THUMPD3,

displaying strong cytoplasmic signal, furthermore it appeared that both THUMPD3 and ALKBH8 were able to significantly enhance TRMT112 cytoplasmic expression while reducing TRMT112 amount in the nucleus. In the case of THUMPD3 this effect was especially noticeable. Lastly, TRMT11 endogenous level displayed a hardly detectable cytoplasmic signal raising questions whether the commercial antibody is suitable for immunofluorescence studies. Instead, overexpressed MTase showed a strong cytoplasmic localisation pattern, but contrary to other MTases, it did not have a noticeable effect on TRMT112 protein localisation.

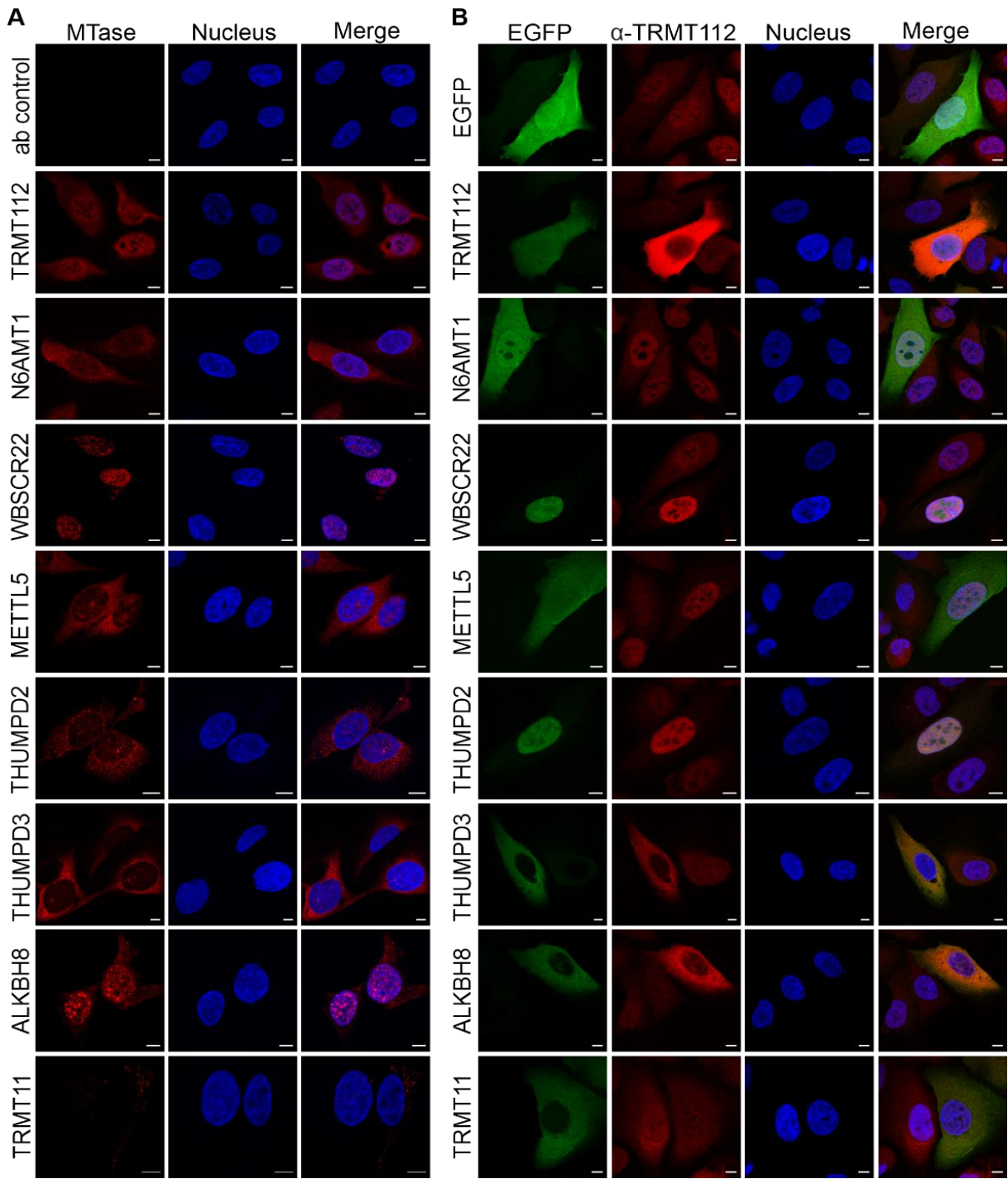


Figure 4. Subcellular localisation of endogenous and overexpressed TRMT112, N6AMT1, WBSCR22, METTL5, THUMP2, THUMP3, ALKBH8 and TRMT11 in U2OS cells. Cells were counterstained with DAPI for DNA labelling. Image sections were captured with Zeiss LSM 710 confocal microscope at 63× magnification. Scale bar, 10 µm. **A.** U2OS cells processed for immunofluorescence with primary antibodies specific to TRMT112, N6AMT1, WBSCR22, METTL5, THUMP2, THUMP3, ALKBH8 and TRMT11 and secondary antibody conjugated with Alexa-568. **B.** U2OS cells were transfected with plasmids coding for EGFP-tagged TRMT112, N6AMT1, WBSCR22, METTL5, THUMP2, THUMP3, ALKBH8 and TRMT11 (revealed thorough EGFP autofluorescence), processed for immunofluorescence with primary antibody specific to TRMT112 and secondary antibody conjugated with Alexa-568.

2.3.2 TRMT112 protein level in cells is tightly regulated and controlled

In previous studies conducted in the Institute of Technology, it has been demonstrated that expression of recombinant WBSCR22 and N6AMT1 proteins together with TRMT112 stabilised both protein in human cells (Öunap *et al.*, 2015; Leetsi *et al.*, 2019). Therefore, during this project additional experiments were conducted to study this in greater detail. For this U2OS cells were transfected with plasmids encoding for EGFP-MTases together with TRMT112-E2Tag construct and the cells were analysed 48 hours later. Figure 5A illustrates, that TRMT112 protein expression was enhanced by co-expression with all MTases except THUMP2. The strongest effect could be observed in the case of N6AMT1, TRMT11 and THUMP3, it was also rather strong in the case of WBSCR22, METTL5 and ALKBH8, even though ALKBH8 had a slightly weaker effect than WBSCR22 and METTL5. It could also be observed that cells transfected with the recombinant TRMT112 protein have reduced endogenous TRMT112 expression levels.

