Biochemical characterisation of tRNA-Asp methyltransferase Dnmt2 and its physiological significance

Von der Fakultät für Energie-, Verfahrens- und Biotechnik der Universität Stuttgart zur Erlangung der Würde eines Doktors der Naturwissenschaften (Dr. rer. nat.) genehmigte Abhandlung

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Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die Dissertation, abgesehen von den ausdrücklich bezeichneten Hilfsmitteln, selbständig verfasst haben.

Stuttgart, den 20.01.14

Raghuvaran Shanmugam

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LIST OF MANUSCRIPTS

1. Jurkowski TP[#], Shanmugam R[#], Helm M, Jeltsch A (2012). Mapping the tRNA binding site on the surface of human DNMT2 methyltransferase. Biochemistry. Jun 5;51(22):4438-44.

[#] Equal contribution by these authors

2. Raghuvaran Shanmugam, Muktak Aklujkar, Matthias Schäfer, Richard Reinhardt, Olaf Nickel, Gunter Reuter, Derek R. Lovley, Ann Ehrenhofer-Murray, Wolfgang Nellen, Serge Ankri, Mark Helm, Tomasz P. Jurkowski, & Albert Jeltsch (2014). The Dnmt2 RNA methyltransferase homolog in *Geobacter sulfurreducens* specifically methylates tRNA-Glu "Nucl. Acids Res., in press"

3. Raghuvaran Shanmugam, Jacob Fierer, Steffan Kaiser, Francesca Tuorto, Frank Lyko, Mark Helm, Tomasz P. Jurkowski, & Albert Jeltsch (2014). Cytosine methylation of $tRNA^{Asp}$ by DNMT2 has a role in translation of Asp-tagged proteins. Manuscript submitted for publication.

LIST OF ABBREVIATIONS

aaRS - Aminoacyl tRNA synthetase

AMP - Adenosine mono phosphate

ATP - Adenosine tri phosphate

AspRS - Aspartyl tRNA synthetase

CFP - Cyan fluorescent proteins

CpT/A - Cytosine-phosphate-thymidine/adenine rich DNA sequence

Dnmt1 - DNA methyltransferase 1

Dnmt2 - DNA methyltransferase 2

Dnmt3 - DNA methyltransferase 3

eIF - Eukaryotic translation initiation factor

FTO - Fat Mass and Obesity-Associated Protein

FAD - Flavin adenine dinucleotide

lncRNA - Long non-coding RNA

NAD - Nicotinamide adenine dinucleotide

Nsun2 - NOP2/Sun RNA Methyltransferase

MEF cells - Mouse embryonic fibroblast cells

M.HhaI - Methyltransferase from Haemophilus haemolyticus

mRNA - Messenger RNA

m^6A - 6-methyl adenosine

m^5C - 5-methyl cytosine

miRNA	-	Micro RNA
MTase	-	Methyltransferase
Poly-Asp	-	Poly Asparate rich region
rRNA	-	Ribosomal RNA
SAH	-	S-adenosyl homocysteine
SAM	-	S-adenosyl methionine
siRNA	-	Small interfering RNA
tRNA	-	Transfer RNA
TRD	-	Target recognition domain
Trm	-	tRNA methyltransferase
YFP	-	Yellow fluorescent proteins

LIST OF FIGURES

ZUSAMMENFASSUNG

Die Methylierung von tRNA spielt eine wichtige Rolle in deren Stabilisierung und ist ebenfalls bedeutend für die fehlerfreie Proteinbiosynthese in den Zellen. In Eukaryoten existieren diverse tRNA Methyltransferasen, darunter auch die tRNA Methyltransferase DNMT2. DNMT2 methyliert $tRNA^{Asp}$ an der Stelle C38 in der Anticodonschleife. Diese Methyltransferase wird auch als „tRNA-Aspartat Methyltransferase 1“ (Trdm1) bezeichnet und ist in Eukaryoten hoch konserviert. In der vorliegenden Arbeit wurde der Mechanismus der Interaktion von DNMT2 mit $tRNA^{Asp}$ erforscht. Weiterhin wurde die Funktion des einzigen prokaryotischen Dnmt2 Homologs, welches in *G. sulfurreducens* gefunden wurde, charakterisiert. Außerdem wurde die physiologische Funktion der C38 Methylierung von $tRNA^{Asp}$ in humanen Zellen untersucht.

Aufgrund fehlender Struktur der Dnmt2 im Komplex mit $tRNA^{Asp}$ sind die molekularen Details der Interaktion zwischen DNMT2 und ihrem Substrat bisher unbekannt. Hierfür sollten in der vorliegenden Arbeit die wichtigen Reste von DNMT2 charakterisiert werden, welche für die tRNA Bindung und Katalyse benötigt werden. Durch gerichtete Mutagenese von 20 konservierten Lysin- und Argininresten von DNMT2, konnte gezeigt werden, dass acht von ihnen die katalytische Aktivität des Enzyms stark beeinflussen. Diese Reste befinden sich auf einer Seite der katalytischen Tasche des Enzyms. Die Bindung der meisten mutierten Enzyme an die tRNA wurde nicht beeinflusst. Daraus lässt sich schließen, dass diese Reste eine wichtige Rolle in der Stabilisierung des Übergangszustands haben. Ein simuliertes Binden, der $tRNA^{Asp}$ an die Tasche des Enzyms, deutet darauf hin, dass DNMT2 hauptsächlich mit dem Anticodon Stamm der tRNA-Asp interagiert.

Das zweite Projekt sollte die Funktion des Dnmt2 Homologs untersuchen. Das Dnmt2 Homolog stammt aus *G. sulfurreducens* (GsDnmt2). Es konnte gezeigt werden, dass GsDnmt2 $tRNA^{Glu}$ mit höherer Effizienz methyliert wurde als $tRNA^{Asp}$. Die molekularen Grundlagen für diese Änderung der Substratspezifität von GsDnmt2 wurde untersucht, und es konnte gezeigt werden, dass die variable Schleife von *G. sulfurreducens* $tRNA^{Asp}$ und $tRNA^{Glu}$ der Eukaryoten eine GG Dinukleotidsequenz

enthalten. Das Vorhandensein dieser GG Dinukleotide hat einen negativen Einfluss auf die Dnmt2 Aktivität. Der Austausch dieser variablen Schleife in Maus $tRNA^{Asp}$ führte zu einer dramatischen Abnahme der Aktivität von humanem DNMT2. Dies zeigt, dass die variable Schleife der tRNA als eine Spezifitätsdeterminante für die Erkennung durch Dnmt2 dient.

Das letzte Projekt befasst sich mit der physiologischen Bedeutung der $tRNA^{Asp}$ C38 Methylierung im Bezug auf die Aminoacylierung und zelluläre Proteinsynthese. Es konnte gezeigt werden, dass die C38 Methylierung die Acylierungsrate von $tRNA^{Asp}$ um das 4-5 fache verstärkt. Gleichzeitig konnte in Dnmt2 knockout MEF Zellen eine Abnahme der Aminoacylierung der $tRNA^{Asp}$ beobachtet werden, was zu einer reduzierten Effizienz in der Proteinbiosynthese von Proteinen mit einer Poly-Asp Sequenz führt. Eine Gen-Ontologie Analyse von Proteinen mit poly-Asp Sequenzen, zeigte, dass eine signifikante Anzahl dieser Proteine mit transkriptionaler Regulation und Genexpressionfunktionen assoziiert sind. Daraus lässt sich schließen, dass der schwache Phänotyp, der unter Stressbedingungen in Dnmt2 KO Zellen beobachtet wurde, mit einer Fehlregulation der Proteinsynthese korreliert sein könnte.

ABSTRACT

Methylation of tRNA plays important roles in the stabilisation of tRNAs and accurate protein synthesis in cells. In eukaryotes various tRNA methyltransferases exist, among them DNMT2 which methylates $tRNA^{Asp}$ at position C38 in the anticodon loop. It is also called tRNA-aspartate methyltransferase 1 (Trdmt1) and the enzyme is highly conserved among eukaryotes. In this work, I investigated the mechanism of DNMT2 interaction with $tRNA^{Asp}$, characterised the function of the only prokaryotic Dnmt2 homolog found in *G. sulfurreducens* and studied the physiological importance of the C38 methylation of $tRNA^{Asp}$ in mammalian cells.

The molecular details of the interaction of DNMT2 and $tRNA^{Asp}$ are unknown due to lack of the co-crystal structure. Here, I characterised the important residues in DNMT2 required for the tRNA binding and catalysis. By site-directed mutagenesis of 20 conserved lysine and arginine residues in DNMT2, I show that 8 of them have a strong effect on the catalytic activity of the enzyme. They map to one side of the enzyme where the catalytic pocket of DNMT2 is located. The binding of most of the mutant enzymes to tRNA was unaffected suggesting a role of these residues in transition state stabilisation. Manual docking of $tRNA^{Asp}$ into the surface cleft decorated by the 8 residues suggested that DNMT2 interacts mainly with the anticodon stem/loop of $tRNA^{Asp}$.

In my second project, I characterised the function of Dnmt2 homolog found in *G. sulfurreducens* (GsDnmt2). Here, I show that GsDnmt2 methylates $tRNA^{Glu}$ more efficiently than $tRNA^{Asp}$. I also report the molecular basis for the swapped substrate specificity of GsDnmt2 and show that the variable loops of *G.sulfurreducens* $tRNA^{Asp}$ and $tRNA^{Glu}$ of eukaryotes contain a -GG- dinucleotide which is not preferred by Dnmt2. Exchange of the variable loop of mouse $tRNA^{Asp}$ to that $tRNA^{Glu}$ led to dramatic decrease in the activity of human DNMT2. This identifies the variable loop of tRNA as a specificity determinant in the recognition by Dnmt2.

In my final project, I investigated the physiological importance of the $tRNA^{Asp}$ C38 methylation in aminoacylation and cellular protein synthesis. Here, I report that C38 methylation enhances the rate of aspartylation on $tRNA^{Asp}$ by 4-5 folds. Concomitant with this, a decrease in the charging levels of $tRNA^{Asp}$ was observed in Dnmt2 knockout MEF cells, which also showed a reduced efficiency in the synthesis of proteins containing poly-Asp sequences. A gene ontology searches for proteins with poly-Asp sequences showed that a significant number of these proteins are associated with transcriptional regulation and gene expression functions. With this I propose that the mild phenotype observed with the Dnmt2 KO cells under stress condition could be correlated to a disregulation of protein synthesis.

Contents

1. INTRODUCTION

Ribonucleic acid shortly called RNA constitutes one fundamental molecule of life in all organisms. These ubiquitous biomolecules play important roles in the process of storing and carrying genetic information, expression of genes and decoding the genetic information (Berg JM, 2002). In addition, RNA constitutes the genome of many viruses, for example influenza, hepatitis C, polio and measles all are RNA viruses (Drake and Holland, 1999; Lohmann et al., 1999; Webster et al., 1992). Unlike double stranded DNA, many RNA molecules are single stranded and they are short lived molecules compared to DNA. RNA molecules are composed of four bases namely, adenine (A), guanine (G), cytosine (C) and uracil (U). They occur in three major forms in all living organisms - mRNA, tRNA and rRNA. In the cellular environment each RNA molecule performs a specialised function. mRNAs are responsible for carrying the genetic information that directs protein synthesis, tRNAs are the carriers of activated amino acids in the protein synthesis and finally the rRNAs (ribosomes) are the protein synthesising factories in a cell (Cooper GC, 2004). In addition to this there are also other regulatory RNA molecules like miRNA, siRNA, piRNA, lncRNA, sncRNA and snoRNAs (Brameier et al., 2011; Griffiths-Jones et al., 2005; Sleutels et al., 2002).

1.1 RNA modifications

The four bases of RNA undergo a variety of post-transcriptional modifications including the addition of various chemical groups like methyl, thiol, isopentenyl group or editing of Adenine to Inosine, Uracil to pseudouridine, etc, (Paris et al., 2012) (Figure 1). RNA modification was discovered 5 decades ago and 160 modifications have been reported to date (Cantara et al., 2011; Machnicka et al., 2013; Yi and Pan, 2011) and in most organisms about 1-2% of the genetic information is dedicated to encode the enzymes needed for RNA modification (Motorin and Helm, 2011). Modified RNA bases are present in all organisms, however their distribution varies among archaea, eukaryotes and prokaryotes (Motorin and Helm, 2011). RNA modifications are found on different cellular RNAs including rRNA, tRNA, mRNA and snRNA and are dynamically regulated. Among the many modifications seen in RNA, the most widely occurring one is the methylation of RNA bases which will be discussed in detail in the next chapter.

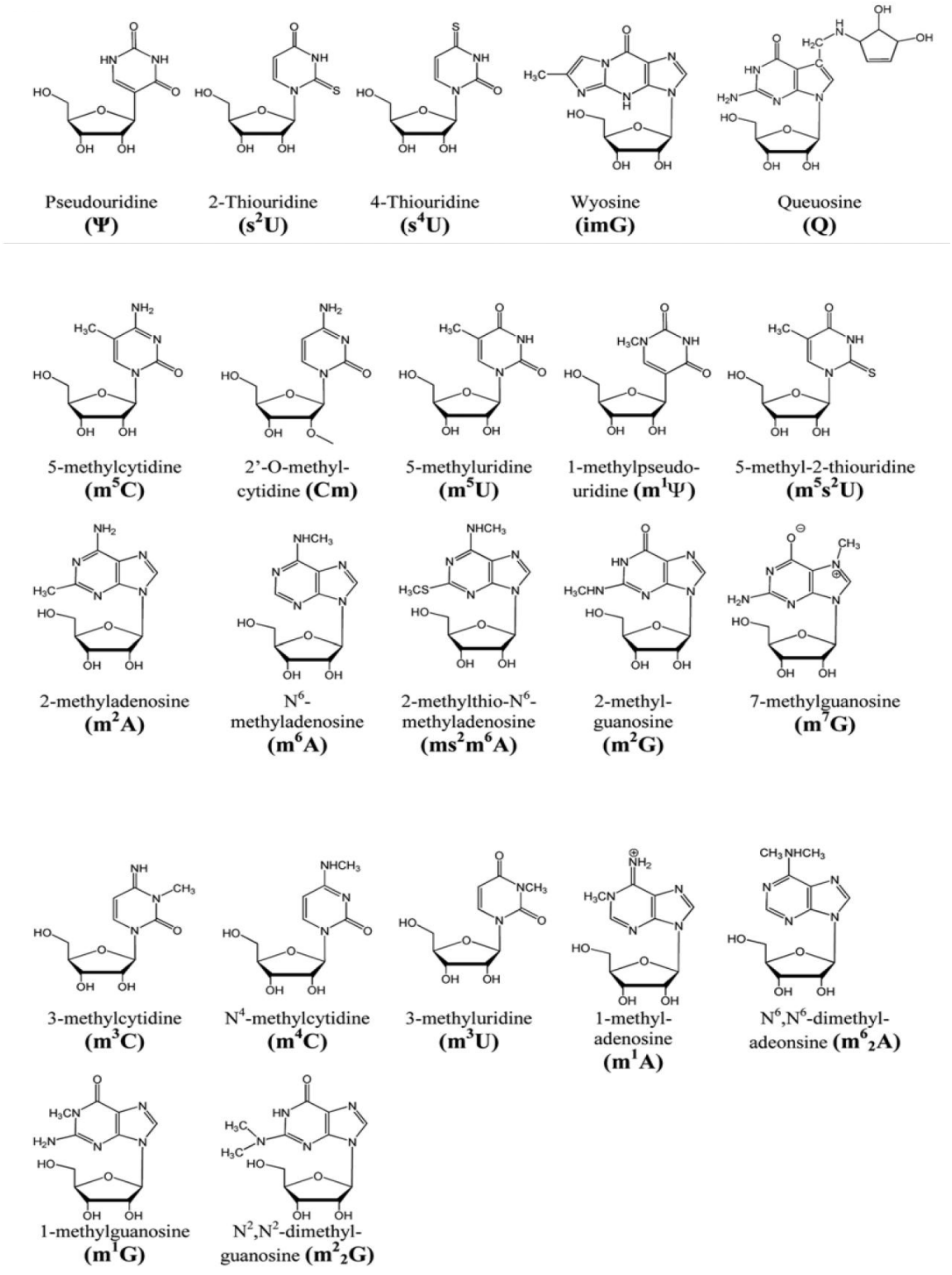

Figure 1. Examples of modified bases found in RNA. Figure Taken from Kellner (2010) and adopted to show only the modified bases.

1.1.2 RNA Methylation

RNA methylation is found in both prokaryotes and eukaryotes (Klagsbrun, 1973). The two most common RNA methylation marks are N6-methyladenosine (m^6A) and 5-

methylcytidine (m^5C) (Niu et al., 2013). These modifications have been studied for decades in mRNAs, tRNAs and in rRNAs. In prokaryotes, RNA methylation serves many functions including stabilisation of RNA structure, specificity determination of protein-RNA interactions, protein translation and antibiotic resistance and also plays a role in cellular stress signalling (Kramer et al., 1988). Development of high throughput RNA-sequencing methods broadened our understanding of how methylation marks are functionally relevant for the embryonic development and influence biological process in eukaryotes, which will be discussed in chapter 1.2 (Sibbritt et al., 2013). RNA methylation was found to be a highly dynamic process that occurs in response to extracellular and environmental cues (Fustin et al., 2013). Very recently, Mettl3 catalysed N6-methyladenosine on mRNA was shown to be demethylated by FTO enzyme (Fu et al., 2013). A variant of this FTO gene has also been discovered in many patients with increased weight or obesity (Gerken et al., 2007). Finally, disfunctioning of RNA methylation is implicated in tumour development like in the case of upregulated Nsun2 protein in mammals (Frye and Watt, 2006). Thus RNA methylation is involved in many important biological functions required for a living organism.

1.1.3 RNA methyltransferases

RNA methyltransferases catalyse the methylation of RNA bases and most of them use S-adenosyl methionine (SAM) as a donor of the methyl group. They are classified into four superfamilies (Motorin and Helm, 2011). First, the Rossmann-fold MTase (RFM) family encompasses most of the known RNA MTases which use SAM as a donor of methyl group (Delk and Rabinowitz, 1975). The second family is the SPOUT MTase family, which catalyses the addition of methyl group to ribosyl moiety in RNA (Anantharaman et al., 2002). The third family of MTases include Radical SAM enzymes, which generate a free radical as a result of the methyltransfer reaction and finally the FAD/NAD-binding family of RNA MTase, which has so far only one identified member (Urbonavicius et al., 2005). Hundreds of homologes of RNA MTase have been identified in different organisms (Motorin and Helm, 2011). However, the focus of this thesis is mainly on the RNA methyltransferase that catalyses the C5-modification in cytosine.

1.1.4 C5-Methyl cytosine, the fifth base

5-methyl cytosine (m^5C) in RNA has a long standing cellular role in eukaryotes. 5-methyl cytosine residues are found both in DNA and RNA. However, they were discovered in the mid of 20th century in DNA (Wyatt, 1950). Later, they were found to have a wide occurrence and 5-methyl cytosines are termed the 'fifth base' in DNA (Hermann et al., 2004). The presence of m^5C modification has been extensively studied in DNA and in eukaryotes it serves a role as an epigenetic modification (Hotchkiss, 1948). m^5C modification is catalysed by SAM dependent DNA/RNA MTases (Figure 2). The m^5C modification exists in several cellular RNAs and controls their functions (Squires et al., 2012), which will be discussed in the subsequent chapter. In recent times novel methods have been developed to map these residues with single base resolution including RNA bisulfite sequencing (Schaefer et al., 2009). Compared to cytosine residues the 5-methyl cytosines are more protected to spontaneous deamination at the N4 residue that subsequently leads to formation of Uracil (Motorin et al., 2010) and this principle is employed in the bisulfite RNA sequencing.

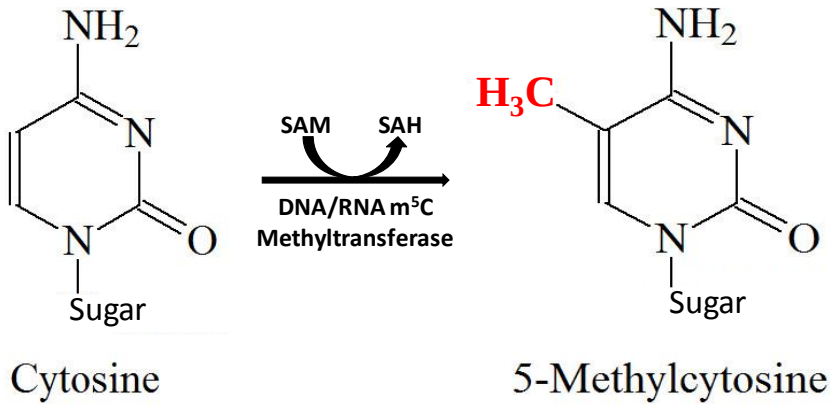

Figure 2. Formation of 5-methyl cytosine from unmodified cytosine in DNA/RNA using co-factor substrate SAM. The methyl group is coloured in red.

1.2 General functions of RNA methylation

1.2.1 Role of rRNA methylation

Ribosomal RNAs (rRNA) are the principle components of both small and large subunits of ribosome and constitute about 60% of ribosomal content. Methylation in rRNA can occur either on the ribosyl moiety or on the bases. There are 22 different methylated nucleosides present in prokaryotic rRNA while it is 10 in eukaryotes (Cavaille and Bachellerie, 1998; Motorin and Helm, 2011). There are two classes of rRNA methyltransferase. The first type of methyltransferase is called stand alone/solitary rRNA MTases. Here, the enzyme itself possesses both target specificity and required catalytic activity. This type of methyltransferase is most commonly found in bacteria for 2'-O-methylation of ribose in rRNAs as well as for the nucleosides methylation. The second type is the guide RNA dependent MTases that contains a protein part and an integral RNA part like in the C/D box snoRNP (Cavaille and Bachellerie, 1998). The enzyme specificity is mediated by the RNA part of the complex and the protein has the enzymatic activity associated with it. These types of MTases are mostly used in archaeal and eukaryotic systems to methylate the ribose at 2'-O position. Methylation of rRNA plays many important roles, for example, m^2G and m^6A in the small rRNA have been found to influence conformational changes in the Watson-Crick base pairing (Kellner et al., 2010; Punekar et al., 2013). Similarly, presence of a methyl-uridine in the A loops of 23S rRNA was reported to stabilize the rRNA folding (Hansen et al., 2002). rRNA methylation is also very important for the translation of mRNA (Basu et al., 2011). For this reason, the sites of methylation in rRNA are highly conserved around the peptidyl transferase center in the large subunit associated rRNA or around the decoding center in the small subunit rRNA (Decatur and Fournier, 2003; Liang et al., 2009; Sirum-Connolly and Mason, 1993). In a report it was shown that deleting the guide snoRNA that target the 2'-O-methylation of 16S rRNA in the decoding center in yeast leads to translational infidelity and reduced translation rate compared to wildtype cells (Baudin-Baillieu et al., 2009). Also, uridine methylation in 23S rRNA is implicated in the stabilisation of codon-anticodon interaction (Baudin-

Baillieu et al., 2009). Another important role of the rRNA methylation is in the generation of bacterial resistance against antibiotics and here methylation occurs mostly in either 16S or 23S rRNA (Long et al., 2006; Macmaster et al., 2010). For example, Cfr MTase mediates methylation of adenine-2503 in the 23S rRNA in *Staphylococcus* spp to make them resistant for chloramphenicol, florfenicol, clindamycin and Phenicols (Kehrenberg et al., 2005; Long et al., 2006).

1.2.2 Role of mRNA methylation

Eukaryotic mRNAs undergo extensive modification at their 5' ends. One of the best studied modifications is the methylation of the terminal guanine residue at the 5' end (m^7G) leading to the formation of a structure called 5'cap (Figure 3). The presence of 5' cap structure is very important for most eukaryotic mRNAs, because this modification assists in the ribosomal recognition of mRNAs. The series of events happening at 5' cap region includes transfer of guanine at the terminal position of mRNA, methylation of guanine at N7 position and the ribose at position 1 and 2 of mRNA and methylation of adenine at position 1 of the mRNA (Cowling, 2010). This complex modification is introduced by a machinery containing triphosphatase, guanylyltransferase and MTases activities along with RNA polymerase II. All the activities can be present in a single complex or as multiple subunit in a complex (Pillutla et al., 1998).

The universally conserved role of mRNA cap methylation is in translation initiation. During mRNA translation, the eIF4E binds with high affinity to the 5'cap (m^7G) that leads to the recruitment of eIF4F which facilitates the 43S initiation complex to start translating the mRNA (Paterson and Rosenberg, 1979; von der Haar et al., 2004). Another important function of the 5'cap is to support transcriptional elongation of mRNA by recruitment of other elongation factors (Sims et al., 2004). 5' cap structure also helps in the splicing of introns from the mRNA, in a report it was shown that removing the 5'cap decreases the efficiency of removal of 5'proximal intron from the mRNA (Ohno et al., 1987). Other less understood roles of the 5' cap methylation include, roles in the nuclear export of mRNAs in higher organism like *Xenopus* (Jarmolowski et al., 1994), in the structural stabilisation of the mRNA against exonuclease activities present in cells (Murthy et al., 1991) and in the polyadenylation

of mRNA at the 3' end (Camper et al., 1984; Flaherty et al., 1997). Apart from the cap methylation, another common methylation mark is the m^6A, mostly seen in the 3'UTR (Meyer et al., 2012). This modification has been implicated in the miRNA binding.

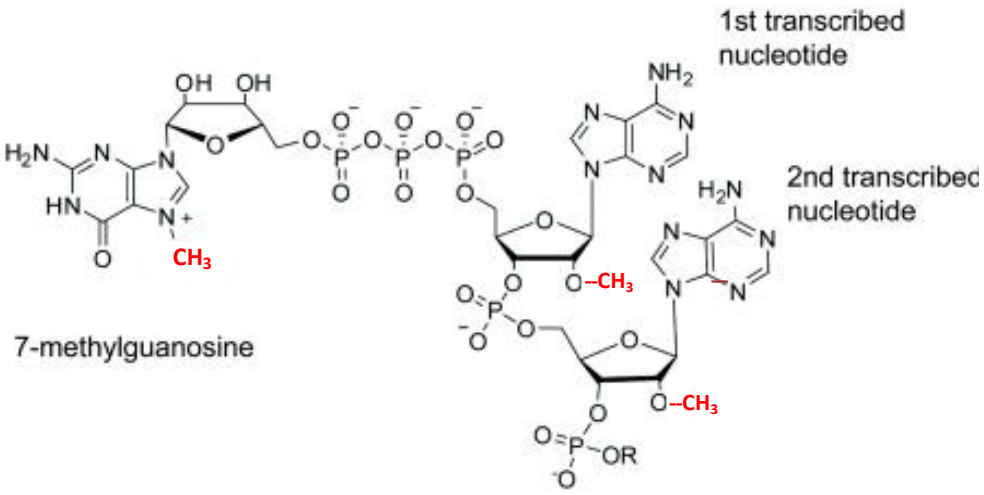

Figure 3. The structure of 5' cap consisting of 7-methyl guanine present in all eukaryotic mRNAs. The methyl groups are coloured in red. Figure taken from Cowling (2010).

1.2.3 Role of methylation of regulatory RNAs

Methylation of regulatory, non-coding RNAs have been described in recent times (Squires et al., 2012). This includes miRNA and siRNA in plants, lncRNA and piwi RNA in *Drosophila* and animals. The miRNAs and siRNAs are frequent targets of 3' uridylation that acts as a signal for degradation of these RNAs (Heo et al., 2008). However, methylation of these RNAs at the 2'-O ribose in the 3' terminal by HEN1 was shown to stabilise them against degradation in plants (Li et al., 2005; Yang et al., 2006; Yu et al., 2005). The methylation acts by preventing 3'-5' exonuclease activity on these RNAs. In Arabidopsis, HEN1 deletion leads to miRNAs that have 3' end uridylation and are found to be varied in size rather than 21-24 nucleotides (Li et al., 2005). A similar role was also discovered for the *Drosophila* ortholog DmHen1, however this protein methylates piRNA which are involved in transposon silencing in the germ line cells (Horwich et al., 2007). Lack of 2'-O methylation at the 3' end of piRNAs led to functional perturbation in transposon silencing. Examples for methylation of long non coding RNA (lncRNA) include C5-cytosine methylation in the H1 region (C1683) of the HOTAIR lncRNA

(Amort et al., 2013). This region is located proximal to the histone demethylase LSD-1 binding site and proposed to be involved in LSD-1 binding. Likewise, XIST which is required for the X-chromosome inactivation in female mammals also undergoes multiple C5-cytosine methylation at repeat region 8 (R8) that modulates its interaction with the PRC2 complex (Amort et al., 2013).

1.2.4 Transfer RNA (tRNA) and tRNA modification

Transfer RNAs (tRNA) are typically 72 nucleotide long RNA molecules synthesised by RNA polymerase III. tRNAs carry activated amino acids and incorporate them into the growing poly peptide chain during protein translation. They fold into a specialised structure consisting of four different stem/loops namely, acceptor stem, D-loop, anticodon loop and T-loop (Willis, 1993). Unlike other RNAs, tRNAs have a specialised L-shaped structure (Figure 4), which is accommodated in the P-site and A-site of the ribosomes. tRNAs act as an adapter between the mRNA and ribosomes. All tRNAs across all species are pos-transcriptionally modified, which includes addition of the –CCA nucleotides to the 3' end of the tRNA (Aebi et al., 1990; Xiong and Steitz, 2004), dihydroxyuridine formation in the D-loop, conversion of uridine to pseudouridine, presence of ribothymidine (T) in the T-loop and methylation of many nucleosides at various positions (Trifunac and Krasna, 1974). One of the very important structural features of tRNA is the presence of the anticodon loop, which determines the specificity of the tRNA towards the cognate aminoacyl-tRNA synthetases. The anticodon in the tRNA base pairs with the triplet codon present in the mRNA and delivers the amino acid into A-site of the ribosome. Although, tRNAs are highly specific for each amino acid there is more than one tRNA for a single amino acid in most higher organism, which bind to alternate codons in mRNA, but incorporate same amino acid. These tRNAs are called 'isoacceptors'. Similarly, tRNAs from different genes can differ in their sequence, but specify the same anticodon sequence and these molecules are called 'isodecoders' (Goodenbour and Pan, 2006). In most organisms, there are several tRNA genes (http://gtrnadb.ucsc.edu), for example, in *Saccharomyces cerevisiae* there are 288 genes reported to code for different tRNAs and in humans the number crosses 500 genes. tRNAs are essential for error-free protein biosynthesis, for this reason they

need to undergo quality control steps after their biogenesis in the nucleus and transport to the cytoplasm. There are two quality control processes that act on pre-tRNAs that are aberrantly processed. The nuclear surveillance pathway acts on pre-tRNAs that are devoid of the 1-methyladenosine modification in the T-loop and degrade them by 3'oligoadenylation and 3' exonuclease activity (LaCava et al., 2005). For example, lack of such modification in the initiator-tRNA^{Met} has been reported in rapid degradation of this tRNA (Kadaba et al., 2006). After the transport into cytoplasm, tRNA lacking specific modifications are recognised by the rapid tRNA decay pathway and degraded by the 5'-3' exonuclease activity of Rat1 and Xrn1 (Chernyakov et al., 2008). A single tRNA is capable of decoding more than one codon in an mRNA due to presence of a wobble base in the anticodon (Aldinger et al., 2012). This wobble base undergoes modification, for example, in few tRNAs it is changed to Inosine (I) or Queuosine (Q), which are capable of non Watson-Crick base paring with different nucleotides (Meier et al., 1985; Murphy and Ramakrishnan, 2004; Senger et al., 1997).

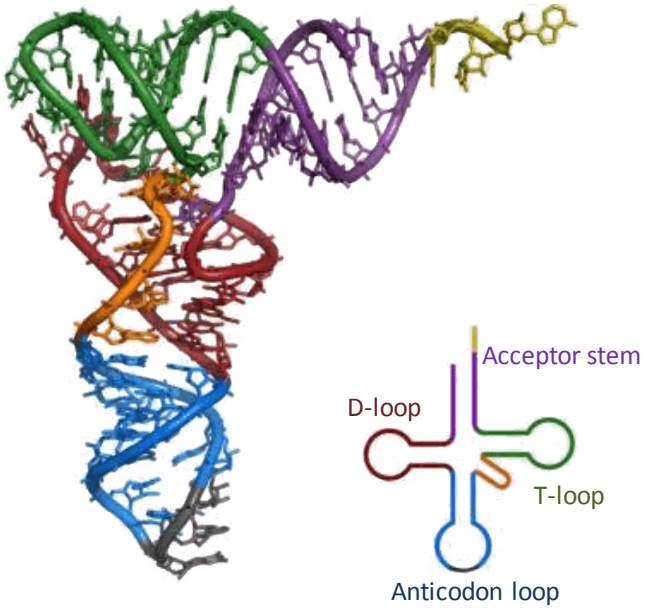

Figure 4. The tertiary structure of tRNAs. The different loops are coloured as indicated. Figure taken from PBD entry 1ehz deposited for X-ray structure of yeast tRNA^{Phe} by Shi (2000).

1.2.5 Methylation of tRNAs and its role

Methylation of tRNA is a well studied modification present in most organisms. Most of these methylations support the function of tRNAs in either of the two ways,

structural stabilisation or by increasing the translational accuracy during the mRNA decoding process (Motorin and Helm, 2010; Nau, 1976). Methylation of bases outside of the anticodon loop and methylation of ribose are mostly associated with a structural stabilisation of tRNA (El Yacoubi et al., 2012). For example, methylation of G18 at the D-loop was shown to stabilise the $tRNA^{Phe}$ and $tRNA^{Met}$ against RNase digestion both in prokaryotes and in eukaryotes (Kumagai et al., 1982). Another classical example for methylation dependent stabilisation of tRNA is the 1-methyladenosine at position 58 in the $tRNA^{iMet}$ which was described previously (Kadaba et al., 2006). It has been reported very recently that in yeast deletion of the *Trm4* and *Trm8* methyltransferases leads to a rapid degradation of $tRNA^{Val(AAC)}$. The increased rate of degradation is due to lack of the m^7G46 and m^5C modifications (Alexandrov et al., 2006). Similarly, temperature sensitivity of tRNA was attributed to the lack of methylation at specific sites, including Um44, $m^{2,2}G26$ (Kotelawala et al., 2008). tRNA $m^1A57/58$ methyltransferase was also shown to be essential for the cell growth and survival under high temperature (Guelorget et al., 2010). In addition, *Trm2* in yeast has been reported to take part in the tRNA maturation, although the modification catalyzed by this protein is non-essential (Johansson and Bystrom, 2002). Another universally conserved modification in eukaryotes is the N(2),N(2)-dimethylguanosine-26 introduced by *Trm1P* enzyme (Purushothaman et al., 2005). It has been experimentally shown, that deletion of *Trm1p* leads to severe growth defect by affecting the metabolism and functionality of the tRNAs.

Methylation in the anticodon loop of tRNAs plays many important roles. Most of the modifications happen at position 34 or position 37 in the anticodon loop. By ribosomal foot printing, 2'O-methylation of guanosine at the wobble position 34 (Gm34) in $tRNA^{Phe}$ was shown to be critical for the ribosomal P-site binding (Ashraf et al., 2000). In a recent report, yeast *Trm*7 was shown to methylate C32 and N34 of $tRNA^{Phe}$, $tRNA^{Trp}$, and $tRNA^{Leu(UAA)}$ (Guy et al., 2012). The authors were able to show that 2'-O-methylation of N(34) of the substrate tRNAs directs the modification of m^1G37 (1-methylguanosine) to yW (wyebutosine) and that *Trm*7 deletion leads to severe reduced growth phenotype in cells. The 1-methyl guanine modification at position 37 is highly conserved in eukaryotic tRNAs with anticodons that start with a ‘C’

and this modification has been linked to prevention of translational frameshifts (Bjork et al., 1989). Anticodon modification has also been reported to influence stress response and DNA damage response pathways (Begley et al., 2007). For example, *Trm*9 in yeast was shown to catalyse methyltransfer to the uridine wobble base of $tRNA^{Arg(UCU)}$ and $tRNA^{Glu(UUC)}$. This modification helps in efficient readout of wobble codons for arginine and glutamate amino acids. When cells lacking wobble base methylation were treated with methyl methanesulfonate (MMS), a DNA damaging agent, translational arrest of proteins involved in DNA damage response occurs due to inefficient readout of arginine (AGA) and glutamate (GAA) amino acids that constitute a large proportion of these proteins. While in wildtype cells, translational enrichment of mRNA from genes coding for translation elongation factor 3 (YEF3) and the ribonucleotide reductase (RNR1 and RNR3) large subunits occurs due to efficient readout of AGA or GAA codons following gene specific codon usage pattern. A similar phenomenon was also reported for the human ABH8 gene (Fu et al., 2010).

1.3 Mammalian C5-cytosine DNA methyltransferases

Eukaryotic DNA methylation is a very important epigenetic modification and has a long standing history. In mammals, the promoters of many genes are occupied by 5-methylcytosine that constitutes one epigenetic modification (Jeltsch, 2002). Methylation of DNA is catalysed by DNA methyltransferase using SAM as a methyl group donor (Cheng, 1995; Cheng and Roberts, 2001). Figure 5 represents the classification of mammalian DNA methyltransferases (Figure 5).

DNMT1 also known as maintenance methyltransferase was the first biochemically characterized DNA methyltransferase in mammals (Yoder et al., 1997). As the enzyme shows preference for hemi methylated DNA it has been implicated in the maintenance of DNA methylation pattern after each successive round of DNA replication (Gowher et al., 2005a; Goyal et al., 2006; Hermann et al., 2004). It has a structurally distinguishable C-terminal and N-terminal domain. The C-terminal domain contains all the characteristic motifs required for an active methyltransferase while the N-terminal part contains domains required for its localization, specificity and

suppression of de novo methylation activity. In mice deletion of DNMT1 gene was shown to be lethal as it is required for proper development (Li et al., 1992).

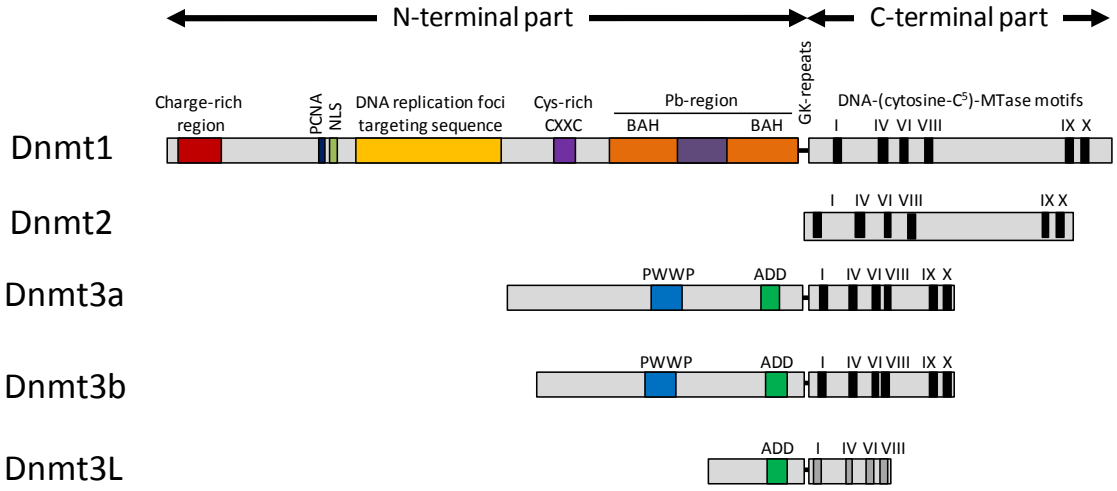

Figure 5. Structure and domain arrangement of mammalian DNA methyltransferase. The DNA methyltransferase catalytic motifs are coloured in black. {Figure Courtesy: Tomasz Jurkowski}.

The DNMT3 family of DNA methyltransferases consist of DNMT3a, DNMT3b and DNMT3L. Among these, DNMT3a and 3b are catalytically active and DNMT3L has no catalytic activity, however it regulates the activity of Dnmt3a and Dnmt3b (Cheng and Blumenthal, 2008; Gowher et al., 2005b). The members of this class participate in establishing the de novo DNA methylation pattern during embryonic development that is maintained throughout the life of an animal (Bird, 2002; Okano et al., 1998). The catalytically active members of this family contain a C-terminal domain having all the motifs typical for an active methyltransferase and an N-terminal domain that specifies the localization of these enzymes (Bestor, 2000).

In mammals, these two classes of DNMTs are responsible for the DNA methylation pattern that varies in a spatio-temporal pattern during different developmental stages (Chow and Brown, 2003; Reik et al., 2001). In a multi-cellular organism, like human, DNA methylation patterns determine the gene expression and identity of cells that constitute an organ system (Jaenisch and Bird, 2003; Levenson and Sweatt, 2005; Reik, 2007). Abnormalities in DNA methylation can lead to disorders like Rett syndrome, ICF syndrome and also cause cancer (Kriaucionis and Bird, 2003; Xu et al., 1999).

DNMT2 was initially assigned as a member of the mammalian DNA methyltransferase family because of their amino acid sequence similarities to other active DNA methyltransferases. However, DNA methylation activity was reportedly weak or absent for this enzyme (Hermann et al., 2003; Okano et al., 1998; Yoder and Bestor, 1998), which remains to be a controversial issue, described in more detail in chapter 1.4.5 of this thesis. In the year 2006, Dnmt2 was shown to catalyse the methylation of $tRNA^{Asp}$ at C38 position (Goll et al., 2006) which was unexpected at that time. The members of the Dnmt2 family are widely conserved among eukaryotes suggesting an important role in cellular processes. The main focus of this thesis falls on characterising this protein.

1.4 Mammalian C5-cytosine tRNA methyltransferase

There are two C5-cytosine tRNA methyltransferases that have been discovered in mammals, which are Dnmt2 and Nsun2. The latter enzyme has been biochemically shown to methylate the C34 position of $tRNA^{Leu}$ in higher eukaryotes and represent the mammalian homolog of *Trm*4 gene from yeast (Brzezicha et al., 2006). This modification has been linked to germ cell differentiation in mouse testis. Nsun2 knockout mice are viable, however, male mice are sterile (Hussain et al., 2013b). Apart from tRNA methylation, NSUN2 has been reported to methylate other RNAs like p16 mRNA and vault non-coding RNA (Hussain et al., 2013a; Zhang et al., 2012). The other methyltransferase DNMT2, which is recently renamed as tRNA-aspartic acid methyltransferase (TRDMT1) will constitute further part of this thesis.

1.4.1 Discovery and structure of Dnmt2 proteins

Dnmt2 was first discovered in 1995 in fission yeast, where the homolog was named Pmt1. At this time the only other known and biochemically characterised eukaryotic DNA methyltransferase was Dnmt1. Because Pmt1 is very similar to other prokaryotic and eukaryotic m^5C-methyltransferases the authors investigated its activity and found that Pmt1 homolog is not capable of methyltransferase activity in vitro and that *Schizosaccharomyces pombe* lacks any detectable methylation in its genome. This led

the authors to a speculation that the enzyme may act as a RNA methyltransferase (Wilkinson et al., 1995). Similarly, mouse Dnmt2 was also shown to be deficient for DNA methylation activity (Okano et al., 1998). The human DNMT2 enzyme was later discovered and was found to be a small globular protein and contain all the characteristic amino acid motifs found in other active DNA MTases but it lacks the N-terminal regulatory domain which is present in other eukaryotic DNA MTase (Bestor, 2000; Van den Wyngaert et al., 1998). In 2001, the structure of DNMT2 with bound AdoHcy (SAH) was solved at 1.8 Å resolution (Dong et al., 2001). The structural determination was obtained for recombinant DNMT2 protein, which lacked a poorly conserved region of 47 amino acids from residue 190 to 238. The crystal structure of DNMT2 (Figure 6) shows that it has one large domain and one small domain consisting of Rossmann fold motifs. The large domain contains the catalytic motifs and the co-factor binding site in an open conformation. This domain is made of eight-β stranded sheets surrounded by three α-helices on one side and 4 α-helices on the other side. The small domain in the crystal structure consists of four antiparallel β sheets surrounded by five α-helices. The overall structure and size of DNMT2 resemble that of the bacterial methyltransferase M.HhaI (Dong et al., 2001). The authors were also able to show that DNMT2 binds to the DNA in a denaturant resistant nature and its binding to the SAH was comparable to M.HhaI. In spite of all these binding properties, authors were unable to show DNA methyltransferase activity in vitro or in vivo for DNMT2.

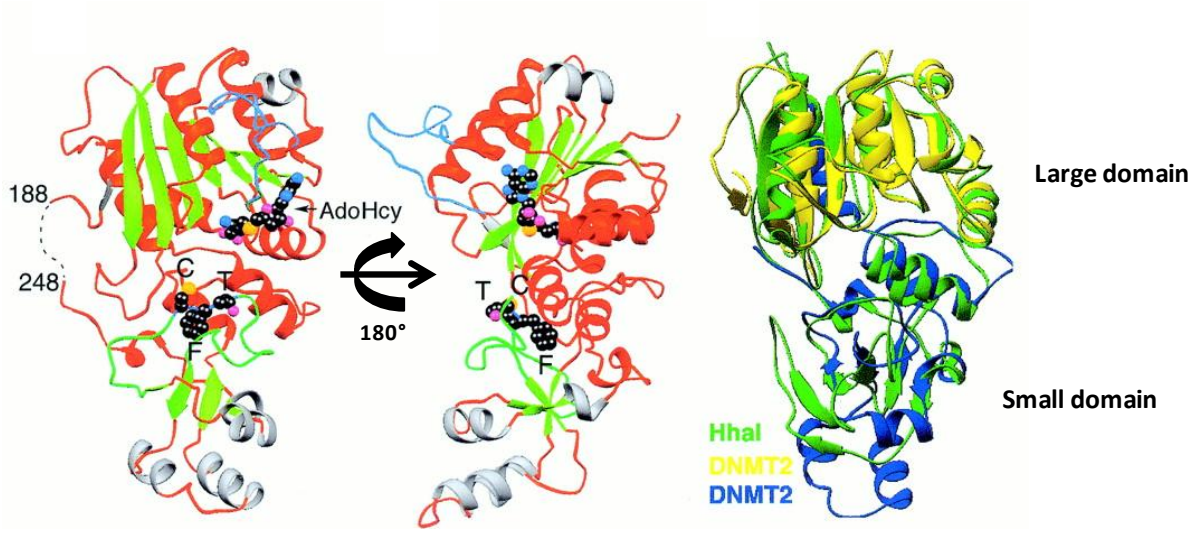

Figure 6. A ribbon model of the DNMT2Δ47–AdoHcy complex. The left panel represents the front view of the complex and the center panel indicates the backview of the complex. α Helices and loops are colored in red (unique helices of DNMT2 in gray), strands in green, the catalytic loop in light blue and the target recognition domain (TRD) loop in green. The bound AdoHcy and CFT motif of the TRD are shown as balls, with carbon atoms in black, nitrogen atoms in light blue, oxygen atoms in magenta and sulfur atoms in orange. B) Superimposition of DNMT2Δ47, colored yellow (residues 1–188) and blue (residues 248–391), and M.*Hha*I (pdb 1HMY), colored green. Figure taken from Dong (2001).