Next it was studied whether increasing TRMT112 protein level in cells also affects the endogenous level of its partner methyltransferases. For this U2OS cells were electroporated with increasing amounts (0,25 µg to 4 µg of DNA) of TRMT112-E2tag plasmid construct and cells were analysed 48 h post transfection. As can be seen in figure 5B, the amount of exogenous TRMT112 gradually increased, but at one point the level of endogenous TRMT112 level started to drop. Together with results from figure 5A, this illustrates that the cell does not distinguish between recombinant TRMT112 or endogenously expressed protein. It could be argued that when the recombinant protein reaches a certain level, the cell starts to use that instead of the naturally expressed TRMT112. Meanwhile, no significant changes in the endogenous levels of the WBSCR22, N6AMT1, METTL5 and ALKBH8 were detected. It should be noted that TRMT11, THUMP2 and THUMP3 protein levels could not be studied due to lack of commercially available antibodies for Western blot analysis.

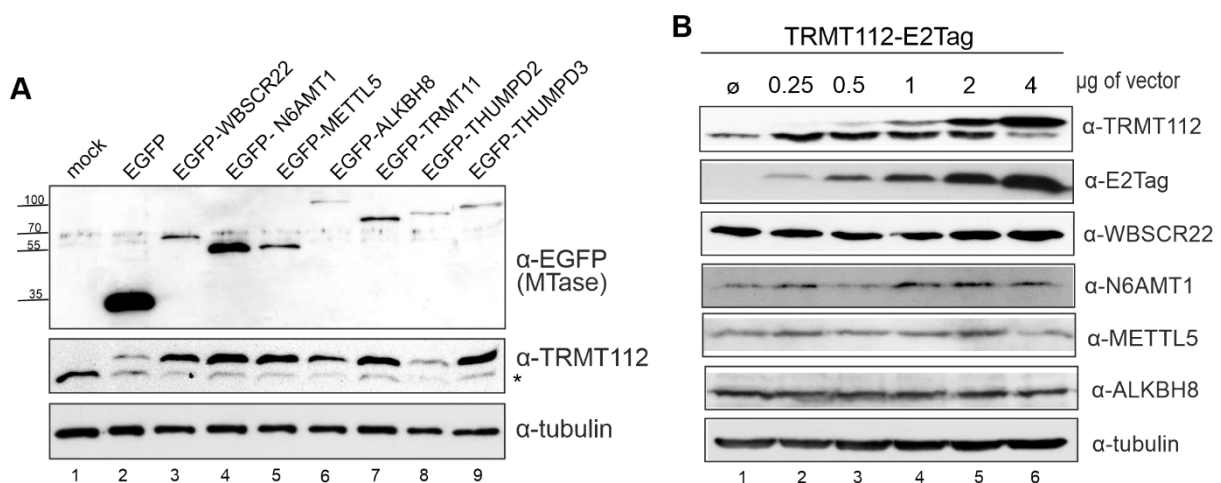


Figure 5.A. TRMT112 protein expression is stabilised by co-expression with its partner methyltransferases. U2OS cells were co-transfected with plasmids coding for TRMT112-E2Tag and EGFP-tagged WBSCR22, N6AMT1, METTL5, ALKBH8, TRMT11, THUMP2, THUMP3 as well as EGFP alone. Cells were analysed 48 hours later with Western blot using antibodies against EGFP, TRMT112 and tubulin. Images of results with anti-TRMT112 depict both recombinant (upper line) and endogenous (lower line marked with *) protein.

Figure 5.B. Increasing TRMT112 protein level in cells does not affect the endogenous level of its partner methyltransferases. U2OS cells were transfected with increasing amounts (0,25 μg to 4 μg of DNA) of TRMT112-E2tag plasmid construct and cells were analysed 48 h later with Western blot using antibodies against TRMT112, E2tag, WBSCR22, N6AMT1, METTL5, ALKBH8 and tubulin.

2.3.4 TRMT112 expression and stability is strongly affected by single amino acid mutations

Recent studies have revealed that TRMT112 interacts with N6AMT1 and METTL5 in a similar way through a large area (van Tran *et al.*, 2019; W. Li *et al.*, 2019). To study in more detail whether TRMT112 interacts with all of its partner MTases in a similar way, several amino acids located on the surface where TRMT112 forms its complex with the partner MTases were mutated and constructed into a E2Tag plasmid vectors (performed by Margit Mutso). In the plasmid constructs, non-polar amino acids Leu8, Met45 and Ile113 responsible for forming hydrophobic interactions with N6AMT1 and METTL5 ((van Tran *et al.*, 2019; W. Li *et al.*, 2019) were changed to other non-polar amino acids tryptophan (L8W), alanine (M45A) and phenylalanine (I113F). Hydrogen bond forming amino acids Thr5 and Lys48 were replaced with alanine (T5A, K48A), but Ser10 with phenylalanine (S10F). In addition, three more amino acids Glu50, Glu92 and Phe107 were mutated to alanine (E50A, E92A, F107A) and one double mutant construct K48A+E92A was made.

First the effect of amino acid mutations on TRMT112 expression level in U2OS and HEK293 cells was studied. U2OS and HEK293 were transfected with wt TRMT112, its

mutants and empty pQM vector and the cell samples were analysed 48 h post transfection using antibodies against E2Tag and TRMT112 itself. Images of results with anti-TRMT112 depict both recombinant (upper line) and endogenous (lower line marked with *) protein. As seen in figure 6, T5A, M45A, E92A, F107A and double mutation K48A+E92A significantly reduced the recombinant TRMT112 expression in U2OS as well as HEK293 cells. I113F also exhibited a reduced expression in both cell lines, but not as significantly as T5A, M45A, E92A, F107A or the double mutant. Meanwhile L8W, K48A and E50A mutations did not have a noteworthy effect on the expression level of TRMT112 compared to the wt protein in both U2OS and HEK293 cell lines. On the other hand, S10F appeared to enhance and stabilise TRMT112 expression in U2OS cells but not in HEK293.

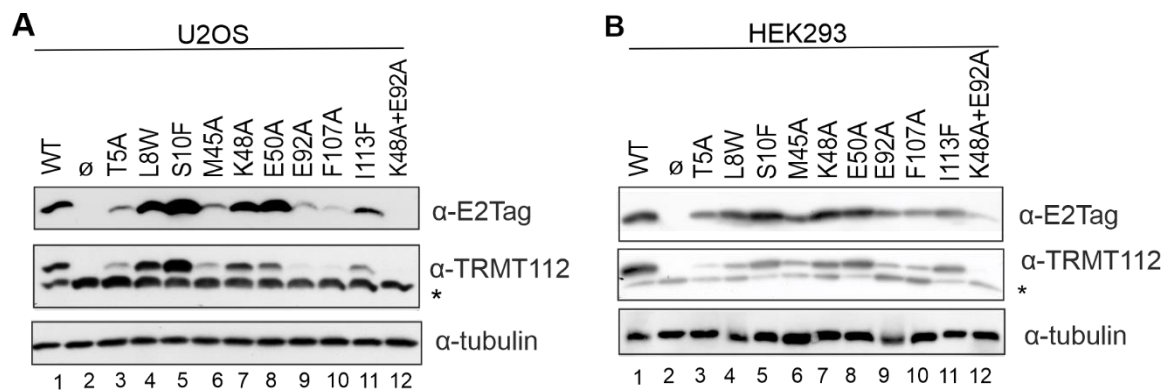


Figure 6. TRMT112 expression and stability is strongly affected by single amino acid mutations. U2OS (A) and HEK293 (B) cells were transfected with E2tagged plasmids encoding for wt TRMT112 (line 1), TRMT112 containing single amino acid mutations (T5A (line 3), L8W (line 4), S10F (line 5), M45A (line 6), K48A (line 7), E50A (line 8), E92A (line 9), F107A (line 10), I113F (line 11), K48A+E92A (line 12)) and empty pQM vector (line 2). Samples were analysed 48 h later using antibodies against E2Tag, TRMT112 and tubulin. Images of results with anti-TRMT112 depict both recombinant (upper line) and endogenous (lower line marked with *) protein.