1.4.2 Dnmt2 homologs in different species

Members of Dnmt2 family were found in diverse organisms ranging from yeast to humans. Sequence comparison of all the Dnmt2 homologs showed the presence of the characteristic DNA methyltransferase amino acid sequence motifs (motif I-X) (Jeltsch et al., 2006). In addition to this, members of Dnmt2 family in all species contain a unique Cys-Phe-Thr (CFT) motif in the target recognition domain (TRD). Although Dnmt2 is widely conserved, in some of the organisms like *Drosophila* and *Dictyostelium*, Dnmt2 represents the only known DNA MTase homolog (Hung et al., 1999; Katoh et al., 2006). For this reason, these species are considered as the best model to study the potential DNA methylation function of Dnmt2. Dnmt2 was shown to be differentially expressed during development in *Drosophila* and *Dictyostelium*. Northern blot analysis demonstrated that Dnmt2 expression is very high in *Drosophila* larval stage and tends to get weaker during the embryogenesis and eventually ceases out in the adult stage of this organism (Hung et al., 1999). Similarly, a developmentally restricted expression of DnmA was also reported in *Dictyostelium* (Kuhlmann et al., 2005). Later, *EhMeth* the Dnmt2 homolog in *Entamoeda histolytica* which also does not contain additional Dnmt-related proteins was shown to be a nuclear matrix associated protein and its interaction with EhMRS2 was confirmed by yeast one hybrid system (Banerjee et al., 2005). EhMRS2 is a nuclear-scaffold attachment region that participates in genome organisation and it was shown to be methylated at the 3' end by *Ehmeth*.

1.4.3 Methyltransferase activity of Dnmt2

After the initial discovery of Dnmt2, much work was done to detect a DNA methyltransferase activity of this enzyme. A weak in vitro methylation of DNA by DNMT2 was reported in the year 2003, where the methylation was found to be approximately 0.7% of the total cytosines on a PCR fragment containing 34 CG sites (Hermann et al., 2003). In the same report, the consensus sequence for Dnmt2 target site was suggested to be -ttnCGga(g/a)-. In *Dictyostelium*, the Dnmt2 homolog DnmA was shown to methylate some of the retrotransposons like DIRS-1 and Skipper (Kuhlmann et al., 2005). By knocking out DnmA gene the authors reported increased expression and mobilisation of retro transposable elements. The authors also found that 0.2% of the total cytosine in *Dictyostelium* is methylated by DnmA, which is comparatively less than the methylation level observed with other DNA MTases. The work with *Ehmeth* enzyme also led to the discovery of a weak DNA methylation by this enzyme (Banerjee et al., 2005; Fisher et al., 2004; Harony et al., 2006). However, few other studies reported absence of any detectable DNA methylation by Dnmt2 under both in vitro and in vivo conditions (Okano et al., 1998; Raddatz et al., 2013; Yoder and Bestor, 1998). This DNA methylation activity of Dnmt2 enzyme still remains to be controversial due to recent reports in *Drosophila* showing DNA methylation by Dnmt2, which will be discussed in chapter 1.4.5 of this thesis. Thus more studies are needed to validate DNA methylation by Dnmt2 before any conclusions are drawn.

Finally, in the year 2006, in a seminal paper Goll and her coworkers (2006), showed Dnmt2 protein to methylate $tRNA^{Asp}$ from different species including mouse, *Drosophila* and *Arabidopsis*. Using tandem mass spectrometry the authors confirmed the site of methylation in $tRNA^{Asp}$ to be C38 in the anticodon loop (Figure 7). The $tRNA^{Asp}$ methylation by Dnmt2 was later confirmed in many organisms, including *Dictyostelium, Entamoeba histolytica, Artemia franciscana* and *zebrafish* (Becker et al., 2012; Feng et al., 2007; Muller et al., 2013; Rai et al., 2007; Tovy et al., 2010). Following the discovery of Dnmt2 catalytic activity, motif IV and motif VI were found to be catalytically very important as they are involved in the active site formation and stabilisation of enzyme-substrate complex, described in the subsequent chapter.

Mutation of the cysteine residue in this motif led to a strong reduction in the catalytic activity of the enzyme and binding to the substrate (Jurkowski et al., 2008). Recently, the substrate specificity of Dnmt2 has been extended to other tRNAs in few organisms. For example, bisulfite analysis of total RNA from *Drosophila* showed methylation of $tRNA^{Gly}$ and $tRNA^{Val}$ at C38 position in the anticodon loop by Dnmt2 (Schaefer et al., 2010). Likewise, the pmt1 protein in *S.pombe* was shown to methylate endogenous $tRNA^{Glu}$ though comparatively weaker than $tRNA^{Asp}$. DnmA of *Dictyostelium* was also found to methylate its $tRNA^{Glu}$ in vitro but not in the cell (Becker et al., 2012; Muller et al., 2013). Nonetheless, the physiological relevance of the C38 methylation of tRNAs has not been deciphered in any organism.

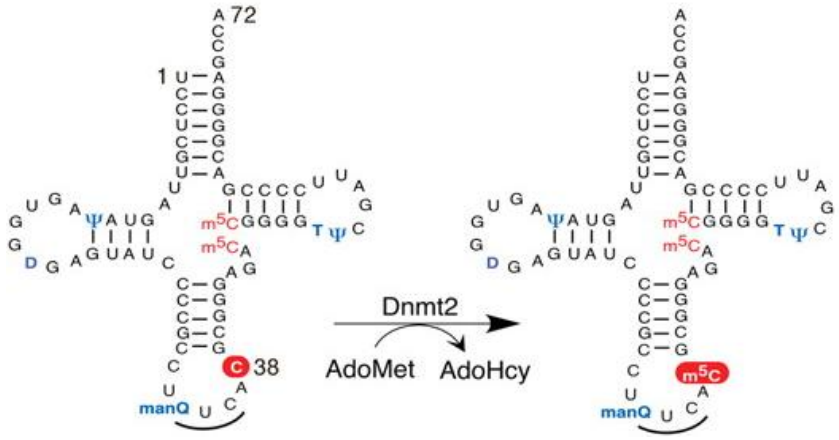

Figure 7. Schematic representation of DNMT2 mediated methylation of $tRNA^{Asp}$. Dnmt2 uses Adomet (SAM) as a co-factor in the methyltransfer reaction to cytosine 38 of $tRNA^{Asp}$. The cofactor product is released in the form of S-adenosyl homocysteine (SAH). Figure taken from Goll (2006).

1.4.4 Catalytic mechanism and evolution of Dnmt2

RNA and DNA C5-cytosine methyltransferase catalysis methylation at 5^{th} carbon in the aromatic ring of cytosine bases. The catalytic mechanism of DNA MTases was discovered first and thought to be universal for all m^5C MTases (Wu and Santi, 1987). Generally, in m^5C-DNA methyltransferases a covalent enzyme-substrate intermediate is formed by an initial nucleophilic attack of the SH group from the cysteine residue located in the motif IV (GPPC) on the C6 carbon atom of the target cytosine, subsequently a nucleophilic attack on the methyl group of the cofactor SAM occurs. At

this time the protonation of the N3 atom in the cytosine is facilitated by the glutamate residue located in the motif VI (ENV) of the enzyme. This is followed by deprotonation of the C5 carbon in the cytosine leading to the beta elimination of the cysteinyl group at position C6. This produces the free enzyme along with S-adenosyl homocysteine and the C5 methylated cytosine (Chen et al., 1991; Jeltsch, 2002; Kumar et al., 1994). The discovery of a different catalytic mechanism for RNA MTases came as a surprise, when it was shown in Fmu, a 16S rRNA m^5C-RNA methyltransferase, the sulfur atom (SH) for the initial nucleophilic attack comes from a cysteine residue in the motif VI (TCS) leading to formation of a covalent bond at C6 position of the base in the target RNA (Liu and Santi, 2000). This is followed by methylation of the C5 position using SAM and the enzyme is regenerated by subsequent beta elimination. In this mechanism, an aspartate residue from the motif IV (DAPC) provides stability for the complex during transition state (Bujnicki et al., 2004). Later, the cysteine residue from motif IV was also shown to be involved in the catalysis (King and Redman, 2002). Thus the catalytic mechanism of DNA and RNA MTases differs mainly in the use of active site cysteine residue for the methyltransfer reaction. Interestingly, although Dnmt2 has been shown to be a tRNA methyltransferase it was also found to have motif IV and motif VI of a typical DNA MTase type with -PCQ- and -ENV- respectively. In the year 2008, it was demonstrated that DNMT2 in fact follows a DNA MTase catalytic mechanism (Jurkowski et al., 2008). The experimental proof came from mutagenesis of the conserved cysteine residue (C79) in the PCQ motif of DNMT2, that led to a complete loss of methylation in $tRNA^{Asp}$. Similarly, exchange of the Glutamate (E119) residue in motif VI rendered the enzyme inactive. Another cysteine (C292) which is conserved in the CFT motif of Dnmt2 family had only a moderate effect on the catalysis. This observation clearly demonstrated that Dnmt2 methylates tRNA using a DNA MTase like catalytic mechanism (Figure 8).

The Dnmt2 enzyme is present in most eukaryotes but its homologs are present in only few bacterial species, like *Geobacter sulferruducens* and *Geobacter metallireducens* (Goll et al., 2006; Jurkowski and Jeltsch, 2011; Ponger and Li, 2005). This could suggest a horizontal gene transfer of Dnmt2 between eukaryotes and the prokaryote (Ponger and Li, 2005). However, Goll and her coworkers suggested a

different concept proposing that all eukaryotic DNA MTases could have evolved from a common Dnmt2-like ancestral gene (Goll et al., 2006). In order to investigate this proposal, a phylogenetic analysis including 2300 prokaryotic and eukaryotic MTases was made (Jurkowski and Jeltsch, 2011). Based on the phylogenetic tree constructed from bootstrap values of various methyltransferases, Dnmt2 was found not to be derived from any RNA methyltransferase found in prokaryotes or eukaryotes. In the 3D clustering of MTases based on the observed Blast similarity scores, Dnmt2 showed a clear separation from other eukaryotic DNA MTases indicative of its independent origin and not being an ancestral gene for the eukartoyic DNA MTases.

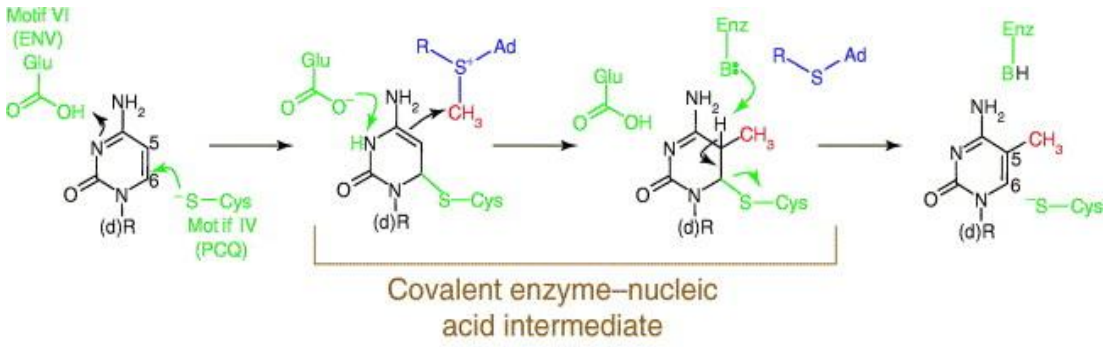

Figure 8. General catalytic mechanism of the Dnmt2 enzyme. The motifs involved are colored green, the co-factor SAM is colored in purple and the methyl group in red. Figure taken from Jeltsch (2006).

1.4.5 Dnmt2 in fly- A solitary methyltransferase

Drosophila melanogaster contains only a single copy of DNA methyltrasferase which is homologous to Dnmt2 (Hung et al., 1999). Due to this Dnmt2 function can be well assessed in this organism. A number of earlier reports showed presence of DNA methylation in *D. melanogaster* (Field et al., 2004; Gowher et al., 2000; Lyko, 2001; Lyko et al., 2000; Marhold et al., 2004a; Marhold et al., 2004b; Phalke et al., 2009; Weissmann et al., 2003). The first evidence of DNA methylation came from the work of Lyko et al. (2000), when they showed existence of 5-methyl cytosine in the genomic DNA extracts of *Drosophila* embryos (0-4 hours). The authors also reported that most of the methylation occurs in CpT/A regions and found that proportion of 5-methyl cytosine decreases in the adult stage of the flies. Immediate appearance of another report from our lab also suggested existence of DNA methylation in this organism

(Gowher et al., 2000) and a follow up work re-confirmed the site specificity of DNA methylation at CpT/A regions (Kunert et al., 2003). Following this, methylation of DNA in *Drosophila* had been implicated in the maintenance of chromatin condensation and histone modifications (Weissmann et al., 2003). In search of the candidate methyltransferase responsible for the DNA methylation in *Drosophila*, Lyko et al. (2000b) identified a putative Dnmt2 homolog in this organism and suggested a role in DNA methylation. However, the biochemical evidence for the Dnmt2 mediated methylation of DNA in *Drosophila* came from the work of Kunert (2003). By combined immunofluorescence and genomic bisulfite sequencing, the authors showed that overexpression of Dnmt2 leads to hypermethylation of genomic DNA at CpT/A sites. Later in 2009, came direct evidence that showed *Drosophila* Dnmt2 methylates genomic DNA at retrotransposons during early embryogenesis (Phalke et al., 2009). By knocking out Dnmt2 in *Drosophila,* the authors found aberrant overexpression of Invader4 retrotransposons that mimics a Suv4-20 null phenotype. They found methylated cytosines in the 5' LTR of the Invader4 elements in wildtype stains, which was absent in the knockout stains. However, the DNA methylation activity of dDnmt2 remained controversial, because a comment to this work showed absence of any DNA methylation at the retrotransposons by dDnmt2 (Schaefer et al., 2010). Later, dDnmt2 was also shown to mediate silencing of Rt1b retrotransposon through recruitment by SETDB1 protein to the heterochromatic regions in the genome with CpA sequence (Gou et al., 2010). Dnmt2 was also shown to silence tumour suppressor gene retinoblastoma family protein 1 (Rb) in imaginal discs. One the other hand, a recent study tested the function of Dnmt2 mediated DNA methylation and showed absence of DNA methylation by Dnmt2 (Raddatz et al., 2013). In this work, the model organism *Drosophila* and a triple knockout (Dnmt1, 3a and 3b) mouse both of which have only Dnmt2 homolog as a representative DNA MTase were used to comprehensively analyse the methylation content of the whole genome by bisulfite sequencing. Through this scrutinising analysis the authors could not detect any DNA methylation activity associated with Dnmt2. Based on these contradicting findings, additional work will be needed to clarify the role of Dnmt2 in the DNA methylation of *Drosophila* genome and in DNA methylation in general.

However, report from Goll (2006) showing $tRNA^{Asp}$ specific methylation by *Drosophila* Dnmt2 has changed the focus of Dnmt2 function in *Drosophila*, when $tRNA^{Asp}$ lacking the C38 modifications were found to be more accessible to Ribo-endonucleases, like Angiogenin (Schaefer et al., 2010). The authors showed that incubation of tRNA isolated from wildtype and dDnmt2 knockout strains with angiogenin, led to increased fragmentation of tRNA isolated from knockout strains than the wildtype strains due to lack of dDnmt2 catalysed modification. In the same work, the authors found $tRNA^{Gly}$ and $tRNA^{Val}$ to be additional substrates for dDnmt2 and a similar fragmentation of these substrates could be shown in the absence of C38 methylation. This suggests that Dnmt2 helps to maintain the stability and the steady state levels of the tRNAs and helps to minimise tRNA degradation by endonucleases.

Very recently it came to light that fragmentation of tRNA leads to change in the expression of few genes (Durdevic et al., 2013b). It was experimentally shown that the tRNA-derived fragments from Dnmt2 KO cells are preferentially processed by Dicer-2 and loaded into the RISC complex. These fragments are then capable of inhibiting the Dicer-2 activity on its natural substrates leading to misregulation of siRNA pathway dependent genes. The authors showed that the mus308 transcript was upregulated in Dnmt2 knockout flies after heat shock due to reduction in the eis2.1 expression, which regulates the Mus308. As tRNA fragmentation is more pronounced under heat shock condition in *Drosophila*, this phenomenon was shown to have a consequential effect on the expression of stress response genes.

Another unexpected role of Dnmt2 in the non-random sister chromatid exchange of X and Y chromosomes was also discovered in *Drosophila* strains in recent times (Yadlapalli and Yamashita, 2013). The authors clearly showed by CO-FISH analysis that in asymmetric cell division of *Drosophila* male germline stem cells (GSCs), the daughter GSCs inherited the sister chromatid of the Y chromosome, whose template strand contained the (GTATT)6 satellite, while the gonialblasts inherited the sister chromatid whose template contained the complementary (AATAC)6 sequence. A similar bias was also observed in the X chromosome segregation. In mutant Dnmt2 flies this bias or nonrandomised sister chromatid segregation was lost and further it

was shown that presence of Dnmt2 in the parents primed the segregation pattern, which is maintained through embryogenesis and in adult tissues.

Recently, dDnmt2 was also reported to be essential for the antiviral defence of RNA virus and innate immune response in heat shocked strains (Durdevic et al., 2013a). This mechanism was shown to be related to the binding of Dnmt2 to the genome of single strand RNA virus like *Drosophila* C virus. In Dnmt2 mutant flies, the level of viral RNA genomes increased more than 100 folds and the immune response against these viral particles was reported to be slow and inefficient to remove them from the host genome during the acute phase of infection.

A chapter of my thesis will focus on the role of C38 modification in the aminoacylation of $tRNA^{Asp}$ and its consequent effect on the protein synthesis, which will also suggest a role of this modification in increasing the fitness of the organism.

1.4.6 Dnmt2 and RNA epigenetics

In mammals, DNA methylation mediated epigenetic inheritance has been known for many decades and the responsible DNA MTases have been classified into either DNMT1 or DNMT3 family (Goll and Bestor, 2005; Law and Jacobsen, 2010). The Dnmt2 family proteins have escaped the conventional function of DNA methylation and have been shown to be a tRNA methyltransferase (Goll et al., 2006). Thus Dnmt2 function remained unconventional compared to the conventional functions of other DNMTs in maintaining the epigenetic inheritance. However, recently it was shown from the group of Lyko that Dnmt2 play an essential role in the non Mendelian inheritance of certain traits for example fur coloration in the tail and feet of mice (Kiani et al., 2013). In this analysis, Kit mRNA (Kit2123–2150) was micro injected into the fertilised eggs of two $Dnmt2^{-/-}$ or two wildtype parents and subsequently analysed by bisulfite sequencing for any detectable methylation marks. The results revealed two novel sites of methylation in the RNA isolated from wildtype embryos that is absent in the $Dnmt2^{-/-}$ embryos. Hence, DNMT2 mediated methylation of Kit mRNA was reported to enable the fur coloration in the offsprings. Likewise, DNMT2 was also shown to be required for the para mutations of Sox9/miR-124 which results in an overweight phenotype of

offsprings in mice. However, these argumentations need further validation, in particular the ability of DNMT2 to methylate the described targets needs to be shown.

1.4.7 Bacterial homolog of Dnmt2

In my thesis, I undertook efforts to characterise the function of a Dnmt2 homolog present in the bacterial species called *Geobacter sulfurreducens*. This organism is a member of *Geobacter* and a δ-Proteobacterium. *G.sulfurreducens* lives in the subsurface environment under anaerobic conditions and it uses acetate and metal ions to produce energy. The speciality of this organism is the ability to use Fe(III) ions as the terminal electron acceptor in the energy metabolism. Similarly, it can also utilise other metal ions like vanadium and radioactive uranium to produce insoluble metal ions and this property makes it very suitable for the bioremediation of uranium in contaminated water bodies (Esteve-Nunez et al., 2005). Another potential role of this *Geobacter* spp is their ability to generate electricity by oxidizing organic substrates, which can be harvested and deployed in electrical instrumentation (Bond et al., 2002). The complete genome of the *Geobacter* species has been sequenced and reported to contain a single chromosome of 3,814,139 base pairs (bp) (Methe et al., 2003). There are 3466 predicted protein-encoding ORFs. Interestingly, the genome of this organism is different from other metal reducing anaerobes. For instance, *G. sulfurreducens* shares only two genes with another metal ion–reducing γ-Proteobacterium *Shewanella oneidensis* and 50% of the electron transport proteins in *G. sulfurreducens* have no homolog in other species. *Geobacter* spp also contains cytochrome proteins, catalase, superoxide dismutase, ruberythrin, and protoporphyrinogen oxidase that participate in the oxygen radical scavenging and this organism uses aerobic metabolism to remove complex carbon compounds (Cypionka, 2000; Lin et al., 2004). *G. sulfurreducens* contains a full set of genes encoding proteins for Krebs cycle wherein the citrate synthetase has a eukaryotic origin (Methe et al., 2003). In addition, there is also a Dnmt2 homolog present in this species. In the genome of this organism, a total of 48 tRNA genes are encoded, of which there are 2 identical gene copies for $tRNA^{Asp}$ (Chan and Lowe, 2009).

1.5 Aminoacylation and Aminoacyl-tRNA synthetase

Aminoacylation is the first step in protein synthesis. Since one aim of this work was to investigate the influence of Dnmt2 mediated tRNA methylation on aminoacylation and translation, these processes will be introduced here briefly. The aminoacylation process is responsible for the production of acylated (charged) tRNAs that are delivered to the ribosomes during the process of protein synthesis. In the cell, each tRNA is recognized by a specific aminoacyl tRNA synthetase that adds the specific amino acid to the 3' end of tRNA in the acceptor stem. The amino acids carried by the tRNAs are incorporated into the growing polypeptide chain by sequence specific binding of the anti-codon in the tRNA to the triplet codon present in the mRNA (Berg JM, 2002). The aminoacylation reaction is initiated by the activation of the specific amino acid by aminoacyl-tRNA synthatase (aaRS) enzyme, which uses ATP and leads to the formation of an aminoacyl-adenylate. The aminoacyl moiety is subsequently transferred to the specific tRNA to produce the aminoacyl-tRNA and AMP is released (Schimmel and Soll, 1979). The aminoacyl tRNA synthetases are highly specific towards their substrate tRNAs and amino acids. For each of the 20 amino acids present in any living cells, there is a specific aminoacyl tRNA synthetase (Giege, 2006). These synthetases are classified into two types based on their mechanism and complexity. Class I enzymes are either monomeric or dimeric and aminoacylate the 2'OH of adenine of the CCA end of the tRNA. This class includes the enzymes specific for arginine, cysteine, glutamic acid, glutamine, isoleucine, leucine, methionine, tyrosine, tryptophan and valine. The class II enzymes are either di or tetrameric complexes and aminoacylate the 3' OH of the adenosine nucleotide of the CCA end. This class includes synthetases specific for alanine, asparagine, aspartic acid, glycine, histidine, lysine, phenylalanine, proline, serine, and threonine. All these 20 aminoacyl tRNA synthetases possess specific regions that interact with the identity determinants present on their specific tRNAs (Berg JM, 2002). Often these determinants include bases present in the anti-codon loop that facilitates recognition and efficiency of aminoacylation. For example, 2-thiouridine modification of $tRNA^{Glu}$ on the first base of the anti-codon was reported to be a positive determinant for the glutamylation by GluRS in E.Coli. The

unmodified $tRNA^{Glu}$ was found to be 100 fold less efficient than the modified $tRNA^{Glu}$ (Sylvers et al., 1993).