2.3.5 TRMT112 mutants confirm many similarities, but also reveal several differences while interacting with its partner methyltransferases

Next U2OS cells were co-transfected with two plasmid vectors, encoding for TRMT112 or its mutants and EGFP-MTases. 48 h post transfection immunoprecipitation assay with GFP_Trapp beads was performed using EGFP tagged MTase as a bait. EGFP-MTase proteins were able to interact with both, recombinant and endogenous TRMT112 proteins and immunoprecipitate them from the cell lysates through the GFP_Trapp binding. In Figure 7, upper three images of each panel represent input where soluble fraction of cell lysates was analysed with anti-EGFP, anti-E2Tag and anti-TRMT112 antibodies. IP GFP-trap section (lower three images) shows the

immunoprecipitated proteins after the GPF_Trapp assay analysed with the same antibodies as input section. Images of results with anti-TRMT112 depict both recombinant (upper line) and endogenous (lower line marked with *) protein.

During this experiment a strong stabilization effect through co-expression of TRMT112 and its partner methyltransferase could be observed once more. Only exception here was THUMPD2 (Figure 7G) which overall exhibited a very low signal, however when comparing co-transfection with WT and empty pQM vector signals, a mild stabilisation effect could still be observed.

Analysis of the TRMT112 mutation effects revealed numerous similarities along with several differences in the way TRMT112 interacts with its partners. The amino acids Leu8, Met45 and Ile113 of TRMT112, as previously mentioned, are directly involved in hydrophobic interactions with N6AMT1 and METTL5 (van Tran *et al.*, 2019; W. Li *et al.*, 2019). In this experiment, mutations L8W and M45A had a strong impact on METTL5 interaction (Figure 7C; lines 4, 6) and WBSCR22 (Figure 7B). These mutations had a less significant impact on ALKBH8 (Figure 7D, E) and appeared to have no effect at all on N6AMT1 and TRMT11 interaction (Figure 7A). I113F mutation had no effect on either of them (Figure 7A, B, C, D, lines 11). On the other hand, all three mutation L8W, M45A and I113F had a significant effect on THUMPD2 and THUMPD3 interactions (Figure 7F, G, lines 4, 6, 11). Hydrogen-bond forming amino acid threonine 5 replacement with alanine (T5A) strongly affected the stability of TRMT112 in cells (Figure 7) as well as interactions with most methyltransferases (Figure 7A, C, D, F, G; lines 3). Only one that appeared to slightly interact with T5A mutant was ALKBH8 (Figure 7B, line 3). In contrast, when another polar amino acid serine 10 was replaced with phenylalanine (S10F), it enhanced the stability of TRMT112 protein alone (Figure 7H, line 5). When co-expressed with EGFP-MTases, S10F had a positive effect on ALKBH8 interaction, did not significantly affect the interactions with N6AMT1, METTL5, ALKBH8, TRMT11 and THUMPD3, but had a negative effect on the TRMT112-WBSCR22 and TRMT112-THUMPD2 complex formation (Figure 7, lines 5). The K48A mutation had no effect on N6AMT1, WBSCR22, METTL5, TRMT11 and ALKBH8, but had a very strong negative effect on THUMPD2 and THUMPD3 (Figure 7, lines 7). E92A appeared to have a mild negative effect on THUMPD2 and THUMPD3 (Figure 7; lines 9), however together with K48A it had a significant negative impact on THUMPD2, THUMPD3 as well as TRMT11 (Figure 7; lines 12). Mutation E50A is not localised on the interface surface of TRMT112-MTase interaction and did not have a negative effect in any case (line 8). Lastly, replacement of the phenylalanine in position 107 with alanine (F107A) negatively influenced the interaction with THUMPD2, THUMPD3, but had a less noticeable effect in the case of METTL5 and

WBSCR22 (Figure 7G, F, B, C, lines 10). On the other hand, F107A mutation had no effect in the case of N6AMT1, ALKBH8, TRMT11 (Figure 7A, D, E, lines 10). The summary of immunoprecipitation results is shown in Table 6. All experiments were repeated at least three times.

Table 6. Summary of TRMT112 mutation analysis.

Interaction with TRMT112											
	WT	T5A	L8W	S10F	M45A	K48A	E50A	E92A	F107A	I113F	K48A+E92A
N6AMT1	++	-	++	++	++	++	++	++	++	++	++
WBSCR22	++	-	+	+	+	++	++	++	+	++	++
METTL5	++	-	+	++	+	++	++	++	+	++	++
ALKBH8	++	+	+	++	++	++	++	++	++	++	++
TRMT11	++	-	++	++	++	++	++	++	++	++	-
THUMPD2	++	-	-	-	-	-	++	+	-	-	-
THUMPD3	++	-	-	++	-	-	++	+	-	-	-

++ no effect; + negative effect; - significant negative effect

In conclusion, based on the data obtained from the TRMT112 mutation analysis, TRMT112-interacting MTases can be divided into three groups based on the impact different mutations had on the TRMT112-MTase interaction. The first group contains N6AMT1, which was hardly affected by any of the mutations, second group consists of METTL5, WBSCR22, ALKBH8 and TRMT11, which all were affected by certain mutations, some in a similar pattern, some differently, and lastly third group includes THUMPD2 and THUMPD2, which were affected by many mutations.

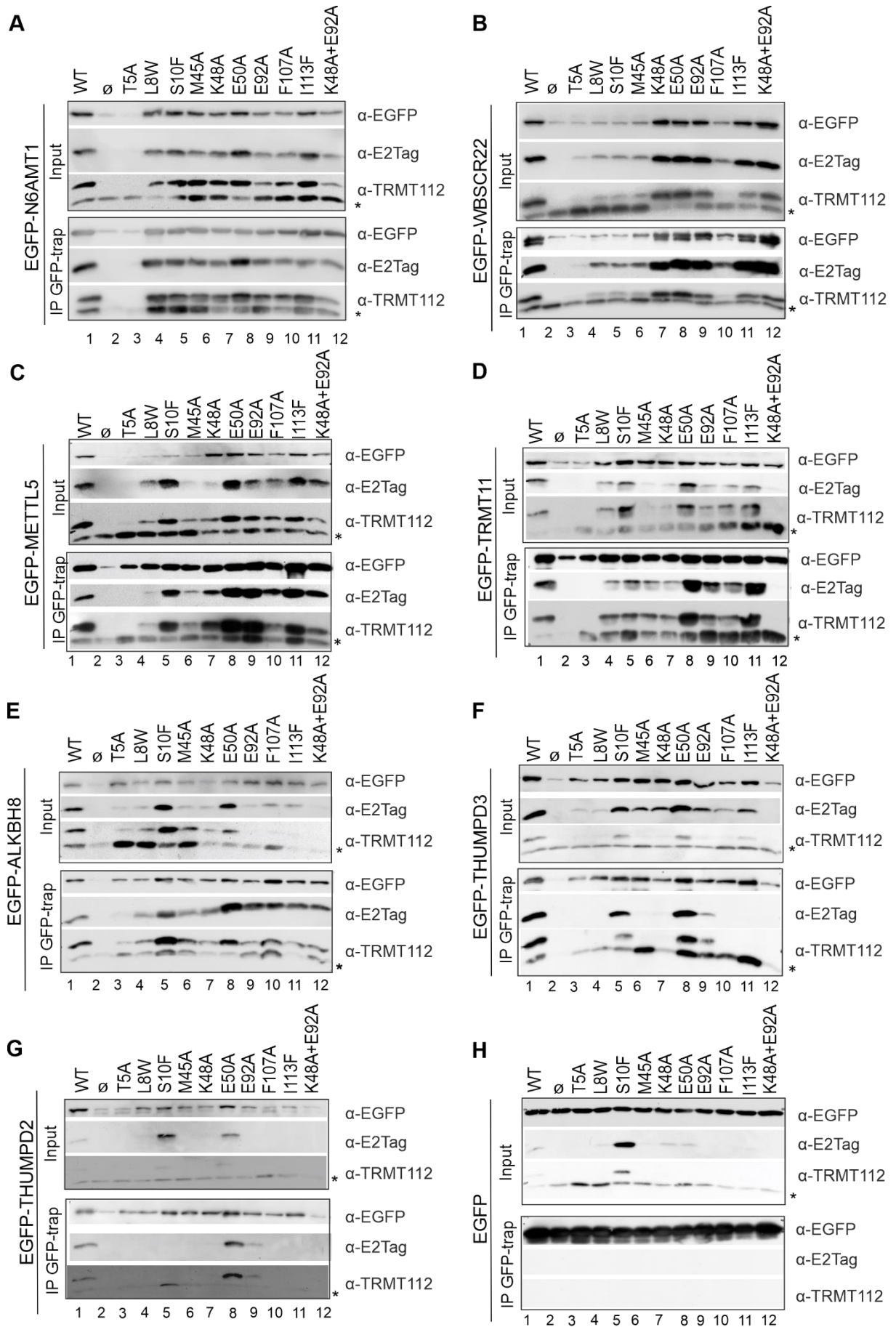


Figure 7. TRMT112 single amino acid mutant interactions with N6AMT1, WBSCR22, METTL5, TRMT11, ALKBH8, THUMP2, THUMP3 confirm similarities, but also

reveal several differences in the way TRMT112 interacts with its partners. U2OS cells were transfected with plasmids encoding for EGFP (*H*) and EGFP-tagged N6AMT1 (*A*), WBSCR22 (*B*), METTL5 (*C*), TRMT11 (*D*), ALKBH8 (*E*), THUMP2 (*F*), THUMP3 (*G*) along with E2tagged plasmids encoding for wt TRMT112 (line 1), TRMT112 containing single amino acid mutations (T5A (line 3), L8W (line 4), S10F (line 5), M45A (line 6), K48A (line 7), E50A (line 8), E92A (line 9), F107A (line 10), I113F (line 11), K48A+E92A (line 12)) and empty pQM vector (line 2). 48 h later immunoprecipitation assay with GFP_Trap beads was performed using EGFP-tagged MTase as a bait and samples were subsequently analysed with Western blot. Upper three images of A-H panel represent input total soluble cell lysate, while lower three images represent soluble fraction of cell lysates after the GFP_Trap assay. Both sections were analysed using antibodies against EGFP, anti-E2Tag and anti-TRMT112. Images of results with anti-TRMT112 depict both recombinant (upper line) and endogenous (lower line marked with *) protein.

2.3.6 Partial genomic knockout of TRMT112 using CRISPR/Cas9 system significantly reduces WBSCR22 protein level in human cells

In order to study the interactions of TRMT112 and its partner MTases further, it was attempted to create a TRMT112 knockout cell line using the CRISPR/Cas9 gene editing system. U2OS cells were transfected with Cas9 and guide RNA containing plasmids targeting TRMT112 exon 1, 2 and 4. During this experiment a full knockout cell line isolation proved to be unsuccessful, nonetheless two partial knockout cell lines were obtained, first one with a plasmid targeting exon 2, while the other one targeting exon 4 (Figure 8, lines 2 and 3).

Both partial knockout cell lines were confirmed by Western blot analysis with cell lysates containing 2×10^6 live cells. As can be seen from Figure 8, both cell lines exhibit different amounts of TRMT112 protein level, with exon 2 knockout (line 2) displaying significantly less amount of TRMT112 protein compared to exon 4 (line 3) knock out.

Next WBSCR22, N6AMT1 and METTL5 protein level was analysed in order to study whether genomic knockout of TRMT112 affects their protein level in the cell similarly to previously described results using TRMT112 knockdown with siRNA (Öunap *et al.*, 2015; Zorbas *et al.*, 2015; Leetsi *et al.*, 2019; van Tran *et al.*, 2019). Figure 8 illustrates that TRMT112 exon 2 knockout cell line (line 2) exhibited a significant reduction of WBSCR22, less noticeable reduction of METTL5 and had hardly any change in N6AMT1 expression. Exon 4 knockout cell line TRMT112 protein level was higher compared to exon 2 knockout and had hardly any effect on WBSCR22, METLL5 and N6AMT1 protein level (line 3).