2. AIMS

2.1 Mapping of the DNMT2-tRNA^{Asp} interaction points

One important aim of this thesis was to characterize the amino acids involved in the interaction of DNMT2 enzyme to its substrate tRNA^{Asp}. Lack of a co-crystal structure of DNMT2-tRNA complex impedes our understanding of the substrate recognition mechanism and enzyme-substrate complex formation. So, this project was designed to characterise the effect of an alanine exchange of selected, conserved, basic residues on the activity and binding of DNMT2 to its substrate tRNA^{Asp}. In total 20 amino acids would be mutated to alanine. All the mutant enzymes would be analysed for the proper folding as wildtype DNMT2 enzyme. Further the mutant enzymes would be studied for their tRNA^{Asp} binding ability to see the effect of introduced mutation. Following this, the tRNA methylation activity of all these mutant enzymes would be investigated and also the binding of the mutants to SAM will be analysed. With the experimental results, the study would provide insight into the molecular interactions between DNMT2 and tRNA^{Asp} by proposing a model of tRNA^{Asp} docked on the 3D crystal structure of DNMT2 enzyme.

2.2 Elucidating the role of the bacterial Dnmt2 homolog

The second objective of this project was to characterise the substrate specificity and biological role of a Dnmt2 homolog present in the bacterial species called *Geobacter sulfurreducens*. The project also add value to our Dnmt2 consortium studying the function of Dnmt2 protein in different species which already includes *D. melanogaster, S. pombe and D. discoideum, E. histolytica* and *M. musculus*. In this project, the *Geobacter* Dnmt2 gene was cloned to express and purify the protein for checking its methylation activity on tRNA^{Asp}. The methylation activity of the recombinant GsDnmt2 protein would be studied on all tRNA substrates from *G. sulfurreducens* with cytosine at 38th position. In this project, the methylation of endogenous tRNA^{Asp} and tRNA^{Glu} from *G. sulfurreducens* would be analysed to show the substrate specificity of the GsDnmt2 enzyme. Further, the project would aim to connect the outcomes of the

experiments with eukaryotic Dnmt2 (mainly human DNMT2) substrate recognition and support the horizontal gene transfer of GsDnmt2 gene in *G. sulfurreducens*. In addition, this project would uncover a role of $tRNA^{Asp}$ varible loop on the substrate recognition by human DNMT2 enzyme.

2.3 Investigation of the effect of $tRNA^{Asp}$ methylation in protein translation

The third aim of this project was to elucidate the physiological significance of Dnmt2 mediated C38 methylation on $tRNA^{Asp}$. Towards this aim, the project would investigate the influence of the C38 methyl group on aminoacylation of $tRNA^{Asp}$ by aspartyl-tRNA synthetase. Followed by this, the in vivo level of charged $tRNA^{Asp}$ in wildtype and Dnmt2 knockout mouse embryonic fibroblast cells would be examined. To find a physiological connection for any changes in the charging level of $tRNA^{Asp}$, the rate of synthesis of fluorescent reporter constructs with a poly-Asp sequence would be analysed in the cells. Further, it is one main aim of this project to find a direct molecular connection of the C38 methylation on endogenous protein synthesis. For this, selected endogenous proteins containing poly-Asp sequence motifs would be analysed for the steady state level in wildtype and Dnmt2 KO cells. The gene expression and the half-life of the selected proteins would be analysed to support our hypothesis on the role of DNMT2 in synthesis of poly-Asp containing protein. With the experimental results, the study would propose a model to account for the reduced stress response seen with Dnmt2 KO cells (Schaefer et al., 2010) and also to support a molecular connection for reduced synthesis of overall protein level in Dnmt2 and Nsun2 double knockout cells which was recently reported (Tuorto et al., 2012).

3. RESULTS

3.1 Mapping the tRNA Binding Site on the Surface of Human DNMT2 Methyltransferase

(Manuscript 1 in the attachment of this thesis)

Jurkowski TP[#] & Shanmugam R[#] (2012). Biochemistry. ([#] equal contribution).

In order to systematically characterise the residues important for the tRNA binding in DNMT2, 20 conserved basic amino acid residues as shown in figure 1 of manuscript 1 were selected and they were mutated to alanine by site-directed mutagenesis. The mutant proteins were expressed and purified to near homogeneity. In order to check the proper folding of the mutant proteins, circular dichroism analysis was performed for all the mutants and that of wildtype DNMT2. The results as shown in Supplemental figure 1b of manuscript 1 indicated that folding of all the mutant proteins were similar to that of wildtype DNMT2 protein. After confirming the proper folding of all the mutants, tRNA methylation kinetics were carried out for all the mutant proteins and the wildtype. The results as shown in the figure 3 of manuscript 1 indicated that 8 of the alanine exchange mutants had a huge decrease in the catalytic activity which will be elaborated in the discussion chapter 4.1.1 of this thesis. Similarly, four other mutants showed moderate decline (2-4 fold) in the methylation activity. The remaining eight mutant proteins showed better or comparable to wildtype activity in methylation (Figure 3b of manuscript 1). Afterwards, tRNA binding was evaluated for all the mutants. As shown in figure 2a of manuscript 1, the results indicated that binding of most of the mutant proteins to $tRNA^{Asp}$ was comparable or better than the wildtype enzyme. However, few of the mutants showed decreased binding to $tRNA^{Asp}$ which included R84A, R95A and R371A. The effects will be detailed in the discussion part of the thesis 4.1.2. Following this, SAM binding was analysed for the mutants with reduced activity and the results indicated that SAM binding was not altered for any of the mutants (Figure 2b of manuscript 1). Based on the experimental data, modelling of $tRNA^{Asp}$ onto the surface of DNMT2 protein was done manually. The manual modelling gave interesting insights in to the substrate recognition mechanism of DNMT2 protein

which will be described in the discussion section 4.1.3 of the thesis and indicated that DNMT2 undergoes a conformational change during the reaction progress and the side where the catalytic pocket is located plays an important role in the recognition and binding of $tRNA^{Asp}$ during the methylation reaction.

3.2 The Dnmt2 RNA methyltransferase homolog in *Geobacter sulfurreducens* specifically methylates $tRNA^{Glu}$

(Manuscript 2 in the attachment of this thesis)

Raghuvaran Shanmugam et al., "Nucl. Acids Res., in revision"

In order to characterise the function of Dnmt2 in the prokaryote *Geobacter sulfurreducens,* the gene coding for the protein (GsDnmt2) was cloned and the recombinant GsDnmt2 protein was purified to homogeneity on Ni-NTA column. In order to create a catalytic mutant, the enzyme's active site cysteine (C74) was mutated to alanine by site-directed mutagenesis. tRNA methylation kinetics was performed with GsDnmt2 wildtype and active site mutant protein on $tRNA^{Asp}$ from *D. melanogaster* and *M. musculus*. As shown in figure 1c on manuscript 2, the results showed that GsDnmt2 is active on these tRNAs while the active site mutant lost its activity. Following this, $tRNA^{Asp}$ from *Geobacter sulfureducens* was used in methylation and showed only a very weak activity of the enzyme on its cognate substrate. This observation has led to the suggestion that GsDnmt2 methylates other tRNA species in *G. sulfurreducens*. By multiple sequence alignment, five *G. sulfurreducens* tRNAs were identified to contain cytosine at position 38 in their sequence which are $tRNA^{Asp}$, $tRNA^{Glu}$, $tRNA^{His}$, $tRNA^{Val}$ and $tRNA^{Ala}$. All these tRNAs were in vitro transcribed and methylation kinetics were performed with GsDnmt2 wildtype and active site mutant proteins. The result indicated that $tRNA^{Glu}$ is more preferentially methylated compared to $tRNA^{Asp}$ (Figure 2 of manuscript 2). The results finally indicated that GsDnmt2 has altered substrate specificity which will be addressed at molecular context in the discussion section 4.2.2 of the thesis. The observation of changed substrate specificity of GsDnmt2 was followed by the specificity analysis of various Dnmt2 proteins from eukaryotic phyla. For this, Dnmt2 proteins from *D. discoidium, S. pombe, D.*

melanogaster and *H. sapiens* were purified and the methylation on *G. sulfureducens* $tRNA^{Asp}$ and $tRNA^{Glu}$ were determined showing that all the eukaryotic Dnmt2 proteins have a preference for $tRNA^{Asp}$. However, the GsDnmt2 protein methylated $tRNA^{Glu}$ preferentially than $tRNA^{Asp}$ suggesting an evolutionary model for the swapped substrate specificity of GsDnmt2 as described in the discussion section 4.2.2 of the thesis. Further in vivo methylation of GsDnmt2 also showed that it methylated endogenous $tRNA^{Glu}$ (Figure 3 of manuscript 2). After the substrate specificity change was confirmed both at in vitro and in vivo level, the molecular connection behind this remained elusive. To solve this puzzle, sequence dissimilarities between the preferred and non-preferred substrate $tRNA^{Asp}$ and $tRNA^{Glu}$ by eukaryotic Dnmt2 and GsDnmt2 respectively were compared. As shown in figure 6 of manuscript 2, the structural comparison showed a region of dissimilarity in the variable loop of preferred and non-preferred substrates. In all the non-preferred substrates including $tRNA^{Glu}$ of eukaryotes and $tRNA^{Asp}$ of *G. sulfurreducens,* a -GG- dinucleotide is present in the variable loop while it is absent in all the preferred susbtrate tRNAs. Based on this, a mutant murine $tRNA^{Asp}$ with variable loop from a murine $tRNA^{Glu}$ was prepared and methylated in vitro. The results shown in figure 7 in manuscript 2 indicated a dramatic loss of methylation activity by both GsDnmt2 and human DNMT2. These results finally indicate that the variable loop play a role in the recognition of tRNA by Dnmt2 proteins which will be described in more detail in the discussion section 4.2.2 of the thesis.

3.3 Cytosine methylation of $tRNA^{Asp}$ by DNMT2 has a role in translation of Asp-tagged proteins

(Manuscript 3 in the attachment of this thesis)

Raghuvaran Shanmugam et al., Manuscript in preparation

The influence of C38 methylation of $tRNA^{Asp}$ was investigated by studying the in vitro aminoacylation kinetics of methylated and unmethylated $tRNA^{Asp}$ by aspartyl tRNA synthatase. The results from this kinetics showed that C38 methylated $tRNA^{Asp}$ was a better substrate compared to unmethylated $tRNA^{Asp}$ (Figure 2a and 2b of manuscript 3). This suggests a molecular interaction of the C38 methyl group on the

amino acid residues of AspRS proteins which is described in the discussion section 4.3.1 of the thesis. A follow up to this differential aminoacylation rate in vivo, a reduced fraction of charged $tRNA^{Asp}$ was detected in Dnmt2 KO cells when compared with the wildtype cells, which is in agreement with the in vitro results (Figure 2c of manuscript 3). To analyse the effect of differential rate of aminoacylation on protein synthesis, fluorescent reporters namely YFP and CFP containing a N-terminal Asp_6 sequence were transfected in to wildtype and Dnmt2 knockout cells. As shown in figure 3b and 4b of manuscript 3, a reduction in the synthesis of Asp_6-YFP/CFP in the Dnmt2 KO cells as compared to wildtype cells was observed. To show a physiological significance of the findings with fluorescent reporter proteins, candidate cellular proteins with a poly-Asp region in their sequence were selected and their expression in wildtype and Dnmt2 KO cells were analysed. As shown in figure 5a of manuscript 3, synthesis of poly-Asp protein were reduced in Dnmt2 KO cells indicative of a novel translation control mechanism that is described in more details in the discussion section 4.3.2 of the thesis. Additional experiments indicated that this effect was not due to altered protein degradation in Dnmt2 KO cells (Figure 5c of manuscript 3). Gene ontology analysis of all the mammalian poly-Asp containing proteins showed (Table 1 of manuscript 3) a significant number of such proteins in pathways related to gene expression and transcription regulation. This gave a correlation to the phenotype of Dnmt2 KO flies and cells under stress condition that will be considered in the discussion section 4.3.3 of the thesis in a more detailed manner.

4. DISCUSSION

4.1 Mapping the interactions of DNMT2-tRNA^{Asp}

4.1.1 Mutation in the conserved lysine and arginine residues of DNMT2 reduces its activity

Enzymes have evolved to recognise their specific substrates using particular residues located in their structure. The details of such interacting regions and the residues involved are best revealed by co-crystal structures of the enzyme-substrate complex. Previously, a crystal structure of DNMT2 was published by Dong et al. (2001), but it included only the co-factor product SAH bound to the enzyme and not the tRNA substrate due to lack of a defined substrate for Dnmt2 at that time. It became obvious from the crystal structure of Dnmt2 and that of the well-known bacterial MTase M.HhaI that they share many regions similar in structure. The structural comparison of these two MTases gave important clues about the amino acid residues that might participate in the binding of nucleic acid substrate to Dnmt2. The superposition of these two MTase revealed the region between 289-295 of DNMT2 and 247-253 of M.HhaI are well overlaid and this region might be responsible for the substrate binding (Dong et al., 2001). It was indeed shown from our lab that C292 which is conserved only in Dnmt2 family proteins is involved in the tRNA binding and its disruption may cause partial loss of tRNA binding (Jurkowski et al., 2008). The C292 residue in DNMT2 is homologous to the Thr250 of M.HhaI, which is important for its DNA binding. However, other amino acids involved in the tRNA binding of Dnmt2 are not known. The present study gives insight into the residues involved in the tRNA^{Asp} binding of Dnmt2. By sequence alignment of different Dnmt2 proteins from lower eukaryotes and mammals, highly conserved lysine and arginine residues were selected and all these residues were exchanged to alanine. The idea behind this mutagenesis approach was that the positively charged lysine and arginine residues provide electrostatic attraction for the tRNA phosphodiester backbone. Exchanging them to an uncharged amino acid like alanine interrupts that force, which could help to map residues involved in the Dnmt2 interaction with tRNA^{Asp}. In total, 20 basic amino acids were selected for mutation which include a R84 located in the vicinity of motif IV, which harbours the

catalytic cysteine residue. Another highly conserved residue was R95, located near to the putative catalytic pocket on the 3D structure of the protein. Apart from this, the lysine and arginine residues located in the target recognition domain of the protein were selected. This refer to R288, R289 and K295 of the protein. The other residues were K122, K168, K196, R240, K241, K251, K254, K271, R275, K346, K363, K367, R369, R371 and K387 (Figure 1a of manuscript 1). On the 3D structure of the protein these residues are grouped to form surface grooves, consisting of the positively charged side chain exposed on the outside of the protein (Figure 9). The results indicated that 8 alanine mutants had profound effect (0-25% activity of wildtype) on the catalytic activity of the protein, indicating a potential effect of these residues on the interaction of $tRNA^{Asp}$. These residues are R84, R95, K122, R275, R289, R295, K367 and R371. In addition, 4 more other mutants had <50% of the activity of the wildtype enzyme and they are K251, R288, K241 and R369. In the three dimensional structure of DNMT2, these residues are located on one side of the protein where the catalytic pocket is situated. The mutants that had better or wildtype-like activity are located on the back side of the protein. In the 3D structure the residues K275, K367, R371 and R95 are located in close proximity to co-factor binding site and mutation of these residues could potential harm SAM binding leading to strong effect on catalysis. To test this hypothesis, the co-factor binding ability of all the mutants with strong effect on the activity was checked. By two different ways, it was shown that co-factor SAM binding was unaffected in all the mutants which showed drastic reduction in their activity towards $tRNA^{Asp}$. This indicated that mutation of the previously mentioned residues affect the $tRNA^{Asp}$ binding either in the ground state or in the transition state of the enzyme substrate complex.

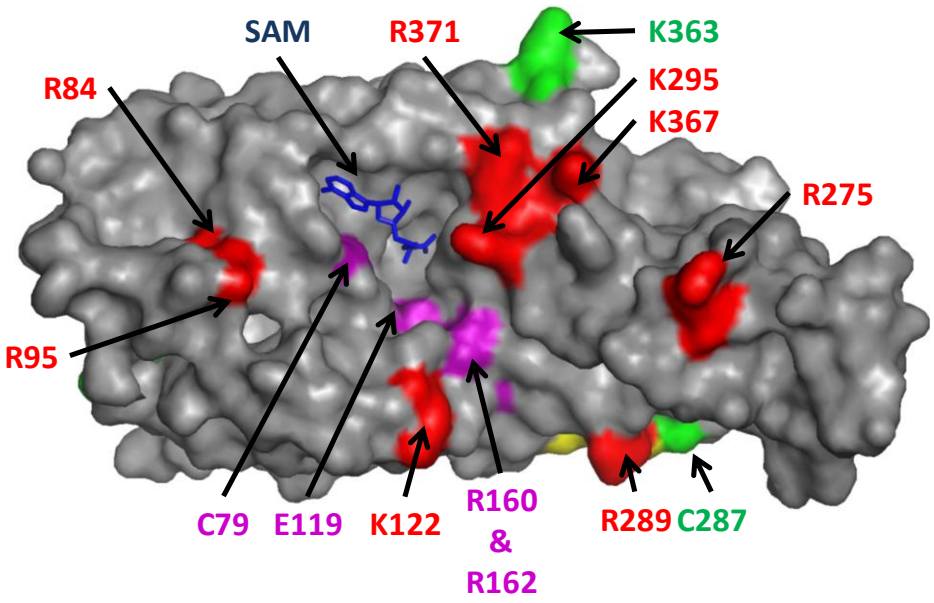

Figure 9: 3D structure of DNMT2 protein indicating the location of residues involved in the catalytic activity of the enzyme (red colour indicates the conserved basic residues and purple colour indicates the catalytic residues). The co-factor SAM is coloured in blue and is placed in the catalytic pocket of the protein.

4.1.2 Effect of mutations in the conserved residues on the enzyme-substrate complex formation at the transition state

In an enzymatic reaction, the enzyme binds the substrate in the ground state follwed by excitation to a transition state where the actual enzymatic process takes place. At both states, the enzyme substrate complex is stabilised by interactions involving the amino acid residues of the protein with the substrate. Introducing mutations in such amino acids may render the enzyme inefficient to catalyse the reaction. While describing the crystal structure of DNMT2, a model was proposed to account for its DNA binding by Dong et al. (2001). By superimposing DNMT2 on active M.HhaI MTase, it was proposed that DNMT2 upon binding its substrate, could adopt a closed confirmation like other DNA MTase and in this conformation the basic residues around the catalytic pocket would form a surface cleft, consisting of positively charged lysine and arginine residues, which stabilise the enzyme-substrate complex (Dong et al., 2001). The data here showed that a number of such residues (K275, K367, R371, K122, R289, R275, R84 and R95) are located along this groove and disturbing the positive

charge on them by alanine substitution had severe effect on the catalytic activity of the enzyme. To understand the influence on $tRNA^{Asp}$ binding for these mutations, tRNA binding experiment was conducted. The result showed that $tRNA^{Asp}$ binding of the mutant R371A was affected by about 50% compared to the binding efficiency of the wildtype protein. R84A and R95A also showed slightly reduced binding to $tRNA^{Asp}$ but retained approximately 73% and 80% of the wildtype binding respectively. Collectively, these three residues stabilises the binding of $tRNA^{Asp}$ to the DNMT2 enzyme in the ground state. The $tRNA^{Asp}$ binding of all other mutants were equal or better than the wildtype enzyme. However, these residues have potential role in the stabilisation of enzyme-substrate complex in transition state, where the actual methyl transfer takes place. This is supported by a previous report from the co-crystal structure of yeast AspRS with $tRNA^{Asp}$ (Ruff et al., 1991). The authors predicted that the anticodon stem/loop of $tRNA^{Asp}$ undergoes a large conformational change upon binding to the protein to unstack the bases of anticodon and this conformational change was stabilised by the interactions of enzyme amino terminal residues rather than the sequence of $tRNA^{Asp}$. A similar conformational change would be induced by DNMT2 upon its binding to $tRNA^{Asp}$ and stabilization of this unfavourable confirmation of the bound $tRNA^{Asp}$ was mediated through K275, K367, K122, R289 and R275 residues, which lay on same side of DNMT2 where the active center of the enzyme is found. Also, this might be the reason for a significant reduction in the catalytic activity of these mutant enzymes, even though they did not contribute to the ground state binding of the $tRNA^{Asp}$.

4.1.3 The Anticodon stem/loop of $tRNA^{Asp}$ contributes crucial contacts to DNMT2

Another aim of this project was to create a model of DNMT2 binding to $tRNA^{Asp}$ and to characterise the contact regions between DNMT2 and $tRNA^{Asp}$. To this end, $tRNA^{Asp}$ was manually docked on the surface of DNMT2 and an open-source biological modelling tool 'PyMol' was used to visualise the contact regions. In the mutagenesis approach, it was shown that eight positively charged amino acids along a single surface cleft of the DNMT2 protein have a role in $tRNA^{Asp}$ binding and mutations in these

residues caused a drastic decrease in the enzyme activity. With this information, $tRNA^{Asp}$ was manually docked to this region of DNMT2. To this end, the $tRNA^{Asp}$ structure was retrieved from the PDB entry (1ASY) deposited for the AspRS-$tRNA^{Asp}$ co-crystal complex. In this complex, the enzyme was shown to contact both the acceptor arm and the anticodon arm of the $tRNA^{Asp}$. However, this kind of dual interaction is not feasible with DNMT2 owing to the size and shape constraints of the putative tRNA binding pocket in the DNMT2 protein. Therefore, for the modelling of the DNMT2-$tRNA^{Asp}$ complex, the anticodon stem/loop of the $tRNA^{Asp}$ was placed in the putative binding pocket, in such a way to position the target base in close proximity to the catalytic cysteine residues in the active center (figure 10). As DNMT2 has been previously shown to follow DNA methyltransferase like catalytic mechanism from our lab (Jurkowski et al., 2008), the rotation of the target base in the catalytic pocket was considered. This model suggest that only one end of the $tRNA^{Asp}$ could be accommodated on DNMT2 surface and this region comprises the anticodon stem/loop, the variable loop and a part of D-loop of the L-shaped $tRNA^{Asp}$ while the acceptor arm and T-loop of the $tRNA^{Asp}$ lay outside of the protein (figure 10). In agreement with this, the data here showed that mutating the lysine and arginine residues on the back side of the protein do not led loss of catalytic activity, because, $tRNA^{Asp}$ do not make any interaction in this region.

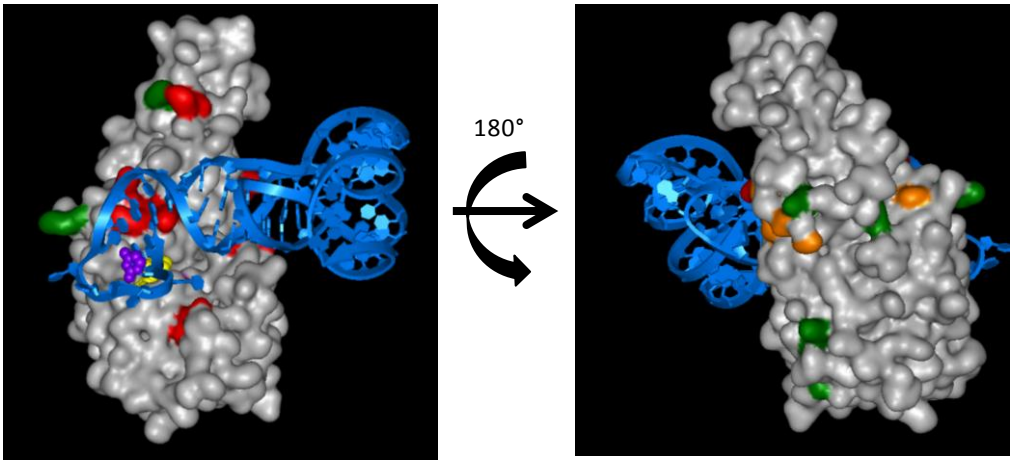

Figure 10. Manual docking of $tRNA^{Asp}$ on DNMT2 protein. The residues contacting tRNAs are coloured red and those do not contact are coloured green and orange.

Following this model of DNMT2-tRNA^{Asp}, elucidiating the interaction, two new crystal structure of Dnmt2 homologs appeared, one from *E. histolytica* (*EhMeth*) from the group of Ficner (2012) and another from *Spodoptera frugiperda* (*sf*Dnmt2) from the group of Zhong (2013). The crystal structure of *EhMeth* was found to be similar to the human DNMT2 protein, however the active site loop in *EhMeth* was well defined (Schulz et al., 2012). The authors proposed that active site loop comprising the residues between 80-100 of the *EhMeth* is involved in the substrate binding, which is in agreement with the current model showing that mutation in the R84 and R95 have a substantial effect on the catalytic activity of DNMT2 enzyme. However, the involvement of residue R99 and R226 in substrate binding proposed by the authors cannot be explained by the current model. In addition, the crystal structure of *sfDnmt2* protein from *Spodoptera frugiperda* supported the current model proposed in this study (Li et al., 2013). In the *Sf*Dnmt2 crystal structure, the authors showed that the catalytic loop protrudes out from the catalytic domain with a positive charge surface to facilitate substrate binding. The crystal structure of both *EhMeth* and *Sf*Dnmt2 are in agreement with current model, proposing conformational change of the enzyme upon substrate binding. This suggests that the model of DNMT2- tRNA^{Asp} can be generalised for other homologs of the proteins.

4.2 Function of Dnmt2 homolog in *Geobacter sulfurreducens*

4.2.1 Additional targets of Dnmt2 and methylation of tRNA^{Glu} by *Geobacter* Dnmt2 (GsDnmt2)

The Dnmt2 enzyme is highly conserved in eukaryotes. However, based on multiple sequence alignments of various Dnmt2 enzymes, a single homolog of this enzyme was found in a prokaryote called *Geobacter sulfurreducens* (Goll et al., 2006; Jurkowski and Jeltsch, 2011), which is named here as GsDnmt2. In the present work the function of this enzyme was biochemically characterised and showed that it has a different substrate specificity than all other Dnmt2 enzymes, because the Dnmt2 protein in many eukaryotes were found to methylate tRNA^{Asp} at C38 position (Becker

et al., 2012; Goll et al., 2006; Jurkowski et al., 2008; Muller et al., 2013; Schaefer et al., 2010), in contrast, this work shows that GsDnmt2 protein prefers methylation of $tRNA^{Glu}$ more than $tRNA^{Asp}$. It was also shown experimentally that the site of tRNA methylation by this enzyme is the same C38 position as in the eukaryotic homologs. Mutation of the cysteine residue in the catalytic motif (C74) of GsDnmt2 led to a complete loss of activity, in agreement with the role of this cysteine in the catalysis (Jurkowski et al., 2008). In recent time, additional substrates for some of the eukaryotic Dnmt2 proteins have been discribed. For example, methylation of $tRNA^{Glu}$ by the *S. pombe* Dnmt2 homolog Pmt1 has been reported both in vitro and in vivo (Becker et al., 2012). However, $tRNA^{Glu}$ methylation was weaker than $tRNA^{Asp}$ methylation by this enzyme. $tRNA^{Glu}$ methylation was also reported for the DnmA proteins, which is a homolog of Dnmt2 present in *D. discoidium*. However, the activity was observed only in vitro (Muller et al., 2013). To this end, GsDnmt2 methylation on all other C38 containing tRNAs of *G. sulfurreducens* was tested and it was surprisingly found to have high level of methylation on $tRNA^{Glu}$ compared to $tRNA^{Asp}$ by GsDnmt2. Following this, the methylation profile of eukaryotic Dnmt2 enzymes from human, *Drosophila*, *D. discoidium* and *S. pombe* was investigated. In all of them, a strong methylation of $tRNA^{Asp}$ was observed when compared with methylation of $tRNA^{Glu}$. In agreement with previous reports, a weak methylation of $tRNA^{Glu}$ was seen with Pmt1 and DnmA proteins in this analysis (Becker et al., 2012; Muller et al., 2013). $tRNA^{Asp}$ and $tRNA^{Glu}$ methylation in *G. sulfurreducens* by bisulfite analysis showed $tRNA^{Glu}$ methylation at C38 position. In addition to the C38 methylation in $tRNA^{Glu}$, methylation at position C62 in the T-stem was also observed but the candidate enzyme responsible for this methylation is yet to be identified in this organism.

The results of this analysis showing altered substrate preference for GsDnmt2 suggests expanded substrate recognition for Dnmt2 proteins. A similar finding was recently reported (Swinehart et al., 2013), which showed expanded substrate specificity for *Trm*10 enzyme, which catalyses modification of G9 base in 9 different tRNAs. The authors found in vitro methylation on noncognate tRNA which cannot be explained by the in vivo pattern of modified tRNAs and showed that overexpression of *Trm*10 can catalyse modification of G9 base in a variety of tRNAs other than the 9

reported tRNAs in vivo. The modification of G9 base by *Trm*10 was shown to be affected by the presence of other modified bases in the tRNA. However, Dnmt2 methylation of $tRNA^{Asp}$ has been shown to be independent of other modified bases in the tRNA, as in vitro transcribed tRNAs are methylated efficiently by this enzyme (Jurkowski et al., 2008; Muller et al., 2013). Nevertheless, the altered substrate specificity of GsDnmt2 would suggest presence of other RNA targets for GsDnmt2 enzyme that might be involved in pathways other than tRNA processing.

4.2.2 Identity determinants of Dnmt2 and variable loop effect on Dnmt2 methylation

Substrate specific tRNA recognition by methyltransferase is mediated by different structural determinants of the substrate and sequence of the enzyme. For example, the bacterial *TrmA* catalyses methylation of a variety of tRNAs at position U54 which is present in the T-loop. Even though, the methylation site is located in the T-loop of the tRNA, crystal structure of *TrmA* revealed crucial contacts with the U17, G18 and G19 residues in the D-loop (Alian et al., 2008). Similarly, the crystal structure of the eukaryotic *Trm*5 in complex with its substrate $tRNA^{Leu}$ showed functional interactions of the enzyme with the D-loop and T-loop for the methylation occurring in the anticodon loop at position G37 (Goto-Ito et al., 2008). This shows the dependence of methyltransferase on different structural elements of the substrate for its catalytic activity. Nonetheless, involvement of T-loop and acceptor stem may not be applicable in the case of Dnmt2 proteins as the previous model (Jurkowski et al., 2012) of DNMT2-$tRNA^{Asp}$ suggested that these regions do not make any functional interaction with the Dnmt2 protein. This suggests that other regions of $tRNA^{Asp}$ contain structural determinants for Dnmt2 recognition. However, recent report indicate that anticodon stem/loop alone is inefficient to be methylated by Dnmt2 enzyme (Schulz et al., 2012), suggesting that additional elements outside of the anticodon stem/loop is required for proper folding of the $tRNA^{Asp}$ and its methylation by Dnmt2. In the modelling of DNMT2- $tRNA^{Asp}$, it was suggested that one end of the $tRNA^{Asp}$ containing the anticodon stem/loop, variable loop and a part of the D stem/loop make contacts with DNMT2 (Jurkowski et al., 2012).

This gives a starting point to investigate the other identity determinant regions in $tRNA^{Asp}$, that helps in its specific recognition by Dnmt2 enzymes. To this end, a multiple sequence alignment of the $tRNA^{Asp}$ and $tRNA^{Glu}$ from different species was made (Figure 11) for comparison of the sequence variabilities in anticodon stem/loop and in the region of variable loop. This sequence alignment gave interesting observation that the length of the variable loop in all the eukaryotic $tRNA^{Asp}$ was restricted to 4 nucleotides, while the $tRNA^{Asp}$ of *Geobacter sulfurreducens* had an increased length of 5 nucleotides. It also showed that $tRNA^{Glu}$ of most eukaryotes which are non preferred substrates for Dnmt2 possess a -GG- dinucleotide in the variable loop. The same sequence feature is also present in the $tRNA^{Asp}$ of *Geobacter sulfurreducens.* The variable loop of $tRNA^{Glu}$ in *Geobacter sulfurreducens* resembles the $tRNA^{Asp}$ of most eukaryotes. This sequence dissimilarity could explain the inefficient methylation of GsDnmt2 on $tRNA^{Asp}$. To make it better, the sequence of $tRNA^{Glu}$ from many eukaryotes and *Geobacter sulfurreducens* were compared. This analysis provided a molecular connection for the swapped specificity of GsDnmt2.

Okamoto et al. (2004) showed the length of the variable loop as a determinant for the methylation at position G46 by transfer RNA (m^7G46) methyltransferase in *Aquifex aeolicus* (Okamoto et al., 2004). In this present work, a similar role was shown for the variable loop sequence on the methylation of $tRNA^{Glu}$ by GsDnmt2. The study also led to investigate the exchange of the variable loop of the *Geobacter sulfurreducens* $tRNA^{Glu}$ with the variable loop of murine $tRNA^{Asp}$. This tRNA mutant now possessed a -GG- dinucleotide in the variable loop and showed decreased methylation by GsDnmt2 compared to the normal $tRNA^{Glu}$. A similar decrease was also observed for human DNMT2 when mutant murine $tRNA^{Asp}$ with variable loop of murine $tRNA^{Glu}$ was used. Here, the effect was even more pronounced than in case in GsDnmt2. The mutant murine $tRNA^{Asp}$ showed only 10% of the wildtype activity. The residual activity seen with mutant $tRNA^{Asp}$ could indicate that human DNMT2 uses specificity determinants outside of the variable loop to a lesser extent.

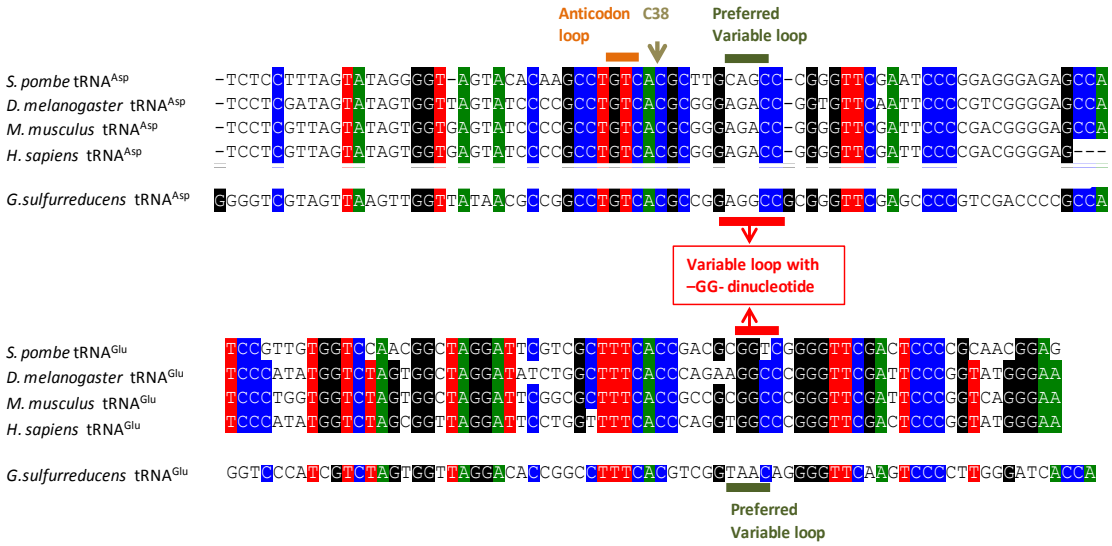

Figure 11. Multiple sequence alignment of $tRNA^{Asp}$ and $tRNA^{Glu}$ from different species. The figure emphasises the variable loop sequence dissimilarities among preferred and non preferred tRNAs by Dnmt2 enzyme.

The result from the variable loop effect on GsDnmt2 activity suggests two new models for the evolution of Dnmt2 proteins. GsDnmt2 enzyme methylates the $tRNA^{Asp}$ of mouse and *Drosophila* but not its own instead it methylates its $tRNA^{Glu}$. One scenario could be, Dnmt2 proteins lost their ability to methylate $tRNA^{Glu}$ as they evolved in eukaryotes. This could be supported by the existence of residual methylation in $tRNA^{Glu}$ by Pmt1, DnmA and dDnmt2 proteins. The second scenario implies that GsDnmt2 has been acquired in the *Geobacter* species by a horizontal gene transfer from eukaryotes. Based on the phylogeny of Dnmt2 protein, the second scenario fits better because these microbes are known for their ability to take up foreign genes (Heuer and Smalla, 2007). In this assumption, GsDnmt2 was acquired by *G. sulfurreducens* however, $tRNA^{Asp}$ with an unfavoured variable loop was not preferred for methylation by this enzyme, while the $tRNA^{Glu}$ with a fitting variable loop is methylated by GsDnmt2 with high preference. This led to stable incorporation of this gene into the *Geobacter*'s genome.

The work also proposes some of the important tRNA structural determinants required by Dnmt2 enzymes for substrate recognition. From the data, it was clear that variable loop determines most of the identity. Work from our collaborators showed the involvement of other residues in the tRNA recognition that includes C32,

C34, A37 and C40 (communicated in (Muller et al., 2013)). However, the current work supports the involvement of C32 and A37 residues in the C38 methylation by Dnmt2 enzyme as these residues are conserved in the $tRNA^{Glu}$ of *G. sulfurreducens* as well, while the involvement of other residues remains to be validated.

Alternatively, the work proposes a distinct tRNA recognition mechanism in human DNMT2 and GsDnmt2 to methylate $tRNA^{Asp}$ and $tRNA^{Glu}$ respectively. In the work of (Christian and Hou, 2007), the authors showed that *Trm*5 of eukarya/archeal and *Trm*D of bacterial are non-orthologous enzymes that catalyse methylation at similar position (G37) in different tRNA. However, the authors proved that for *Trm*5, the identity of the residues on the 3' side of the G37 base is crucial for its activity while for *Trm*D the residues on both 5' and 3' side of the target base determines the specificity. Thus a similar enzyme might depend on diverse structural determinant for its enzymatic activity (Sakaguchi et al., 2012). Similar to this phenomenon, $tRNA^{Asp}$ recognition by human DNMT2 enzyme would depend on the variable loop as well as the identity of the bases in the target recognition motif (C32, C34, A37 and C40) of the $tRNA^{Asp}$. However, $tRNA^{Glu}$ recognition by GsDnmt2 enzyme utilises the variable loop and other unknown identity determinants, because C34 and C40 bases in the target recognition motif are not conserved in this tRNA. The data shown in this work also disfavours the involvement of mannosylqueuosin modification at G34 position in the recognition of Dnmt2 proteins (Goll et al., 2006) as $tRNA^{Glu}$ of *G. sulfurreducens* does not contain G34 and shows methylation by GsDnmt2 while *G. sulfurreducens* $tRNA^{Asp}$ posesses G34 still shows weak methylation by this enzyme. It would be interesting to identify in future, other critical determinants in $tRNA^{Glu}$ of *G. sulfurreducens* and analyse if this could expand the substrates for eukaryotic Dnmt2 proteins.

In conclusion, the study shows that in addition to the anticodon stem/loop of $tRNA^{Asp}$ the variable loop is also recognised by GsDnmt2 and human DNMT2. The work also suggests that, this mode of recognition is common for other eukaryotic Dnmt2 enzymes, which need further experimental validation.

In addition, the present work propose important challenge to identify how these related proteins in prokaryote (GsDnmt2) and eukaryotes (Dnmt2) have evolved mechanisms to recognise different tRNAs. In addition, it would be interesting

to investigate how important is GsDnmt2 in the bacterial species for the cellular homeostasis and whether there are other substrates for GsDnmt2 in the cell.

4.3 Physiological relevance of the C38 methyl group in tRNA^{Asp}

4.3.1 C38 methylation affects the tRNA^{Asp} on its aminoacylation

So far, only very little is known about the physiological relevance of the C38 methylation of tRNA^{Asp}. It has been reported to prevent tRNA^{Asp} fragmentation and increase innate immune response against invading RNA viruses (Durdevic et al., 2013b; Schaefer et al., 2010).

Previously, reports have indicated the importance of tRNA methylation in maintaining the reading frame of mRNA during translation. It was shown that the A/G37 modification in the anticodon loop is very crucial in the maintenance of the reading frame of mRNA during the translation in the P-site of ribosomes (Urbonavicius et al., 2001). In *E. coli* and *S. typhimurium* lack of modification of A37 was shown to increase the frame shifting by 6-7 folds (Hagervall et al., 1993; Qian et al., 1998). Similarly, the G37 methylation of tRNA^{Met} in *Saccharomyces cerevisiae* by *Trm5* enzyme was shown to maintain the reading frame in mitochondrial protein synthesis (Lee et al., 2007). Despite these discoveries a role of Dnmt2 proteins in maintaining the reading frame can be excluded because of the fact that C38 residues occurs at the 2nd position after the anticodon in tRNA and the knockout cells only show mild phenotypes.

A previous report has shown the influence of a single methyl group at G37 on tRNA^{Asp} in preventing its mischarging by other aminoacyl tRNA synthetases (aaRS), suggesting a potential role of tRNA methylation in enhancing the selectivity of tRNA species by different aaRS (Putz et al., 1994). A direct role of C38 interaction of tRNA^{Asp} with AspRS came from the co-crystal structure of the yeast tRNA^{Asp}- AspRS complex. Later, the charging efficiency of AspRS enzyme in *Saccharomyces cerevisiae* was shown to be dependent on the identity of C38 residues in tRNA^{Asp} (Ryckelynck et al., 2003). This led to study the possible role of C38 methylation in the aminoacylation of tRNA^{Asp} by aspartyl-tRNA synthetase (AspRS). The results support few previous findings that showed involvement of tRNA modifications in tRNA charging. In *Salmonella*

typhimurium it was shown that absence of methylation at G37 in $tRNA^{Pro}$ and $tRNA^{Arg}$ reduces the selection of these tRNAs by their respective aaRS enzyme. In a similar way, the pseudouridylation of $tRNA^{Pro}$ at position 38 was also shown to enhance its selection by prolyl-tRNA synthetase (Curran and Yarus, 1989; Li et al., 1997). In line with these findings, this project of my thesis shows that the C38 methylation of $tRNA^{Asp}$ increases its aminoacylation efficiency by the aspartyl-tRNA synthetase by 4-5 folds. This suggests that C38 methylation is recognised by the AspRS. In the crystal structure of yeast AspRS-$tRNA^{Asp}$ complex, the conformation of the complex was shown to be stabilised by the C38 residue and this particular residue was shown to be an identity determinant of the $tRNA^{Asp}$ in addition to the anticodon sequence (Eriani and Gangloff, 1999; Moulinier et al., 2001). In support of the crystal structure and aminoacylation data, this work suggest that the methyl group at C38 position might stack on the G39 residue in AspRS and stabilise the confirmation of the tRNA.

Hypomodification of tRNA have been reported to affect the in vivo charging level of different tRNAs. Alexandrov et al. (2006) showed that lack of $tRNA^{Val}$ modification by *Trm*8 and *Trm*4 could decrease the in vivo level of charged $tRNA^{Val}$ by more than 60% (Alexandrov et al., 2006). A similar phenomenon was reported by Krüger et al. (1998), they showed 40% reduction in the level of charged $tRNA^{Glu}$ in *E. coli* cells, lacking the modification at U34 position on the tRNA (Kruger and Sorensen, 1998). In line with these findings, the present work reveals that the C38 modification also affects the charging level of $tRNA^{Asp}$. In Dnmt2 KO cells lacking the C38 modification, 30-40% reduction in the level of charged $tRNA^{Asp}$ was observed compared to wildtype cells. However, the present work does not exclude rapid degradation of $tRNA^{Asp}$ that might arise from the absence of non essential modification as reported previously for $tRNA^{Val}$ (Alexandrov et al., 2006). Indeed, previous reports have shown role of Dnmt2 in maintaining the steady-state level of $tRNA^{Asp}$, where methylation at C38 position was shown to prevent the endonucleolytic cleavage of this tRNA by Angiogenin. Given the increased susceptibility to degradation and decreased level of charging of $tRNA^{Asp}$ in Dnmt2 KO cells, the physiological effect might arise in the synthesis of specific proteins which will be the focus in the next chapter.

4.3.2 Synthesis of poly-Asp containing proteins in the Dnmt2 Knockout cells is reduced

In the mammalian proteome, synthesis of many proteins are affected by codon biases in the gene. The codon usage/bias refers to the frequency of occurrence of synonymous codons in genes and this has been shown to influence the translation of proteins from their mRNA and regulates embryonic development and tissue specificity (Plotkin et al., 2004; Ren et al., 2007). A recent report in yeast showed, the occurrence of particular codon in mRNAs increases stress response during oxidative condition (Chan et al., 2012). In the report the authors showed that *Trm*4 catalysed modification of $tRNA^{Leu}$ is responsible for the selective enrichment of ribosomal proteins like RPL22A in yeast cells after oxidative stress. This enhances the survival of organisms following cellular stress. The authors also showed that *Trm*4 catalysed methylation of $tRNA^{Leu}$ at the C34 position in the wobble base enhanced the readout of mRNA from genes enriched with TTG codon. In a similar way, Dnmt2 catalysed modification might enhance cell survival after certain stress conditions. Work from our collaborators showed that Dnmt2 mutant flies had reduced viability under stress condition (Schaefer et al., 2010). However, the molecular cause behind reduced viability of the cells remained unexplored. In a recent report, absence of m^5C in mammalian tRNAs was shown to affect the overall protein synthesis (Tuorto et al., 2012). By knocking out Dnmt2 and NSun2 in mice, the authors showed differentiation defect in tissues and cells. Consistently, the authors also observed reduced synthesis of proteins at post-transcriptional level.

The present work elucidates that C38 methylation of $tRNA^{Asp}$ influences the synthesis of proteins with a poly-Asp sequence. In support of this argument, it was shown by use of a fluorescent reporter system, utilising YFP and CFP proteins fused with an N-terminal Asp_6 sequence, that synthesis of these Asp-tagged proteins are reduced in Dnmt2 KO cells. Fluorescent reporter systems are a versatile tool to study the synthesis rate of proteins in cellular environment. The present work used YFP and CFP owing to the fact that individual cells can be analysed for the rate of protein synthesis, whereas use of luciferase and other reporter system does not enable quantification at single cell level. The work also illustrates this effect with endogenous

proteins in Dnmt2 knockout cells. To this end, it was shown that the synthesis of proteins with poly-Asp sequence that includes transcription activation factor 9, transcription factor DP1, Protein SET and Enhancer of zeste 2 are reduced in the Dnmt2 KO cells. Additionally, the cellular degradation of the candidate proteins are shown to be unaffected in Dnmt2 KO cells compared to the wildtype cells. Another possibility for an increased level of protein in cells comes from the gene expression. However, using published data (Tuorto et al., 2012), the expression of the candidate genes are shown to be identical in both cell types suggesting that differential rate of protein synthesis observed in the Dnmt2 KO cells must be due to the inability of the cells to synthesis the pole-Asp protein.