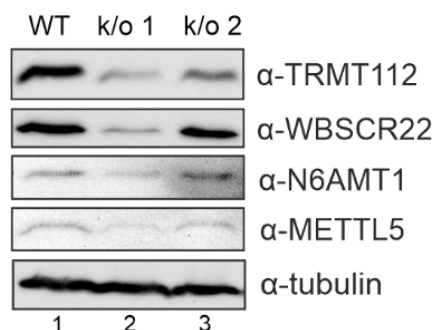


Figure 8. Partial genomic knockout of TRMT112 using CRISPR/Cas9 system significantly reduces WBSCR22 protein level in U2OS cells. Clonal cell lines of TRMT112 partial knockout in U2OS cells were obtained using CRISPR/Cas9 gene editing system targeting exon 2 (line 2) and exon 4 (line 4). Knockouts were confirmed by Western blot analysis using cell lysates containing 2×10^6 live cells. Samples were analysed with antibodies against TRMT112, WBSCR22, N6AMT1, METTL5 and tubulin. Knockout 1 targeting exon 2 (line 2) shows significantly reduced expression of TRMT112 compared to knockout 2 targeting exon 4.

Next subcellular localisation and expression of TRMT112 and its partner methyltransferases was studied in wild type and TRMT112 exon 2 knockout U2OS cells using commercial antibodies against TRMT112 and its partner MTases. Figure 4A has been represented here as Figure 9A to allow side by side comparison of both cell lines. Figure 9 illustrates that TRMT112 shows significant protein level reduction in the partial knockout cells as previously confirmed with Western blot analysis (Figure 9), furthermore a significant WBSCR22 as well as METTL5 reduction can also be seen. Another protein that displayed a significant downregulation in the partial knockout cell lines was THUMPD3, while ALKBH8 protein showed a slight reduction of cytoplasmic signal. In the case of N6AMT1, THUMPD2 as well as TRMT11 no significant expression or localisation changes could be observed, furthermore protein re-localisation was not observed in any case. Nonetheless, in the case of THUMPD2, THUMPD3, ALKBH8 and TRMT11 question remains whether the available antibodies are sufficiently specific for immunofluorescence studies as in Western blot they give a highly unspecific signal with multiple bands, therefore it is not possible to concretely answer the question whether partial genomic knockout of TRMT112 affects their expression and localisation in the cells. In conclusion, based on the data so far it can be argued that partial genomic TRMT112 knockout reduces WBSCR22 protein level and has a negative effect on METTL5 protein level, even though less that in the case of WBSCR22, as it was possible to confirm this using both Western blot and immunofluorescence methods.

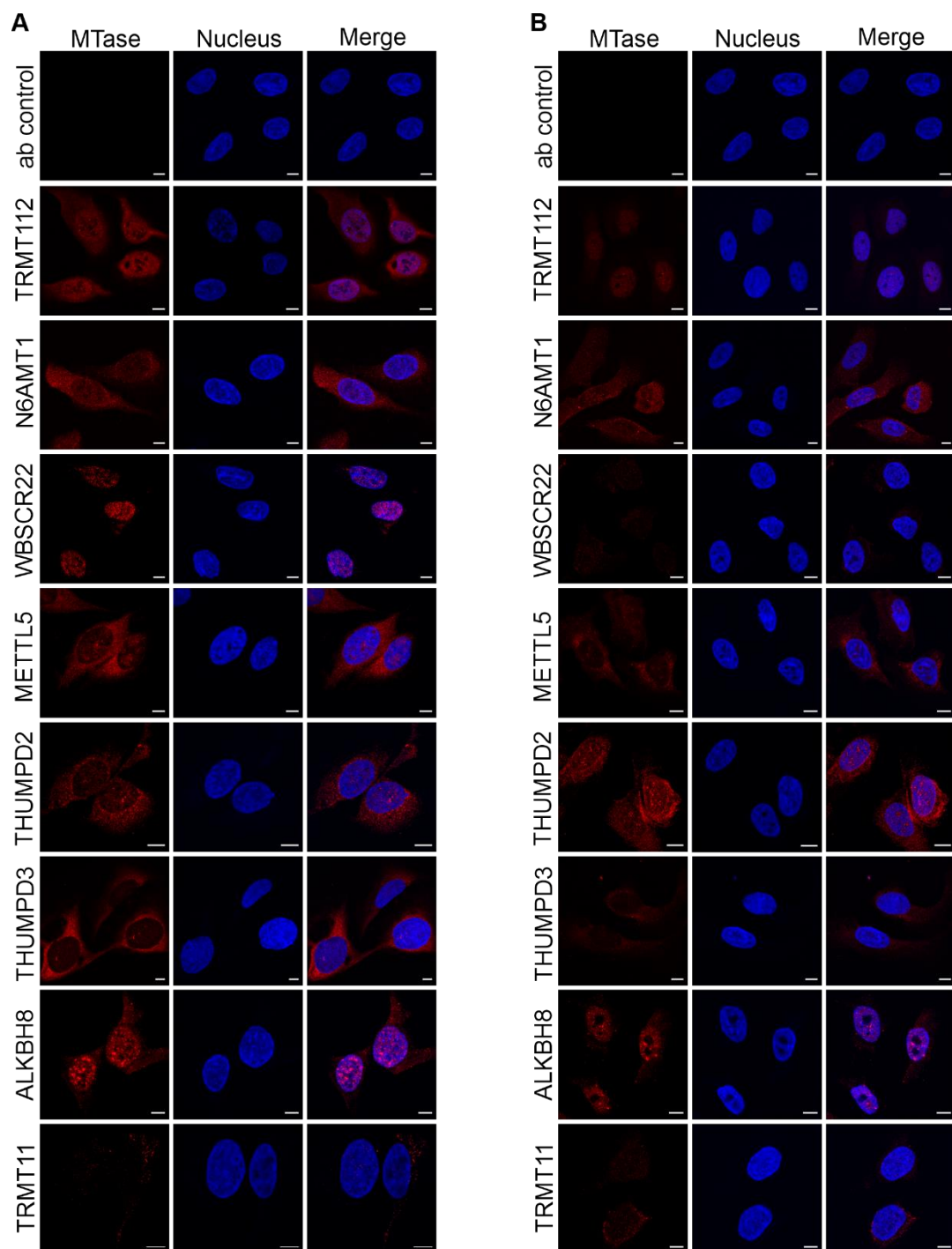


Figure 9. Subcellular localisation of endogenous TRMT112, N6AMT1, WBSCR22, METTL5, THUMPD2, THUMPD3, ALKBH8 and TRMT11 in wild type U2OS cells and U2OS containing partial knockout of TRMT112 gene in exon 2. Cells were counterstained with DAPI for DNA labelling. Image sections were captured with Zeiss LSM 710 confocal microscope at 63× magnification. Scale bar, 10 μm. **A.** Wild type U2OS cells processed for immunofluorescence with primary antibodies specific to TRMT112, N6AMT1, WBSCR22, METTL5, THUMPD2, THUMPD3, ALKBH8 and TRMT11 and secondary antibody conjugated with Alexa-568. **B.** U2OS cell line containing partial knockout of TRMT112 gene in exon 2 processed for immunofluorescence with primary antibodies specific to TRMT112, N6AMT1, WBSCR22, METTL5, THUMPD2, THUMPD3, ALKBH8 and TRMT11 and secondary antibody conjugated with Alexa-568.

2.4. Discussion

During this work the TRMT112-MTase network in mammalian cells in their cellular context was studied. In previous studies TRMT112 has been shown to interact with several methyltransferases and enhance their stability and catalytic activity. A recent SILAC assay from Reet Kurg Lab in Institute of Technology has revealed that TRMT112 interacts with seven methyltransferases – already previously studied N6AMT1, WBSCR22, METTL5 and ALKBH8, two novel methyltransferases THUMPD2 and THUMPD3 specific to human cells, as well as TRMT11, which so far has only been studied in yeast cells (manuscript in preparation). Most of the knowledge about eukaryotic TRMT112 and its orthologues come from studies in yeast, whereas mammalian cells have a markedly more complex internal environment with thousands of different biomolecules where protein function is dependent on epigenetic regulation, interactions with other molecules, availability of substrates as well as compartmentalization.

Firstly, the cellular localisation of TRMT112 and its partners was analysed. Mammalian TRMT112 is localized to both nucleus and cytoplasm of the cell where it interacts and forms complexes with the methyltransferases. It is sometimes difficult to determine the localisation of endogenous proteins as it depends on the specificity of available antibodies. Therefore, to overcome this, EGFP-fused methyltransferases were expressed in cells and compared to their localisation with the signal from available antibodies. The most prominent differences were observed in the case of ALKBH8 and THUMPD2. Furthermore, METTL5 antibody exhibited strong signal in the nucleolus, however the same was not observed with the recombinant METTL5. All mentioned antibodies were polyclonal and potentially not sufficiently specific for use in immunofluorescence assay as they also produce multiple bands during Western blot assay.

During immunofluorescence studies, it was detected that when TRMT112 partner MTases are overexpressed in the cells, endogenous TRMT112 shows increased signal and appears to be able to slightly alter its cellular localisation to follow the localisation of its partner MTase. The data from IF analysis indicate that WBSCR22 and THUMPD2 overexpression increases the amount of TRMT112 in the nucleus while reducing its cytoplasmic expression, but THUMPD3 and ALKBH8 overexpression increases TRMT112 amount in the cytoplasm while reducing its signal in the nucleus. For THUMPD3 this effect was especially noticeable. In the case of N6AMT1, METTL5 and TRMT11 this effect was not as prominent, nonetheless with N6AMT1 and METTL5 overexpression a slight increase in nuclear TRMT112 was observed. Re-localization of recombinant TRMT112 when it is overexpressed simultaneously with exogenous WBSCR22 has also been detected in previous research. Öunap et. al have

showed that mCherry tagged TRMT112 is detected all over the cell, but when co-expressed with EGFP tagged WBSCR22, it relocates to the nucleus (Öunap *et al.*, 2015). One possibility to explain this phenomenon is that when a partner MTase is overexpressed, it is followed by increased TRMT112 protein synthesis in the cell which is then transported to where its partner MTase is localised. It might also be possible that the re-localisation effect emerges from already synthesised free TRMT112 movement in the cell, however, currently it is unknown how much free TRMT112 protein there is in the cells and how much of it is in a complex with other proteins. Another possibility to explain this phenomenon may be that methyltransferases are competing with one other for binding with TRMT112, and WBSCR22, THUMPD2, THUMPD3 and ALKBH8 might be able to outcompete other MTases as they all had a more noticeable effect on TRMT112 re-localisation. Currently it is unknown how stable TRMT112-MTase heterodimers are and whether they do or do not disassociate once they are formed. It may be that TRMT112-MTase complexes in the cell are not stable but instead are dynamic and potentially might be able to disassociate, thus allowing TRMT112 to interact with another MTase if a partner with stronger binding potential is detected. Nonetheless, at this point in research it is not possible to confirm any of these hypotheses as immunofluorescence is only a visual method where the results are based on the specificity of antibodies, therefore further studies are needed to elucidate on this phenomenon.

During previous studies it has been shown that WBSCR22 and METTL5 are metabolically stabilised by TRMT112 (Öunap *et al.*, 2015; Zorbas *et al.*, 2015; van Tran *et al.*, 2019). In additional experiments in Reet Kurg lab, Institute of Technology, it was observed that the recombinant TRMT112 stabilised all seven of its partner MTases when expressed together in the cells (manuscript in preparation). During this study a strong positive feedback loop was observed – expression of MTases stabilised the TRMT112 protein itself in the cells. The only exception here was THUMPD2 which did not appear to enhance the recombinant TRMT112 signal. Interestingly, it was observed that endogenous level of TRMT112 fluctuated when recombinant MTase together with TRMT112 or its mutant were introduced to the cell. Co-expression of recombinant TRMT112 or its mutants together with EGFP-MTases most often reduced endogenous TRMT112, but sometimes enhanced it, this effect was especially visible in case of WBSCR22, METTL5, TRMT11, THUMPD3 and ALKBH8, but less with THUMPD2. This data suggests that endogenous and recombinant TRMT112 are interchangeable in their ability to form complexes with recombinant methyltransferases. The overexpression of recombinant MTase interacts with both exogenous as well as already and newly synthesised endogenous TRMT112, which leads to an overall increased amount of TRMT112 in the cell. It might be only a temporary effect, as previous experiments in Reet Kurg

lab, Institute of Technology using stable cell lines of modified TRMT112 protein, always showed reduced endogenous protein level (manuscript in preparation). Therefore, the data suggests that there is a dynamic and tightly controlled TRMT112-MTase network in the cells where the amount of each MTase depends on the free amount of TRMT112 and vice versa. It is possible that the amount of TRMT112 as well as MTase depends on the amount of available partner protein to form a heterodimer and in time some of the unbound proteins might be degraded. This has previously been shown to be the case with WBSCR22 (Öunap *et al.*, 2015) but more research is needed to confirm whether it is the same with all TRMT112 partners.

However, when gradually increasing only the amount of exogenous TRMT112 in the cell, endogenous level of N6AMT1, WBSCR22, METTL5 and ALKBH8 remained unchanged. This shows that the cell itself cannot increase MTase expression in response to increased TRMT112 amount in the cell. Nonetheless, further research is needed to determine whether increasing amounts of exogenous partner MTase leads to increased TRMT112 expression in the cells.