The results from this analysis provides molecular connection for two previous findings. First, it suggests that the reduction of overall protein synthesis seen in Dnmt2 and NSun2 double knockout mice as shown by Tourto (2012) might be partially connected to the differential synthesis of poly-Asp tagged proteins. The authors also showed decreased levels of polysomes from the double knockout mice. This could be interpreted as the premature release of the ribosomes after they encounter the poly-Asp sequence. As Dnmt2 KO cells have reduced levels of charged $tRNA^{Asp}$, the ribosomes attain a stall on reaching the poly-Asp sequences that creates empty aminoacyl sites in the ribosomes, leading to premature termination of translation. Even though, the results from this project support the general enrichment of protein level from genes coding aspartate rich codons (GAC), this needs to be validated in the future. However, the present work adds up another mechanism of translational control by tRNAs in additions to the recently reported role of tRNA fragments in the inhibition of translation initiation (Ivanov et al., 2011).

Secondly, the results from this work could also explain the molecular connection to the decreased viability seen with Dnmt2 mutant flies under stress conditions, which will be dealt in the next chapter.

4.3.3 Possible functions of Dnmt2 in enhancement of gene regulatory proteins

The work shows that loss of the Dnmt2 protein in cells led to decreased levels of Enhancer of Zeste 2 (EZH2), Transcription factor Dp-1 (TFDP-1), Transcription initiation factor TFIID subunit 9 (TAF9) and Protein-SET (SET). It is interesting to note that all of these are nuclear proteins and are involved in the regulation of gene expression. Ezh2 is a component of the PRC2 complex in which this protein forms the catalytic core. This complex is involved in the expression of a variety of genes that controls embryonic stem cell differentiation and cell survival (Ezhkova et al., 2009; Fan et al., 2011; Lee et al., 2006). TAF9 is an important regulator of general gene transcription and this protein is required for the cell viability (Chen and Manley, 2003; Frontini et al., 2005). Similarly, TFDP-1 was shown to be a component of E2F transcription factor and is involved in both cell proliferation and apoptosis (Chen et al., 2013). This suggests that DNMT2 proteins might help in the survival of cells by maintaining the synthesis of essential proteins in certain physiological conditions. Furthermore, gene ontology analysis revealed that a significant number of proteins containing poly-Asp sequences have roles in the transcription regulation and gene expression.

This observation proposes a new model to explain the mild phenotype seen in Dnmt2 KO cells under stress conditions. The model is, the Dnmt2 KO cells have difficulties in synthesising proteins, which increases the stress response behaviour, due to decrease in the level of standard gene regulatory proteins (poly-Asp proteins). This argument provides a more direct physiological relevance to the stress response mechanism. In addition to this, previously Durdevic et al. (2013b) showed increased $tRNA^{Asp}$ fragmentation in Dnmt2 KO flies exposed to heat shock and also showed that these tRNA fragments are preferentially processed and loaded into Dicer-2 and this persistently interferes with the synthesis of siRNAs affecting the genes controlled by them (Durdevic et al., 2013b). It can be that these two mechanisms co-exist in parallel to control the expression of a set of stress response genes. Nevertheless, it would be a major interest to investigate, if the effect that is observed with selected poly-Asp proteins is applicable to all other such proteins in this category.

Another reason for the inability of the Dnmt2 KO cells to counteract the stress condition might arise due to the inability to increase the level of methylation of

$tRNA^{Asp}$ in cells. Methylation of crucial residues in tRNAs are increased after exposing the cells to unfavourable growth conditions or treatment of H_2O_2 and DNA damaging agents (MMS) (Begley et al., 2007). In a report C34 methylation of $tRNA^{Leu}$ was shown to be upregulated by 70% in yeast cells exposed to H_2O_2 (Chan et al., 2012). This modification stabilises the tRNA and increases its level in response to stress. The same effect was also seen with $tRNA^{His}$ in yeast cells grown under non-permissive temperature and glucose starvation (Preston et al., 2013). However, earlier reports have shown aggregation of Dnmt2 to the stress granules following heat shock in flies and this reduces the activity of Dnmt2 in cells (Schaefer et al., 2010). This condition would lead to decreased steady state level of $tRNA^{Asp}$ during cellular stress. Given the reduced availability of $tRNA^{Asp}$ in cells under stress, charging level of $tRNA^{Asp}$ goes further down that adversely affect the enrichment of poly-Asp proteins leading to reduced viability of cells.

Also, the work proposes a translational control mechanism for proteins with poly-Gly, poly-Val and poly-Glu sequence as described for the poly-Asp proteins, because $tRNA^{Gly}$,$tRNA^{Val}$ and $tRNA^{Glu}$ were found to be the additional substrates for Dnmt2 (Becker et al., 2012; Muller et al., 2013; Schaefer et al., 2010). However, it remains to be shown, if the C38 methyl group might affect the readout of these tRNAs by their respective aminoacyl-tRNA synthetases. In murine proteome, there are about 188 target proteins with poly-Gly and 396 target proteins containing poly-Glu sequence (Obenauer et al., 2003). This posts a major challenge to determine the proteins that can be regulated by Dnmt2 catalysed modification of the respective tRNAs.

Collectively, the results open a new arena for the investigation of Dnmt2 protein on the cellular synthesis of poly-Asp proteins and sought to re-evaluate the molecular connection behind the mild phenotype in Dnmt2 knockouts seen after cellular stress.

5. PUBLICATION LIST AND AUTHOR'S CONTRIBUTION

1. Jurkowski TP[#], Shanmugam R[#], Helm M, Jeltsch A (2012). Mapping the tRNA binding site on the surface of human DNMT2 methyltransferase. Biochemistry. Jun 5;51(22):4438-44.

[#] These authors contributed equally to this study

Shanmugam R, contributed to the design of the study and performed the following experiments: mutagenesis, protein purification, circular dichroism, in vitro transcription, enzyme kinetics, tRNA binding analysis and ANS competition assay. S.R was involved in data analysis and interpretation and manuscript preparation.

2. Raghuvaran Shanmugam, Muktak Aklujkar, Matthias Schäfer, Richard Reinhardt, Olaf Nickel, Gunter Reuter, Derek R. Lovley, Ann Ehrenhofer-Murray, Wolfgang Nellen, Serge Ankri, Mark Helm, Tomasz P. Jurkowski, & Albert Jeltsch (2014). The Dnmt2 RNA methyltransferase homolog in *Geobacter sulfurreducens* specifically methylates tRNA-Glu. "Nucl. Acids Res., in press"

Shanmugam R, contributed to the design of the study and performed all the experiments. Total RNA preparation was provided by M.A. A.E.M provided Pmt1 enzyme. S.R was involved in data analysis and interpretation and wrote the manuscript draft.

3. Raghuvaran Shanmugam, Jacob Fierer, Steffan Kaiser, Francesca Tuorto, Frank Lyko, Mark Helm, Tomasz P. Jurkowski, & Albert Jeltsch (2014). Cytosine methylation of $tRNA^{Asp}$ by DNMT2 has a role in translation of Asp-tagged proteins. Submitted.

Shanmugam R, contributed to the design of the study and performed the following experiments, protein purification, aminoacylation kinetics, in vivo levels of aminoacylation, microscopy, fluorescence reporter protein analysis, western blotting and in vivo protein degradation analysis. J.F prepared the reporter construct. S.R was involved in data interpretation and wrote the manuscript draft.

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7. LIST OF MANUSCRIPTS

1. Jurkowski TP[#], Shanmugam R[#], Helm M, Jeltsch A (2012). Mapping the tRNA binding site on the surface of human DNMT2 methyltransferase. Biochemistry. Jun 5;51(22):4438-44.

[#] Equal contribution by these authors

2. Raghuvaran Shanmugam, Muktak Aklujkar, Matthias Schäfer, Richard Reinhardt, Olaf Nickel, Gunter Reuter, Derek R. Lovley, Ann Ehrenhofer-Murray, Wolfgang Nellen, Serge Ankri, Mark Helm, Tomasz P. Jurkowski, & Albert Jeltsch (2014). The Dnmt2 RNA methyltransferase homolog in *Geobacter sulfurreducens* specifically methylates tRNA-Glu "Nucl. Acids Res., in press"

3. Raghuvaran Shanmugam, Jacob Fierer, Steffan Kaiser, Francesca Tuorto, Frank Lyko, Mark Helm, Tomasz P. Jurkowski, & Albert Jeltsch (2014). Cytosine methylation of $tRNA^{Asp}$ by DNMT2 has a role in translation of Asp-tagged proteins. Manuscript submitted for publication.

MANUSCRIPT 1

Reference for Manuscript 1

1. Jurkowski TP[#], Shanmugam R[#], Helm M, Jeltsch A (2012). Mapping the tRNA binding site on the surface of human DNMT2 methyltransferase. Biochemistry. Jun 5;51(22):4438-44.

MANUSCRIPT 2

The Dnmt2 RNA methyltransferase homolog of *Geobacter sulfurreducens* specifically methylates tRNA-Glu

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Abstract

Dnmt2 enzymes are conserved in eukaryotes, where they methylate C38 of tRNA-Asp with high activity. Here, the activity of one of very few prokaryotic Dnmt2 homologs from *Geobacter* species (GsDnmt2) was investigated. GsDnmt2 was observed to methylate tRNA-Asp from flies and mice. Unexpectedly, it had only a weak activity towards its matching *Geobacter* tRNA-Asp, but it methylated *Geobacter* tRNA-Glu with good activity. In agreement with this result, we show that tRNA-Glu is methylated in *Geobacter* while the methylation is absent in tRNA-Asp. The activities of Dnmt2 enzymes from *Homo sapiens*, *D. melanogaster*, *Schizosaccharomyces pombe* and *Dictyostelium discoideum* for methylation of the *Geobacter* tRNA-Asp and tRNA-Glu were determined showing that all these Dnmt2s preferentially methylate tRNA-Asp. Hence, the GsDnmt2 enzyme has a swapped tRNA specificity. By comparing the different tRNAs, a characteristic sequence pattern was identified in the variable loop of all preferred tRNA substrates. An exchange of two nucleotides in the variable loop of murine tRNA-Asp converted it to the corresponding variable loop of tRNA-Glu and led to a strong reduction of GsDnmt2 activity. Interestingly, the same loss of activity was observed with human DNMT2, indicating that the variable loop functions as a specificity determinant in tRNA recognition of Dnmt2 enzymes.

Key words: RNA methylation, enzyme specificity, protein-nucleic acid interaction, RNA recognition

Short title: *Geobacter* Dnmt2 methylates tRNA-Glu

Introduction

Several chemical modifications of the bases in DNA and RNA have been discovered in the last decades of research, which include methylation, pseudouridylation, hydroxymethylation, formylation and carboxylation (1-4). In eukaryotes, three families of enzymes with strong sequence similarity to DNA-(cytosine C5)-methyltransferases have been found, namely Dnmt1, Dnmt2 and Dnmt3 (5-7). Dnmt2 enzymes are highly conserved with homologs present from lower eukaryotes like *Schizosaccharomyces pombe* to higher eukaryotes like *Homo sapiens* (8,9). These enzymes contain all characteristic motifs characteristic for DNA methyltransferases, but lack the large N-terminal domain found in the Dnmt1 and Dnmt3 enzymes. Later, in a seminal paper by Goll and colleagues, Dnmt2 enzymes were reported to have a robust methylation activity on $tRNA^{Asp}$ at position C38 (6). After the initial discovery of $tRNA^{Asp}$ methylation by Dnmt2, many studies have confirmed this activity in different organisms (10-16). However, even though Dnmt2 methylates tRNA, it utilises a DNA methyltransferase-like catalytic mechanism in the methyl transfer reactions (10), which also explains the conservation of the corresponding amino acid motifs in the catalytic center.

Dnmt2 knockout studies have indicated a connection to stress response and methylation of small RNAs (13,17,18), non-random sister chromatid segregation in stem cells (19), mobile element and RNA virus control in *Drosophila melanogaster* (20,21), paramutation in mice (22) and adaptation to different growth conditions in lower eukaryotes (12,15,16). However, it is still unclear whether these effects stem from the loss of methylation of C38 in $tRNA^{Asp}$ or whether other Dnmt2 targets are responsible for the phenotypes. Many of the studies have also investigated methylation of alternate tRNA substrates by Dnmt2. In *Dictyostelium discoideum*, Dnmt2 was reported to methylate $tRNA^{Glu}$(CUC/UUC) and $tRNA^{Gly}$(GCC) very weakly *in vitro* (15). The *S. pombe* Dnmt2 homolog, Pmt1, was shown to have a weaker methylation activity on $tRNA^{Glu}$ as well (16). *D. melanogaster* Dnmt2 was found to methylate $tRNA^{Val}$(AAC) and $tRNA^{Gly}$(GCC) *in vivo* in addition to $tRNA^{Asp}$ (13). However, in all enzymatic studies, $tRNA^{Asp}$ was the most preferred substrate for Dnmt2. The molecular basis for the specific interaction of Dnmt2 with its tRNA substrates is not yet known. Previously, we have mapped the binding of $tRNA^{Asp}$ to human DNMT2 by mutating several conserved lysine and arginine residues in human DNMT2, and reported that the anticodon stem/loop of $tRNA^{Asp}$ is a main region of contact for human DNMT2 for the methyl transfer reaction by human DNMT2 (23). Supporting this notion, Müller et al. (2013) proposed a role of the C32, A37, and C40 nucleotides in tRNA recognition (15).

A recent phylogenetic analysis revealed that the Dnmt2 gene family most likely was derived from a prokaryotic DNA-(cytosine C5)-methyltransferase (9). As mentioned above, the enzyme is highly conserved in all eukaryotic phyla. Strikingly, there are only few putative Dnmt2 homologs in bacteria, one of which is found in *Geobacter* species (6,9,24), another putative representative is present in *Holophaga foetida* (Suppl. Fig. 1). These enzymes are clearly defined by the presence of a CFT motif (CFI in *Holophaga*), which is characteristic for Dnmt2 enzymes and involved in the tRNA interaction (9,10). A bioinformatics analysis indicated that the *Geobacter* Dnmt2 enzyme likely is the outcome of a horizontal gene transfer from eukaryotes to *Geobacter* (9), which is known for its ability of lateral gene transfer (25,26). This conclusion is supported by the additional identification of a putative Dnmt2 homolog in *Holophaga*, because these bacteria are not closely related to *Geobacter* (*Holophaga* is a member of the Fibrobacteres/Acidobacteria group while *Geobacter* belongs to the δ-Proteobacteria).

As part of a larger initiative aiming to investigate the function of Dnmt2 in many species including humans, mice, flies, and unicellular eukaryotes and to define its biological role, we set out to investigate the biochemical properties and substrate recognition of *Geobacter sulfurreducens* Dnmt2 (GsDnmt2). Since it has been shown that eukaryotic Dnmt2 enzymes are specific for $tRNA^{Asp}$ C38 methylation, our work was started in this direction. However, we surprisingly found that GsDnmt2 specifically methylates *Geobacter* $tRNA^{Glu}$ *in vitro* and *in vivo* and not *Geobacter* $tRNA^{Asp}$. This represents the first example of a Dnmt2 enzyme with a completely switched substrate specificity. Based on this finding, we further identify the variable loop as a sequence determinant in tRNA recognition of *Geobacter* Dnmt2 and the human enzyme.

Materials and methods

Cloning and site-directed mutagenesis

The gene for *G. sulfurreducens*, GsDnmt2 (GSU0227), was amplified from genomic DNA of *G. sulfurreducens* obtained from DSMZ (Braunschweig, Germany) using a primer set flanking the gene (forward 5'-GCC GCA TAT GAG GGC GGT CGA GCT CTT CTG-3' and reverse 5'-GAT CGG AAT TCT CAC CCC TCC TCA GCC GGT AAC-3'). The amplified gene was cloned into pET28a(+) using NdeI and EcoRI sites and successful cloning validated by DNA sequencing. The catalytic site variant C74A of GsDnmt2 was created by the megaprimer site-directed mutagenesis method as described previously

(27). This residue corresponds to C79 in human DNMT2, which is a key catalytic residue, mutation of which has been shown to inactivate the enzyme (10). The primer used for the site-directed mutagenesis was 5'-GTA AGG CTG CGC AGG AGG CGA C-3'. The presence of the desired mutation was confirmed by sequencing. Constructs for other Dnmt2s are as described: human DNMT2 (28), *S. pombe* Pmt1 (16), *D. discoideum DnmA* (15). The gene for *D. melanogaster* Dnmt2 was amplified from cDNA using the following primer pair: 5'-GCG GTG GTG CTC GAG TTA TTT TAT CGT CAG-3' and 5'-GCG GCA GCC ATA TGG TAT TTC GGG TCT TAG AA-3', and cloned into pET28a+ using XhoI and NdeI sites. The cDNA was prepared using RNA isolated from 0-6 hour old embryos of *Drosophila melanogaster* BerlinWild strain. The cloned sequence was found to contain an insertion present in the Isoform A of the *D. melanogaster* Dnmt2 gene, which was subsequently excised by PCR mutagenesis performed with the following primers: 5'-ATG CCC AAT TGG ATG GAC AAA TAG TTG CCG CCT TGG-3' and 5'-ACT AAA TAG TTC TAA GAC CCG AAA TAC CAT-3'.

Protein expression and purification

The wild type and mutant C74A GsDnmt2 were expressed as His_6-tagged proteins in *E. coli* Rosetta 2 (DE3) cells. Protein expression and purification was performed as described previously (28). Briefly, transformed Rosetta 2 (DE3) cells were grown to OD_{600} = 0.6. Protein expression was induced with 1 mM IPTG and cells were further incubated at 37°C for 3 hours. The cells were sonicated in lysis buffer (30 mM potassium phosphate pH 7.5, 500 mM KCl, 0.1 mM DTT, 10 mM imidazole and 10% glycerol). Recombinant proteins were purified on Ni-NTA beads (Genoxon) and eluted with 200 mM imidazole in lysis buffer. The protein was then dialysed against dialysis buffer I (30 mM potassium phosphate pH 7.5, 300 mM KCl, 10% glycerol, 1 mM EDTA, 0.1 mM DTT) and dialysis buffer II (30 mM potassium phosphate pH 7.5, 100 mM KCl, 60% glycerol, 1 mM EDTA, 0.1 mM DTT). A similar procedure was followed for human DNMT2 purification. For *D. melanogaster* Dnmt2, the protein expression was conducted at 28°C. For the DnmA recombinant protein, expression was carried out at 22°C. The amounts of all recombinant proteins were determined by its specific absorbance and the concentrations and purities were verified by Coomassie-stained SDS-PAGE gels.

In vitro transcription of tRNA substrates

The tRNA sequences were derived from the Genomic tRNA data base (http://gtrnadb.ucsc.edu/) (29). The tRNA substrates for methylation reactions were prepared by *in vitro* transcription basically as described (10). The DNA templates for the *in*

vitro transcription were purchased from MWG. The template DNA and primer sequences are given in Supplemental Table 2. The templates were amplified by PCR with T7 and tRNA-specific primers and the yield and purity of the PCR was inspected on a native 8% acrylamide-TPE gels. *In vitro* transcriptions were performed in a total volume of 200 µL containing 50 µL of 4x transcription buffer (200 mM Tris-HCl pH 8.1, 5 mM spermidine, 25 mM DTT, 0.05% TritonX-100, 50 mM $MgCl_2$ and 1 mg/mL BSA), 20 mM NTPs mix, 5 µL of RNasin plus (Promega), 10 µL of T7 RNA polymerase (Fermentas), and 35 µL of PCR amplified template DNA. The reaction was incubated at 37°C for at least 3 hours, followed by DNase I treatment for another 30 min at 37°C. Then tRNAs were separated on a 12% acylamide / 7 M urea gel and the tRNA bands were excised from the gel (Fig. 1b). The tRNA was eluted with elution buffer (50 mM Tris-HCl pH 7.5, 300 mM sodium acetate, 0.5% SDS) overnight at room temperature followed by ethanol precipitation.

In vitro *methylation kinetics*

The *in vitro* methylation reactions of tRNAs were carried out as described before (30) with few modifications. Before the methylation reactions, the substrate tRNAs were refolded by heating to 65°C and slowly cooled to ambient temperature in the presence of 2 mM $MgCl_2$ and 2 U of RNasin plus (Promega). The subsequent methylation reaction was performed with 0.5 µM of the tRNA substrates and 1 µM of the Dnmt2 enzyme in methylation buffer (20 mM Tris-HCl (pH 8), 20 mM NH_4OAc, 2 mM $MgCl_2$, 2 mM DTT, 0.02 mM EDTA). The reactions were started by addition of 0.76 µM [methyl ^{3}H]-AdoMet (Perkin Elmer) and carried out at 22°C with DnmA, 30°C with Drosophila Dnmt2, and 37°C with all other enzymes. The reactions were stopped at different time points by removing 5 µL aliquots and pipetting them into 500 µl of 5% TCA. The samples were then spotted onto a DE81 anion exchange filter paper disc (0.20 mm thick and 1.5 cm in diameter) and the unbound radioactive AdoMet was washed away with 5% TCA and absolute ethanol. The discs were then air dried and the bound radioactivity was measured with a Hidex 300 SL liquid scintillation counter (Hidex, Finland) using Rotiszint eco plus (Roth, Germany). The reaction progress curves of the single turnover kinetics were fitted using eq. 1 which is derived from a single exponential model:

$$CMP_{theo}(t) = f\left(1 - e^{-k_1 t}\right) \qquad \text{eq. 1}$$

With: k_1, single turnover rate constant of RNA methylation [min^{-1}]; f, saturation level of complete methylation [CPM]. To obtain the initial rate of RNA methylation in CMP/min (v_0), eq. 1 was differentiated at t=0, which results in eq. 2:

$$v_0 = f \times k_1 \qquad \text{eq. 2}$$

In many reactions with less active enzyme / substrates combinations a clear linear initial phase was observed. Then, the initial rate constant of RNA methylation was derived directly by linear regression of the reaction progress curve.

tRNA binding analysis

The tRNA binding analysis was performed using 3' end- pCp labelled tRNAs. For labelling, 1 µg of the in vitro transcribed Gs-tRNA^{Asp} and Gs-tRNA^{Glu} were incubated with [5'-P^{32}] Cytidine 3',5'-bisphosphate (pCp) (Hartmann Analytic) and T4 RNA ligase (NEB) in the presence of ligation buffer (50 mM Tris-HCl pH 7.5; 10 mM $MgCl_2$; 1 mM DTT; 1 mM ATP) and 10% DMSO overnight at 4° C. The labelled tRNAs were purified from unincorporated nucleotides by using Micro-Bio-Spin 6 columns (BioRad). The purified tRNAs were refolded and incubated with increasing Dnmt2 concentrations in methylation buffer. After 30 minutes the reactions were spotted onto a nitrocellulose membrane and washed three times with the reaction buffer. The membranes were exposed to X-ray films and the intensity of the spots were measured using ImageJ software. Data were fitted using the Excel solver module to an equation describing a bimolecular equilibrium binding.