The crystal structure analyses of TRMT112 and MTase complexes studied so far have revealed, that TRMT112 interacts with its partners in a similar way. All TRMT112 partners studied so far in human and yeast cells have revealed that TRMT112 binds to the same region of the MTase domain. TRMT112 does not bind close to the active site on the MTases and it appears that TRMT112 is not directly involved in substrate binding and recognition as it does not have a direct affect the chemical structure and binding properties of the MTase (W. Li *et al.*, 2019). Crystal structures have revealed that TRMT112 covers a large hydrophobic area of its partner MT+ase and it is suggested that TRMT112 binding stabilises the partner protein, thus indirectly enhancing the substrate binding of its partner MTase (van Tran *et al.*, 2019; W. Li *et al.*, 2019). Nonetheless, in human cells crystal structures of TRMT112 are available only with N6AMT1 and METTL5 complex, therefore it cannot be ruled out that some unknown differences exist. The data obtained from this study has revealed that certain mutations on different parts of the TRMT112 binding surface affect its MTase partners in different ways resulting in unaffected or reduced binding and even no binding of TRMT112 at all. Therefore, it can be suggested that although it appears that TRMT112 interacts with its partners in a similar way, there are subtle but nevertheless important differences.

Lastly, during this project the attempt to create a human cell line containing full genomic knockout of TRMT112 was unsuccessful, nonetheless a partial knockout cell line was isolated. During the clonal cell line isolation, it appeared that in some colonies, cells divided at first, but soon become apoptotic, therefore not allowing the colony to survive. This raises a question whether TRMT112 is an essential gene in human cells. It has previously been shown that

TRMT112 is not an essential gene in yeast as well as *Arabidopsis thaliana* (Purushothaman *et al.*, 2005; Hu *et al.*, 2010; Mazauric *et al.*, 2010; Figaro *et al.*, 2012), but no previous studies have attempted to knockout TRMT112 gene from human cells. It cannot be dismissed that TRMT112 gene might be vital for human cell survival, therefore it might not be possible to create a full knockout cell line, however at this stage of this research it cannot be confirmed. Nonetheless, it was revealed that even a partial knockout significantly reduces WBSCR22 expression, slightly reduces METLL5 and has hardly any effect on N6AMT1 expression. Due to lack of commercially available specific antibodies for Western blot assay all the other MTase partners could not be tested. Immunofluorescence results suggest that TRMT112 partial knockout significantly reduces THUMP3 expression as well, however it cannot be confirmed at this stage of research as it was not possible to perform Western blot with the available antibodies. It has already previously been shown that knockdown of TRMT112 with siRNA reduces WBSCR22, METLL5 expression but not N6AMT1 expression ((Öunap *et al.*, 2015; Zorbas *et al.*, 2015; Leetsi *et al.*, 2019; van Tran *et al.*, 2019). Data obtained from this study confirms this and suggests that from these three partners, WBSCR22 might be the most sensitive and dependant on TRMT112 level changes and even a partial reduction of TRMT112 greatly reduces WBSCR22 level in the cell.

It is suggested that the primary function of TRMT112 is to stabilise certain methyltransferases while also enhancing their substrate binding (Bourgeois, L  toquart, *et al.*, 2017). This research as well as previous research has demonstrated that down-regulation of TRMT112 significantly reduced WBSCR22 protein level (  unap *et al.*, 2015; Zorbas *et al.*, 2015). Down-regulation of WBSCR22 itself leads to impaired cell growth, which can be explained with defects in rRNA biogenesis, in particular maturation of small ribosome subunits (  unap *et al.*, 2013). It has previously been shown that WBSCR22 homologue *Bud23* is an essential gene in murine cells (Baxter *et al.*, 2020), and as WBSCR22 is stabilised through TRMT112 interaction it is likely that when TRMT112 is permanently knocked out, so is WBSCR22 and therefore mammalian cells are not able to survive. Nonetheless, WBSCR22-TRMT112 complex has additional role in ribosome biogenesis, as its methyltransferase activity is not necessary for pre-rRNA processing (Zorbas *et al.*, 2015). METLL5 is similarly stabilised by TRMT112 interaction, however METLL5 knockdown does not impair cell growth like WBSCR22 does (van Tran *et al.*, 2019). However, it is suggested that METLL5 has an essential role during brain development as bi-allelic frameshift mutation resulting in change in protein secondary structure causes autosomal-recessive intellectual disability (Richard *et al.*, 2019). In the case of both WBSCR22 and METLL5 it may be that the role of TRMT112 is only to stabilise both proteins, but certainly further research is needed to confirm it.

On the contrary, N6AMT1 is metabolically stable without TRMT112 (Leetsi *et al.*, 2019). In this study N6AMT1 was less affected by TRMT112 mutations than the other methyltransferases, furthermore TRMT112 genomic knockout did not have a noticeable effect on N6AMT1 expression. In previous studies it has been reported that TRMT112-N6AMT1 complex has methyltransferase activity towards N5-glutamine in eRF1 and histone H4 lysine (Figaro *et al.*, 2008; Metzger *et al.*, 2019), however its methylation activity towards DNA is still questionable. N6AMT1 also plays a minor role in arsenic methylation by methylating MMA^{II} into the less toxic DMA acid (Ren *et al.*, 2011) and might have a role in clopidogrel pharmacokinetics (Zhong *et al.*, 2017; Li *et al.*, 2018). N6AMT1 is a rather diverse methyltransferase, and it is quite likely that N6AMT1 alone and in a complex with TRMT112 has activity towards substrates that have not yet been detected.

ALKBH8 is another diverse enzyme, that functions both as a methyltransferase and as an oxygenase, however methyltransferase domain is only active when ALKBH8 is in a complex with TRMT112 (Fu *et al.*, 2010; Songe-Møller *et al.*, 2010; van den Born *et al.*, 2011). ALKBH8-TRMT112 complex is responsible for methylating cm5U and cm5s2U at the wobble position of sever tRNAs including tRNA^{Sec} (Fu *et al.*, 2010; Songe-Møller *et al.*, 2010; van den Born *et al.*, 2011; Endres *et al.*, 2015). ALKBH8 is also involved in DNA damage repair, ROS regulation, selenoprotein level regulation and is upregulated in many cancers, although in this case it is unknown whether TRMT112 plays a part. Nonetheless, it could also be said that it is very likely that ALKBH8-TRMT112 complex has more biological activities that are not know yet.

TRMT112 also forms a complex with TRMT11, which has not yet been characterised in human cells. TRMT11 homologue in yeast Trm11 forms a complex with TRMT112 homologue Trm112 where it is involved in tRNA methylation (Purushothaman *et al.*, 2005). Human TRMT11 appears to be involved in carcinogenesis as it is part of a rather common fusion gene together with GRIK2 that has been found in many cancer types (Yu *et al.*, 2014; Yu, Liu, *et al.*, 2019; Yu, Tsung, *et al.*, 2019). Currently it is predicted that TRMT112 has a tRNA methyltransferase activity in human cells, nonetheless, more studies are needed to confirm this and elucidate on other functions of TRMT11 alone and in complex with TRMT112.

TRMT112 partners THUMP2 and THUMP3 are especially interesting, as TRMT112 mutations affect them the most, suggesting that these proteins might form a complex with TRMT112 in a slightly different way than the other MTases. Both THUMP2 and THUMP3 are still uncharacterised proteins that are specific to human cells and both proteins are predicted to bind RNA and have tRNA methyltransferase activity (Uniprot database).