Total RNA isolation from G. sulfurreducens

G. sulfurreducens was grown anaerobically (N_2:CO_2 80:20) at 30°C in NBAF medium with acetate (15 mM) as the electron donor and fumarate (40 mM) as the electron acceptor as previously described (31). Total RNA isolation from *G. sulfurreducens* was carried out with the MasterPure RNA purification kit (Epicentre) following the manufacturer's instructions. The isolated total RNA was treated with DNase I to remove contaminant genomic DNA. RNA concentrations were determined by UV spectroscopy.

tRNA bisulfite sequencing

The tRNA bisulfite sequencing was performed as described previously (32). For this study, tRNA^{Asp}, tRNA^{Glu}, tRNA^{Glu-2} and tRNA^{Val} were selected because they contain cytosine at position 38 in their sequence. Briefly, 3 µg of total RNA was treated with bisulfite following the instructions of the manufacturer (EpiTect Bisulfite Kit, Qiagen). The bisulfite treated RNA was later used to generate a cDNA by reverse transcription using a stem loop primer (5′-CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG TGG TAA CAA AATC-3', 5′-CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG TGG TAA TAA TCCC-3', and 5′-CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG TGG TAA TAA ACAC -3') for tRNA^{Asp}, tRNA^{Glu} and tRNA^{Val}, respectively. The RT-PCR product was further amplified by PCR using a universal stem loop primer 5′-CAC

GAC ACC AGT TGA-3′ and deaminated tRNA specific primers with the following sequence: 5′-TAG TTA AGT TGG TTAT-3′, 5′- GTT TAG TGG TTA GGA-3′, and 5′-TTA GTT TAG TGG GAGA-3′ for $tRNA^{Asp}$, $tRNA^{Glu}$ and $tRNA^{Val}$, respectively. The PCR products were cloned by TOPO-TA cloning (Invitrogen, Germany). Colony PCR was performed to confirm the presence of desired inserts. To determine the RNA methylation, 60 clones were sequenced for each tRNA (33).

Results

GsDnmt2 is an active enzyme capable of methylating C38 in $tRNA^{Asp}$

After successfully cloning the GsDnmt2 gene into the pET28a+ vector, expressing and purifying the protein (Suppl. Fig. 3), its activity was tested using in vitro transcribed tRNAs (Suppl. Fig. 4). It was known from many previous reports that Dnmt2 from all the eukaryotic organisms methylate $tRNA^{Asp}$ (6,10,12-16). To test whether the bacterial Dnmt2 also methylates $tRNA^{Asp}$, *in vitro* transcribed $tRNA^{Asp}$ of *D. melanogaster* was used as the initial substrate. Since tRNA methylation rate of human DNMT2 is in the range of few turnovers per hour (10) and the Dnmt2 enzymes from other species studied here showed similar activity, it was not possible to conduct steady state methylation experiments. Therefore, we followed the initial time course of tRNA methylation reactions under single turnover conditions using excess of enzyme. As shown in Fig. 1A and C, wild type GsDnmt2 methylated $tRNA^{Asp}$ while no activity was detected with the active site mutant (C74A) in which the catalytic cysteine 74 residue, which is located in a highly conserved PCQ motif in Dnmts, was altered to alanine. The corresponding exchange in human DNMT2 has been shown to inactivate the enzyme as well (10). The observation that the C74A variant lost the methylation activity indicates that GsDnmt2 is an active tRNA methyltransferase and capable of methylating *in vitro* transcribed *D. melanogaster* $tRNA^{Asp}$. The next aim was to confirm that the site of methylation by GsDnmt2 is the same as by other members of the Dnmt2 family. For this, a C38U mutant of the *D. melanogaster* $tRNA^{Asp}$ was prepared and methylation reactions were performed with the wild type GsDnmt2 enzyme. A complete loss of methylation was observed with this mutant tRNA, indicating that cytosine 38 is the methylation target site for GsDnmt2. Finally, methylation of murine $tRNA^{Asp}$ by GsDnmt2 was demonstrated as well. In summary, these data indicate that GsDnmt2 methylates $tRNA^{Asp}$ from *D. melanogaster* and *M. musculus* at C38.

GsDnmt2 is weakly active on *Geobacter*-encoded $tRNA^{Asp}$

After confirmation of the activity of GsDnmt2 on tRNA^{Asp} derived from flies and mice, the catalytic activity of this enzyme on *Geobacter*-encoded tRNA^{Asp} was investigated, which shares large similarity with eukaryotic tRNA^{Asp} (Suppl. Fig. 2). For this, *G. sulfurreducens* tRNA^{Asp} (Gs-tRNA^{Asp}) was prepared by *in vitro* transcription and an *in vitro* methylation was performed using the recombinantly expressed wild type GsDnmt2. However, as shown in Fig. 1B and C, GsDnmt2 was only very weakly active on its cognate tRNA^{Asp}. No activity was observed with the C74A mutant protein, confirming that the radioactive signal observed was due to tRNA methylation by wild type GsDnmt2. In addition, the site of methylation was also confirmed to be cytosine 38 in Gs-tRNA^{Asp} by C38U mutation. The weak methylation of Gs-tRNA^{Asp} was not related to the preparation of the tRNA, because the same tRNA preparation was efficiently methylated by Dnmt2 enzymes from other species (see below, Fig. 4). In summary, when comparing methylation of tRNA^{Asp} from flies, mice, and *Geobacter*, GsDnmt2 unexpectedly showed the weakest methylation activity with its own *Geobacter*-encoded tRNA^{Asp}.

GsDnmt2 shows unusual preference for tRNA^{Glu} over tRNA^{Asp}

After the initial observation that *G. sulfurreducens* Dnmt2 showed lower methylation of its *Geobacter*-encoded tRNA^{Asp} than of tRNA^{Asp} from *Drosophila* or *M. musculus*, the question arose whether any other *Geobacter* tRNA might be more preferred as a substrate. To this end, *Geobacter* tRNAs were inspected for the presence of a cytosine at position 38 of the tRNA sequence. Using the UCSC Genomic tRNA Data Base (http://gtrnadb.ucsc.edu/) (29) we identified tRNA^{His} as well as two isodecoders of each tRNA^{Ala}, tRNA^{Glu}, and tRNA^{Val} as Dnmt2 candidate substrates in *Geobacter*, in addition to tRNA^{Asp}. Among them, both isodecoders of tRNA^{Glu} were selected for methylation analysis, because they show high similarity to tRNA^{Asp}, as well as tRNA^{His} and one of the tRNA^{Val} and tRNA^{Ala} sequences (Suppl. Table 1). All of these tRNAs were synthesised by *in vitro* transcription and assayed for methylation by GsDnmt2. Our results showed that tRNA^{Asp} was weakly methylated as stated above (Fig. 2), but the initial reaction rate for methylation of tRNA^{Glu} (tRNA32) was tenfold higher than methylation of tRNA^{Asp}. The methylation of the second isodecoder of tRNA^{Glu-2} (tRNA20) was intermediate between tRNA^{Asp} and the other tRNA^{Glu} and methylation of other tRNAs was not detectable (data not shown). These results motivated us to continue our study using the tRNA^{Glu} (tRNA32) isodecoder. A mutant tRNA^{Glu} carrying a C38U mutation was prepared and we observed that the mutation caused a complete loss of methylation, indicating that the methylation in tRNA^{Glu} happens only at position C38 (Fig. 2). In addition, the C74A mutation in GsDnmt2 led to loss of methylation on tRNA^{Glu} (Fig. 2). The preferential methylation of Gs-tRNA^{Glu} was

also confirmed using a gel based methylation assay (10) in which the methylated tRNA is directly observed (Suppl. Fig. 5).

To test if the substrate preferences of human and *Geobacter* Dnmt2 were based on preferential binding of one type of tRNA, we determined their equilibrium constants of binding to Gs-tRNA^{Glu} and Gs-tRNA^{Asp} (Suppl. Fig. 6). Our data show that the human enzyme binds tRNA about 4-5 times stronger than the *Geobacter* enzyme, which is in agreement with its general higher enzymatic activity (compare Fig. 2B and 4B). However, the binding of the two different substrates was similar in both cases, indicating that ground state tRNA binding does not contribute to specificity of tRNA methylation by Dnmt2. A similar observation has been made previously in a study aiming to identify amino acid residues involved in tRNA binding and recognition of human DNMT2 (23). We conclude that GsDnmt2 shows a striking change in substrate specificity when compared with other Dnmt2 enzymes, which is not based on tRNA binding preferences.

Cellular methylation of tRNAs in *G.sulfurreducens*

As described above, we have found that GsDnmt2 has a different specificity than other Dnmt2 enzymes *in vitro*. Therefore, the cellular methylation patterns of tRNAs in *G. sulfurreducens* were investigated. For this analysis, total RNA was isolated from wild type *G. sulfurreducens* and the cytosine methylation investigated by bisulfite conversion, reverse transcription and cloning of the converted tRNA^{Asp}, tRNA^{Glu} and tRNA^{Val} followed by sequencing of individual clones. As shown in Fig. 3, the methylation at the C38 position of tRNA^{Glu} in wild type *G. sulfurreducens* was 22%. The tRNA^{Glu-2} isodecoder was methylated by 36% at the same site. In contrast, signals for tRNA^{Asp} and tRNA^{Val} C38 methylation were at background levels of about 3%. Hence, both tRNA^{Glu} isodecoders were efficiently methylated *in vitro* and they are also methylated in cells, while tRNA^{Asp}, which showed the weakest *in vitro* methylation among all substrates tested, was not methylated in cells. We conclude that cellular tRNA methylation at C38 mirrors the *in vitro* specificity GsDnmt2. The same observation has been made with human DNMT2. In human cells tRNA^{Asp} but not tRNA^{Glu} is methylated at C38 (34,35), which is in agreement with the *in vitro* specificity of the human DNMT2 enzyme. The incomplete methylation of the tRNA^{Glu} in *Geobacter* is similar to what has been observed in for tRNA^{Asp} in other species by RNA bisulfite (16,32,34). It is possible that the reverse transcription step of the RNA bisulfite method is more efficient with unmodified, immature tRNAs, which may influence the results.

Comparison of methylation specificities of Dnmt2 homologs from various species

We concluded in the last paragraphs that GsDnmt2 strongly prefers to methylate Gs-tRNA^{Glu} not Gs-tRNA^{Asp} both *in vitro* and *in vivo*. However, as shown above, tRNA^{Asp} from other species like *D. melanogaster* and *M. musculus* were good GsDnmt2 substrates. This observation suggested that there may be changes in the *Geobacter* tRNAs that increase methylation of Gs-tRNA^{Glu} but reduce the reaction with Gs-tRNA^{Asp}. Hence, the specificities of Dnmt2 enzymes from other species were investigated for the methylation of *Geobacter* tRNA^{Asp} and tRNA^{Glu} also using the C38U mutant tRNA versions as controls (Fig. 4). Dnmt2 enzymes from different species which represent key eukaryotic phyla were expressed and purified (Suppl. Fig. 3) and their methylation activities were analysed. The assays were done in parallel for a specific enzyme with all tRNA substrates. The results showed that human DNMT2, *S. pombe* Pmt1 and *D. discoideum* DnmA, all preferred the Gs-tRNA^{Asp} substrate (Fig. 4). *D. melanogaster* Dnmt2 also showed some activity on Gs-tRNA^{Glu}, but it still had a clear preference for Gs-tRNA^{Asp} (Fig. 4). The C38U mutations completely abrogated the methylation activity for all the enzymes, confirming that methylation occurred at position C38. Although the results with the other Dnmt2 enzymes cannot be directly translated to their biological role, because the methylation was tested on *G. sulfurreducens* tRNAs, our data clearly demonstrate that the change in the biological target observed with GsDnmt2 is specific for this enzyme among all Dnmt2 enzymes tested here (Fig. 5). These results show that the swap in specificity of GsDnmt2 from Gs-tRNA^{Asp} to Gs-tRNA^{Glu} must be due to changes within the GsDnmt2 protein and the Gs-tRNAs which affect the interaction of GsDnmt2 with *Geobacter* encoded tRNAs. However, these alterations are only efficient when combined, because GsDnmt2 still methylates tRNA^{Asp} from other species and Dnmt2 enzymes from other species prefer methylating tRNA^{Asp} from *Geobacter*.

The variable loop functions as a sequence determinant in the tRNA recognition of Dnmt2

To investigate the molecular reason for the inversion of the substrate preference of the GsDnmt2 enzyme with its matching *Geobacter* tRNAs, we compared the secondary structures of tRNA^{Asp} and tRNA^{Glu} from several species and noticed that Gs-tRNA^{Asp} has a longer variable loop with an extra guanine (Fig. 6). Furthermore, the variable loops of all preferred Dnmt2 substrates (namely tRNA^{Asp} from all species but *Geobacter* and Gs-tRNA^{Glu}) have at least one A at position 45 or 46 of the variable loop, while all the inactive counterparts contain a GG dinucleotide. Based on this, we speculated that the variable loop may be a specificity determinant of GsDnmt2. To test this hypothesis, the variable

loop region of murine $tRNA^{Asp}$ was swapped with that of murine $tRNA^{Glu}$ by an exchange of two nucleotides in the variable loop (AGAC to CGGC). This exchange introduced the GG nucleoside sequence into mutant $tRNA^{Asp}$, which could perturb its methylation by Dnmt2. The mutant tRNA was synthesised by *in vitro* transcription. Equal amounts of the wild type and mutant tRNAs were used in methylation reactions with GsDnmt2 (Suppl. Fig. 4). Strikingly, the alteration of these two bases in the variable loop of murine $tRNA^{Asp}$ led to a strong decrease in the methylation rate (Fig. 7A). This result supports the hypothesis of an involvement of the variable loop in the enzyme-substrate recognition of GsDnmt2. To further extend this finding, methylation reactions were performed with the same substrates using the human DNMT2 enzyme. Here, also, a similar reduction of activity was observed with mutant tRNA compared to wild type (Fig. 7A), indicating that the variable loop is also important for $tRNA^{Asp}$ recognition by human DNMT2. Next we investigated if the variable loop of Gs-$tRNA^{Glu}$ also has a role in recognition. As shown in Fig. 7B, replacement of the original Gs-$tRNA^{Glu}$ variable loop by either Gs-$tRNA^{Asp}$ or murine $tRNA^{Glu}$ resulted in a strong reduction of its methylation by GsDnmt2.These data indicate that the GG dinucleotide in the variable loop functions as a universal anti-determinant for methylation by Dnmt2. However, replacing the variable loop of murine $tRNA^{Glu}$ with the corresponding loop from murine $tRNA^{Asp}$ did not result in a significant increase of its methylation by human DNMT2 (data not shown), indicating that the variable loop is not the only recognition determinant of Dnmt2.

Discussion

Dnmt2 enzymes are conserved in most eukaryotic species (6,9) and methylate $tRNA^{Asp}$ with high activity. Here, the activity of one of the few known bacterial putative Dnmt2 enzymes was studied, which is found in *Geobacter* strains including *G. sulfurreducens* (GsDnmt2). We show that GsDnmt2 actively methylates $tRNA^{Asp}$ from *M. musculus* and *Drosophila* that were used as model substrates in previous work. This result confirms that GsDnmt2 is a member of the Dnmt2 family as expected on the basis of the amino acid alignment. However, the endogenous *Geobacter* $tRNA^{Asp}$ was methylated only very inefficiently. Instead, GsDnmt2 methylates *Geobacter* $tRNA^{Glu}$ at the corresponding site, indicating a complete swap in its specificity (Fig. 5) - a surprising and unexpected result that was confirmed in a methylation analysis of tRNA isolated from *G. sulfurreducens*. There are two isodecoders of $tRNA^{Glu}$ in *Geobacter* (tRNA20, here called $tRNA^{Glu-2}$, and tRNA32, here called $tRNA^{Glu}$), which both were methylated *in vitro* and *in vivo*, although the relative methylation levels of the two isodecoders varied in these two data sets. *In vitro*, $tRNA^{Glu}$ was methylated 3 times faster while in cells the methylation

level of $tRNA^{Glu-2}$ was 1.6 times higher. There could be several explanations for this difference: i) $tRNA^{Glu-2}$ methylation could be less efficient under *in vitro* conditions, ii) additional modifications could modulate the $tRNA^{Glu}$ or $tRNA^{Glu-2}$ methylation *in vivo*, or iii) additional modifications could affect the recovery of methylated $tRNA^{Glu}$ and $tRNA^{Glu-2}$ differently after bisulfite conversion. However, in all data sets both isodecoders of Gs-$tRNA^{Glu}$ were more methylated than Gs-$tRNA^{Asp}$.

We have shown that *Geobacter* Dnmt2 has an about 100 fold swapped specificity for *Geobacter* tRNAs, because it prefers $tRNA^{Glu}$ roughly 10 fold over $tRNA^{Asp}$, while all other Dnmt2 enzymes showed an at least 10 fold preference for $tRNA^{Asp}$ over $tRNA^{Glu}$ (Fig. 5). What could be the molecular basis of the unexpected loss of *Geobacter* $tRNA^{Asp}$ methylation by GsDnmt2 and the corresponding gain of methylation of $tRNA^{Glu}$? So far, various amino acids involved in tRNA recognition of the human enzyme could be mapped (23), but the sequence elements needed on the tRNA side were largely unknown. Initially, the G34 base at the wobble position of the anticodon, which is modified to mannosylqueuosine in eukaryotic cells, was associated with Dnmt2 activity (6). However, the results with *G. sulfurreducens* are not in favour of that model, because Gs-$tRNA^{Asp}$ contains the G34, but it is a very weak substrate, while Gs-$tRNA^{Glu}$, which is the preferred substrate, does not have a G34. By comparing substrate and non-substrate tRNAs of GsDnmt2, it became apparent that all non-substrates contain a GG dinucleotide in the variable loop as a characteristic feature. Indeed, introduction of this sequence into murine $tRNA^{Asp}$ and *Geobacter* $tRNA^{Glu}$ drastically reduced their methylation by GsDnmt2. We cannot rule out the possibility that the two point exchanges in the variable loop might disrupt tRNA folding. However, the initial discovery that the GG dinucleotide is inhibiting GsDnmt2 was made with natural tRNAs, which are all fully functional in protein biosynthesis, which rules out loss of structure in these cases. Therefore, when taken together, our results strongly suggest that the variable loop functions as an important specificity determinant in GsDnmt2. Interestingly, the exchange in the murine $tRNA^{Asp}$ also reduced its methylation by the human DNMT2, indicating that the variable loop is a critical specificity determinant for the tRNA recognition of human DNMT2 as well. However, introduction of the favourable variable loop into murine $tRNA^{Glu}$ did not increase its methylation. Hence, our data indicate that Dnmt2 enzymes use further tRNA-specific sequence determinants in addition to the variable loop. These (so far unknown) contacts mediate the preference of the human DNMT2 enzyme for Gs-$tRNA^{Asp}$, despite the presence of the unfavourable variable loop. Furthermore, they prevent methylation of murine $tRNA^{Glu}$, even after introducing a favourable variable loop. The contribution of additional specificity determinants (besides the GG dinucleotide in the variable loop) to

tRNA recognition by Dnmt2 enzymes is also supported by the observation that Dnmt2 enzymes from other species still strongly prefer Gs-tRNA^{Asp} over Gs-tRNA^{Glu}.

The role of the variable loop as specificity determinant in tRNA recognition by Dnmt2 is not without precedence. Examples for tRNA interacting enzymes, which use the variable loop for tRNA recognition include a bacterial tRNA (m7G46) methyltransferase (36), a prokaryotic tRNA-dependent amidotransferase (37) and the same strategy is also used by several aminoacyl-tRNA-synthetases (38). Recognition of nucleic acids by proteins, in general, is based on direct and indirect readout, i.e. direct contacts of amino acids to nucleobases, which detect the nature of the base, and contacts to the nucleic acid backbone, which detect the structure of the nucleic acid. The GG dinucleotide in the variable loop of the tRNA could interfere with either of these processes. However, the observation that the variable loop of preferred tRNA substrates is only characterized by the absence of the GG dinucleotide but it does not contain a specific nucleotide sequence, might argue against direct base contacts. This suggests that an indirect readout mechanism is operational in which the presence of the GG dinucleotide induces a structural change in the tRNA which then prevents its methylation.

The finding that GsDnmt2 methylates tRNA^{Asp} from other species (as all other members of the Dnmt2 family do) but not from *Geobacter*, provides strong support for the hypothesis that the Gs-Dnmt2 was acquired by horizontal gene transfer, because there is no reason for this enzyme to methylate tRNA^{Asp}, which is not a substrate in *Geobacter* itself. In the horizontal gene transfer view, the activity towards tRNA^{Asp} can be interpreted as an evolutionary relict. Based on our data, one may speculate that initially a Dnmt2 gene with tRNA^{Asp} preference, but also the ability to methylate tRNA^{Glu}, was acquired by *Geobacter*. Precedence for such dual activity can be seen in many species including *D. melanogaster, S. pombe* and *D. discoideum* (13,15,16). Methylation of the Gs-tRNA^{Glu} was favourable, which led to the stable integration of the Dnmt2, whereas methylation of the endogenous tRNA^{Asp} was lost in a co-evolution of GsDnmt2 and the Gs-tRNA^{Asp}. At present, one can only speculate about the role of Dnmt2 in *Geobacter*. In previous studies, tRNA methylation by Dnmt2 was found to improve the stability of the tRNA (13) or affect tRNA charging (Shanmugam et al., manuscript in preparation), and prevent tRNA fragmentation (13). Which of these roles, if any, is important for *Geobacter* tRNA^{Glu} cannot be said at present. The generation of a Dnmt2 knock-out strain was unsuccessful (M. Aklujkar, unpublished information), which may indicate that Dnmt2 is an important gene in *G. sulfurreducens*, which is in line with the evolutionary arguments. This would be an

interesting finding, since Dnmt2 enzymes are non-essential in other model organisms under normal growth conditions (6,12,13,15,21).

Conclusions

The first enzymatic characterisation of the specificity of a bacterial Dnmt2 homolog, the enzyme from *G. sulfurreducens*, revealed that it is the only known Dnmt2 enzyme that does not prefer methylation of $tRNA^{Asp}$. Instead, an unexpected swap of specificity of this enzyme was observed towards methylation of *Geobacter* $tRNA^{Glu}$, which was confirmed *in vitro* and *in vivo*. However, GsDnmt2 still methylates $tRNA^{Asp}$ from other species, and Dnmt2 enzymes from other species still methylate *Geobacter* $tRNA^{Asp}$. We provide an evolutionary scenario that can explain this interesting finding. We noticed that the *Geobacter* $tRNA^{Asp}$ has a modified variable loop, which serves as specificity determinant and causes the loss of methylation activity. Furthermore, we show that this mechanism could be extended to the human enzyme as well, indicating that work with a bacterial Dnmt2 homolog, finally, has led to important insight into the mechanism of the corresponding human enzyme.

Supplementary data are available at NAR online.

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Figure legends

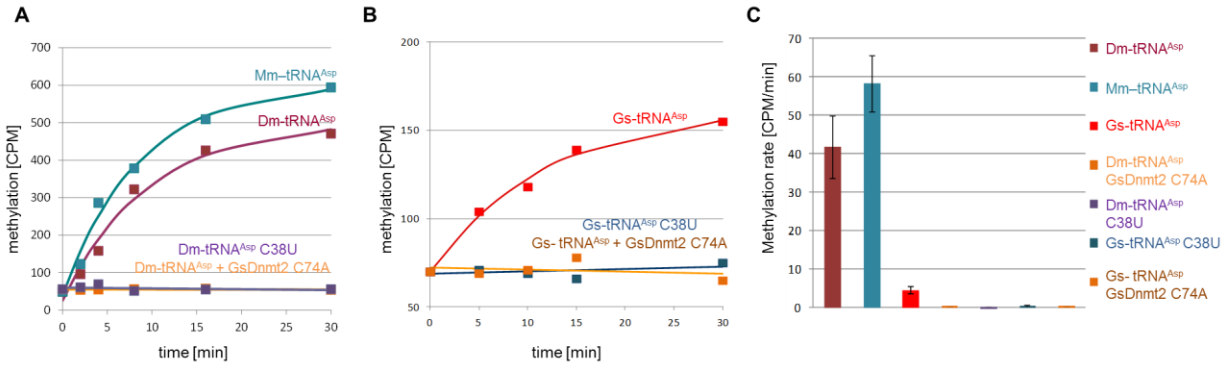


Figure 1: *G. sulfurreducens* Dnmt2 methylates C38 of tRNA^{Asp}. A) Methylation of *D. melanogaster* tRNA^{Asp} and murine-tRNA^{Asp} with the GsDnmt2 enzyme. Control reactions using the catalytically inactive GsDnmt2 mutant C74A and Dm-tRNA^{Asp} C38U mutant show no methylation. B) Methylation of tRNA^{Asp} from *G. sulfurreducens* with GsDnmt2 showing weak methylation. Control reactions with the C38U mutant of the tRNA^{Asp} or the catalytically inactive C74A variant of GsDnmt2 showed no methylation signal. C) Summary of the tRNA methylation data reporting average methylation rates and SEM taken from 2-3 independent experiments.