According to Human protein atlas database, THUMPD2 is a nuclear protein whereas THUMPD3 is a cytoplasmic protein, which is also consistent with the EGPF-fused methyltransferase localisation pattern. Data from previous research from Reet Kurg lab in Institute of Technology shows that THUMPD2 is the weakest interactor with TRMT112, but nonetheless TRMT112 interaction stabilises it. However, TRMT112 is not stabilised by THUMPD2, therefore this is the only TRMT112 partner MTase with which TRMT112 does not have a positive feedback loop. THUMPD3 shows a very similar cytoplasmic location to TRMT11 and ALKBH8 who both have tRNA methyltransferase activity, while THUMPD2 displays a similar localisation pattern with WBSCR22, although THUMPD2 does not accumulate in the nucleoli. The enzymatic activity and functions of both proteins remains to be elucidated in further research.

To conclude, human TRMT112 forms complexes with at least seven different methyltransferases and can be considered as an important “hub” protein that regulates the activity of several of the MTases it interacts with. TRMT112-Mtase complexes are involved in rRNA, tRNA and protein methylation and therefore affect various biological processes such as protein translation, cell cycle regulation, ribosome biogenesis and others. There are still many open questions as to how TRMT112 activity is regulated in the cells and whether it has any additional functions apart from being a methyltransferase co-factor. Moreover, further research is needed on TRMT112 partners to elucidate their functions in human cells. There is a lot of evidence that TRMT112 partners are involved in various human pathologies, therefore further understanding of TRMT112-Mtase heterodimer functions in normal as well as pathological processes could lead them being used as drug targets in future.

Summary

In summary, during this research project TRMT112-methyltransferase network in human cells was studied. TRMT112 is expressed in both the nucleus and cytoplasm where it interacts and form complexes with seven different methyltransferases - N6AMT1, WBSCR22, METTL5, THUMPD2, THUMPD3, ALKBH8 and TRMT11. When MTases are co-transfected into the cells together with TRMT112 a strong positive feedback loop can be observed where TRMT112 stabilises MTases while in return MTases stabilise the TRMT112 protein itself, furthermore, immunofluorescence results indicate that TRMT112 might be able to change its cellular localisation to follow the localisation of certain partner methyltransferases.

Recent studies have revealed that TRMT112 interacts with N6AMT1 and METTL5 in a similar way by covering a large hydrophobic area of the partner protein and partially protecting it from external environment, thereby increasing its solubility and stability. Single amino acid mutations on the TRMT112-MTase interaction surface confirm that there are many similarities in the way TRMT112 binds to all its partners, however several differences can be observed as well. Particularly partners THUMPD2 and THUMPD3 appear to be more impacted by TRMT112 mutations than the other MTases, suggesting that these proteins bind TRMT112 slightly differently than the others. Furthermore, certain mutations in TRMT112 cause the protein to be highly unstable while some enhance its stability and expression level.

CRISPR/Cas9 system was successfully adapted for knocking out TRMT112 gene from human cells, however only a partial TRMT112 knockout clonal cell line was isolated raising the question whether TRMT112 is an essential gene in human cells. Partial TRMT112 knockout significantly reduces WBSCR22 protein level as well as lowers METTL5 protein level but has no impact on N6AMT1 level.

There are still many open questions about the precise mechanism of TRMT112 activity regulation in cells and whether it has any additional functions independently of its role as a co-activator of MTases, not to mention unknown functions of TRMT112 partners. Many members of TRMT112-MTases network are involved in various pathologies, therefore new discoveries will provide a better understanding of the role of methyltransferases in development of diseases and might lead to TRMT112-MTase network members being utilised as drug targets in the future.

TRMT112 poolt aktiveeritavate metüültransferaaside võrgustiku iseloomustus imetajarakkudes

Baiba Brūmele

Resümee

Mitmed metüültransferaasid vajavad metaboolse stabiilsuse ja ensümaatilise aktiivsuse tõttu kofaktorit. Üks sellistest on väike evolutsiooniliselt konserveerunud valk TRMT112, mis interakteerub ja moodustab kompleksi vähemalt seitsme erineva rRNA, tRNA ja valgu metüültransferaasiga. Paljud TRMT112-metüültransferaasi võrgustiku liikmed on seotud erinevate patoloogiatega, seetõttu võimaldavad uued avastused paremini mõista metüültransferaaside rolli haiguste arengus. Hiljutised uuringud on näidanud, et TRMT112 suhtleb oma partneritega sarnasel viisil, kattes partnervalgu suure hüdrofoobse alaga, suurendades seeläbi selle lahustuvust ja stabiilsust.

Antud projekti käigus uuriti TRMT112-metüültransferaasivõrgustikku imetajarakkudes. TRMT112 ekspresseeritakse nii tuumas kui ka tsütoplasmas, kus see interakteerub ja moodustab komplekse seitsme erineva metüültransferaasiga: N6AMT1, WBSCR22, METTL5, THUMP2, THUMP3, ALKBH8 ja TRMT11. Esiteks, kui metüültransferaasid transfekteeritakse koos TRMT112 rakkudesse, moodustub tugev positiivne tagasiside, kus TRMT112 stabiliseerib metüültransferaasi ja metüültransferaasid stabiliseerivad TRMT112 valku, lisaks saab TRMT112 muuta oma rakulist lokaliseerimist, et järgida teatud partneri metüültransferaaside lokaliseerimist. Teiseks, ühe aminohappe mutatsioonid TRMT112-metüültransferaasi interaktsiooni pinnal näitavad, et TRMT112 seondub kõigi oma partneritega sarnasel viisil, kuid on ka mitmeid erinevusi. TRMT112 mutatsioonid mõjutavad kõige rohkem THUMP2 ja THUMP3, mis viitab sellele, et need valgud seovad TRMT112-e veidi teistmoodi kui teised. Lisaks sellele põhjustavad TRMT112 teatud mutatsioonid selle ebastabiilsust, samas kui mõned suurendavad selle stabiilsust ja ekspressioonitaset. Lõpuks, CRISPR / Cas9 süsteemi kasutati TRMT112 geeni väljutamiseks imetajarakkudest, kuid isoleeriti ainult osaline TRMT112 *knockout* rakuliin, mis tekitas küsimuse, kas TRMT112 on imetajarakkudes hädavajalik geen.

TRMT112-metüültransferaase võrgustikku tuleb edasi uurida, sest TRMT112 aktiivsuse reguleerimise täpse mehhanismi kohta on veel palju vastamata küsimusi, samuti on arvatavasti palju tundmatuid TRMT112 partnerite funktsioone nii üksi kui ka kompleksis koos TRMT112-ga.

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