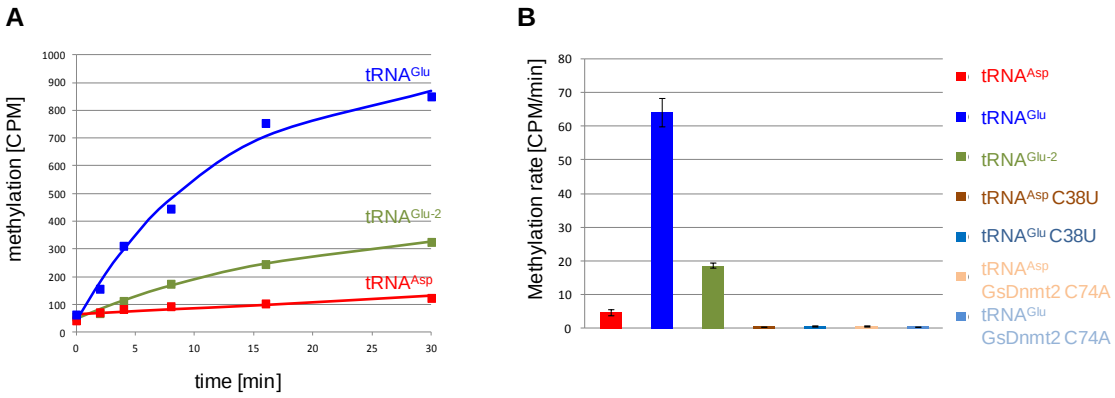


Figure 2: GsDnmt2 prefers methylation of *G. sulfurreducens* $tRNA^{Glu}$ over $tRNA^{Asp}$. A) Examples of *in vitro* methylation of *Geobacter* $tRNA^{Asp}$ and $tRNA^{Glu}$ by GsDnmt2. B) Average methylation rates and SEM based on three repeats of the experiments. Panel B) also includes results of control reactions with $tRNA^{Asp}$ and $tRNA^{Glu}$ C38U variants and with and the catalytically inactive C74A GsDnmt2 protein. The error bars indicates the SEM from at least 3 different experimental repeats.

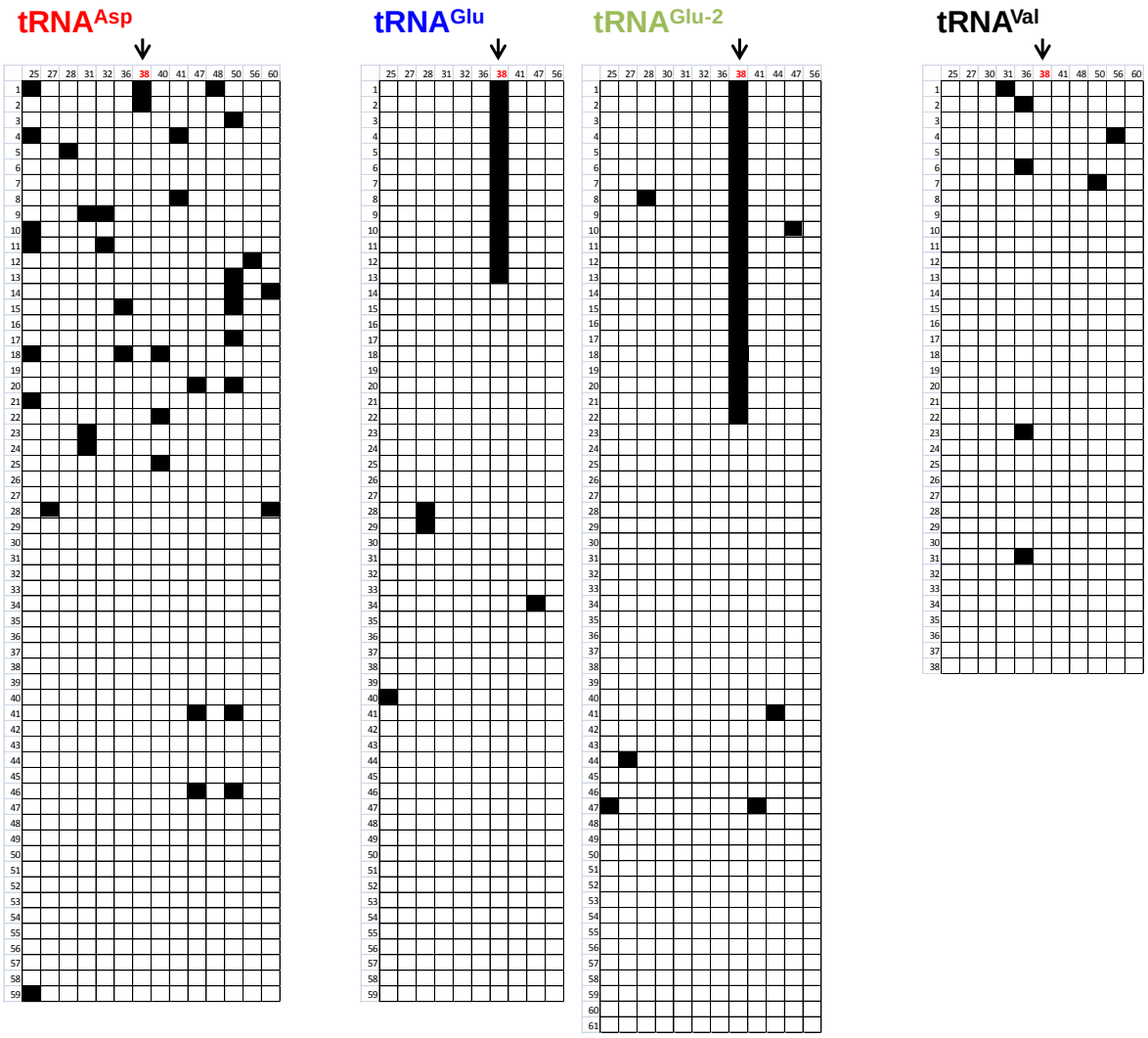


Figure 3: tRNA^{Glu} but not tRNA^{Asp} is methylated at C38 in *G. sulfurreducens*. The figure shows results of RNA bisulfite sequencing of tRNA^{Asp}, tRNA^{Glu}, tRNA^{Glu-2},and tRNA^{Val} from total RNA isolated from *G. sulfurreducens*. The cytosine residues present in the corresponding tRNA are indicated in the top row. Each subsequent row represents an independent clone that was sequenced. Black boxes indicate methylated cytosines, and white boxes indicate unmethylated cytosines. The arrows highlight the C38 positions.

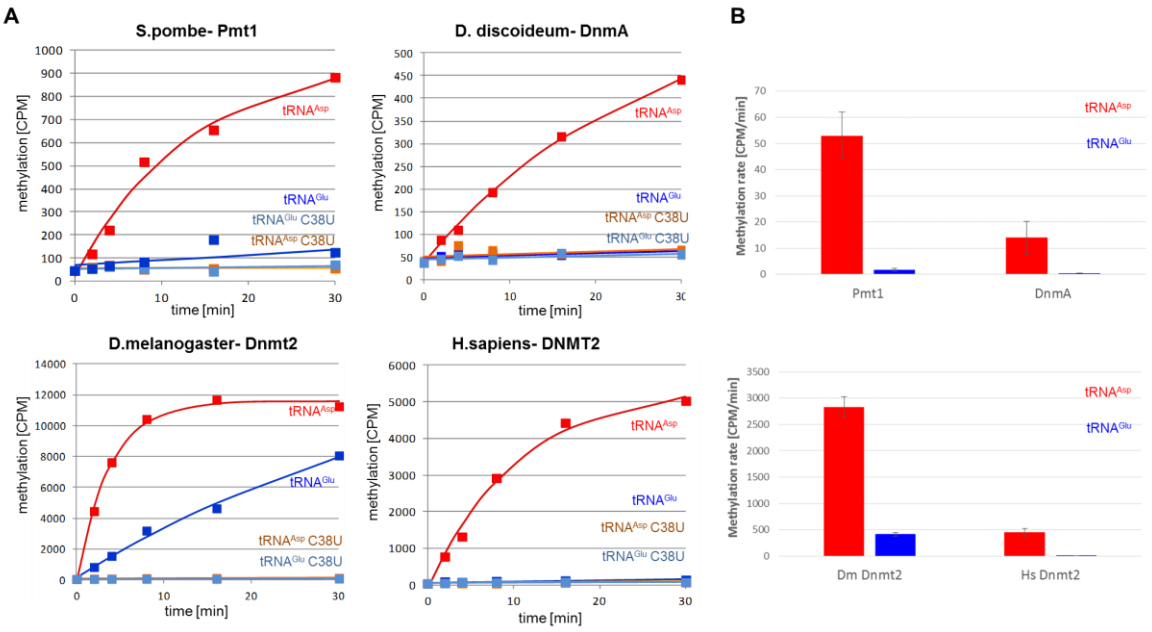


Figure 4: Dnmt2 enzymes from various species prefer methylation of *G. sulfurreducens* $tRNA^{Asp}$. A) Examples of methylation kinetics are shown for wild type Dnmt2 enzymes from *S. pombe* (Pmt1), *D. discoideum* (DnmA), *D. melanogaster* and *H. sapiens* using *in vitro* transcribed Gs-$tRNA^{Asp}$ and Gs-$tRNA^{Glu}$ and their respective C38U variants. B) Average methylation rates and SEM based on two repeats of the experiments.

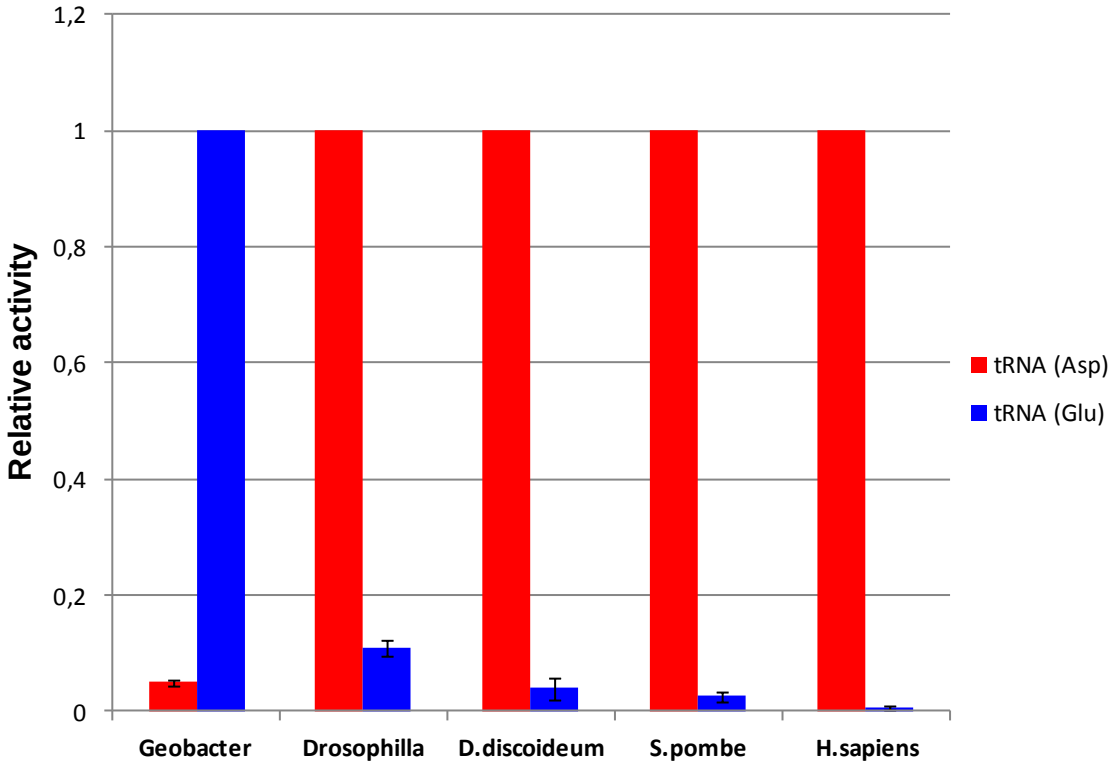


Figure 5: Comparison of the specificities of Dnmt2 homologs from different species for methylation of *Geobacter* $tRNA^{Asp}$ and $tRNA^{Glu}$. Data are replotted from Figs. 2B and 4B and the relative activities of the Asp/Glu pairs were always normalized to the more active substrate for better comparison. Error bars were based on normalized individual experiments and correspond to the SEM of 2-3 repeats.

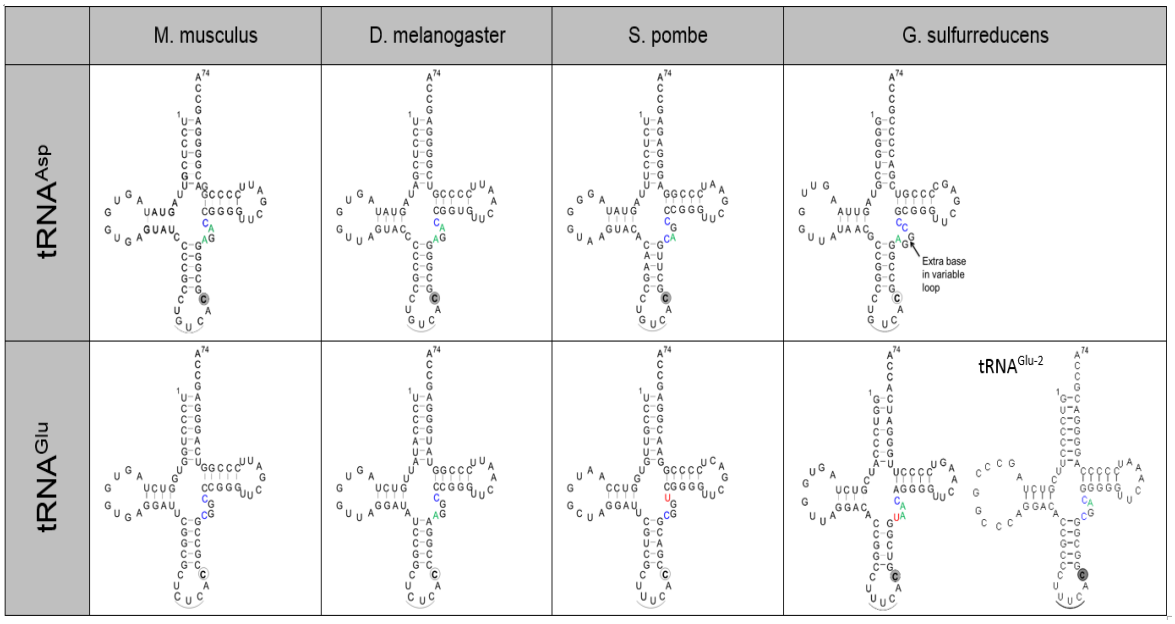


Figure 6: Clover leaf structure of the $tRNA^{Asp}$ and $tRNA^{Glu}$ from different species. The target C38 is printed in bold and highlighted by a grey circle for the preferred substrate tRNA and white circle for the non-preferred one. Bases in the variable loop are colored. The sequences of human $tRNA^{Asp}$ and $tRNA^{Glu}$ are identical to that of murine-$tRNA^{Asp}$ and $tRNA^{Glu}$.

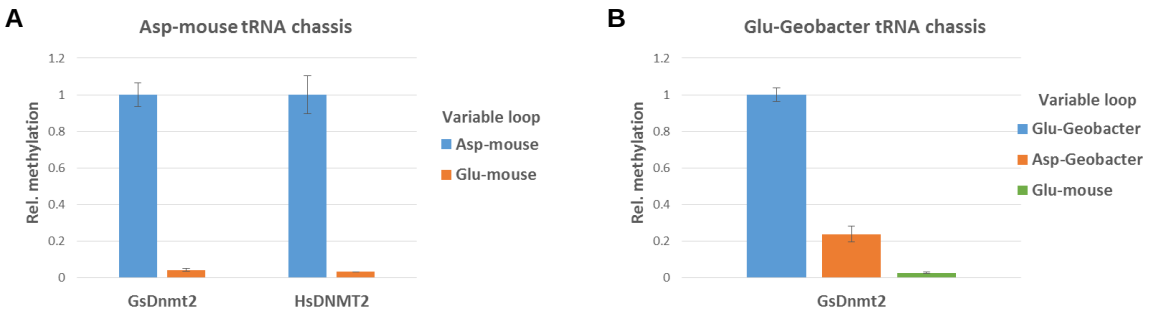
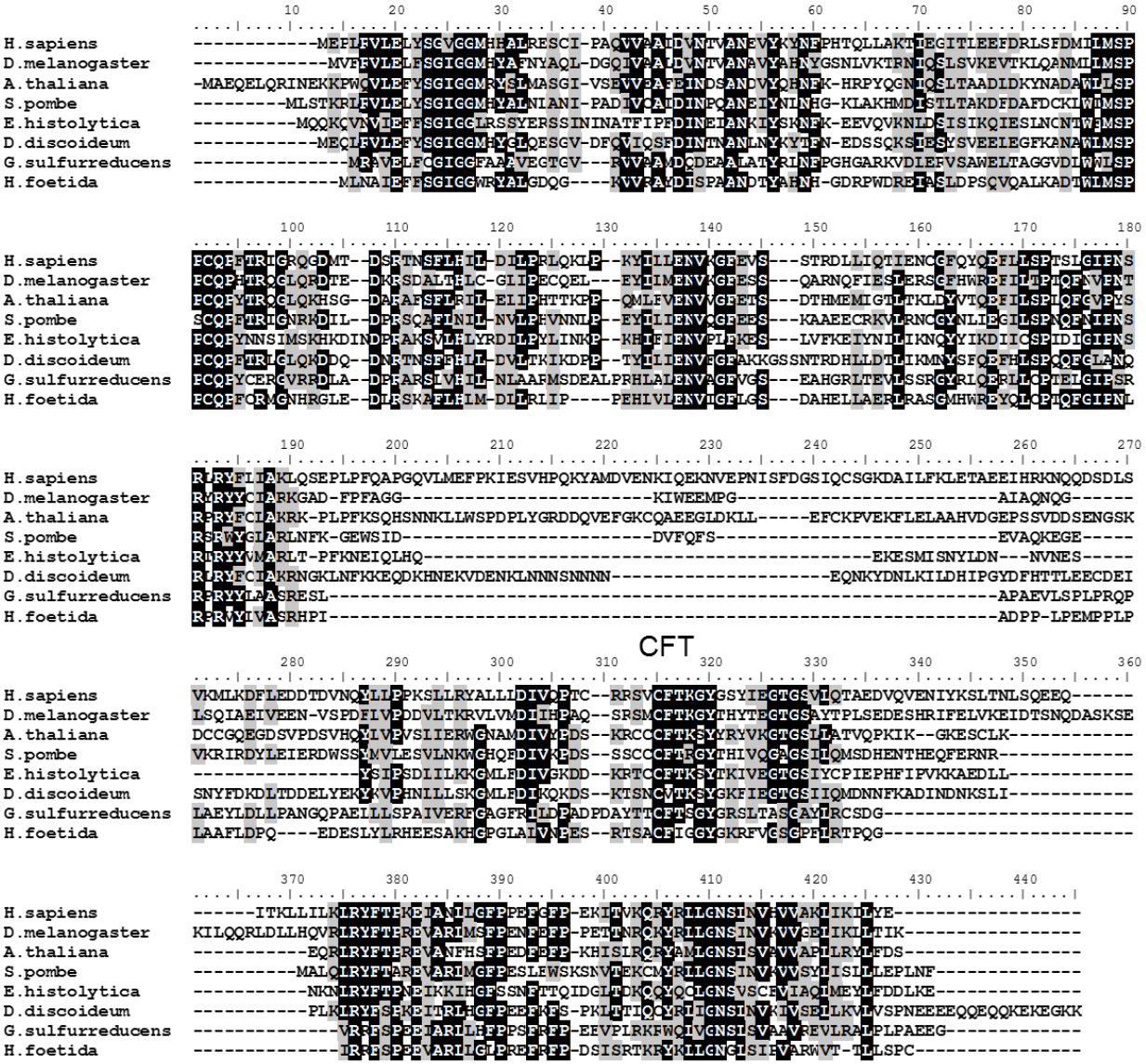


Figure 7: Methylation of tRNA variable loop mutants by GsDnmt2 and human DNMT2. A) Methylation of murine-tRNA^{Asp} with its original variable loop or with the variable loop of murine-tRNA^{Glu} by *Geobacter* Dnmt2 (left pair of bars) or human DNMT2 (right pair of bars). B) GsDnmt2 methylation of Gs-tRNA^{Glu} with its original variable loop or with variable loops from Gs-tRNA^{Asp} or murine-tRNA^{Glu}. The figure shows averages of the methylation rates normalized to the wild type tRNA. Error bars indicate SEM derived from two repeats of the experiments.

The Dnmt2 RNA methyltransferase homolog of *Geobacter sulfurreducens* specifically methylates tRNA-Glu

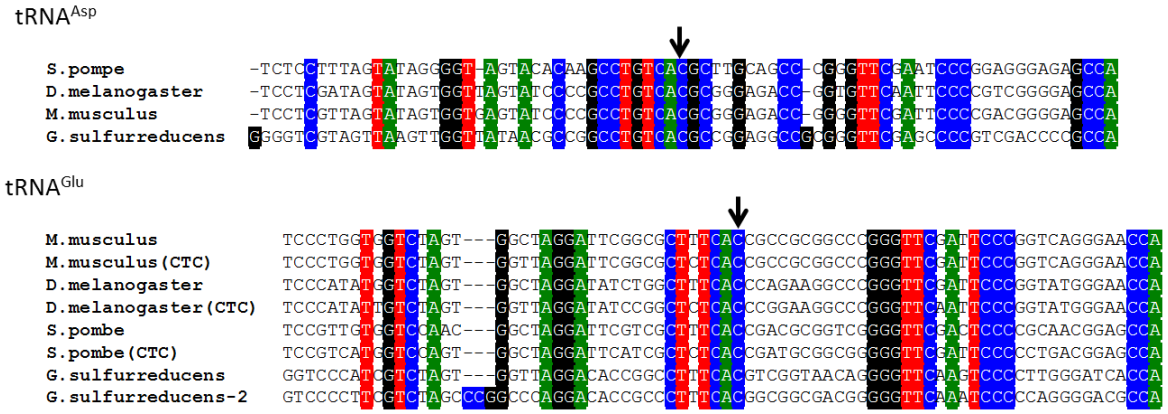
Supplemental information

Suppl. Fig. 1: Amino acid alignment of Dnmt2 enzymes from different species.

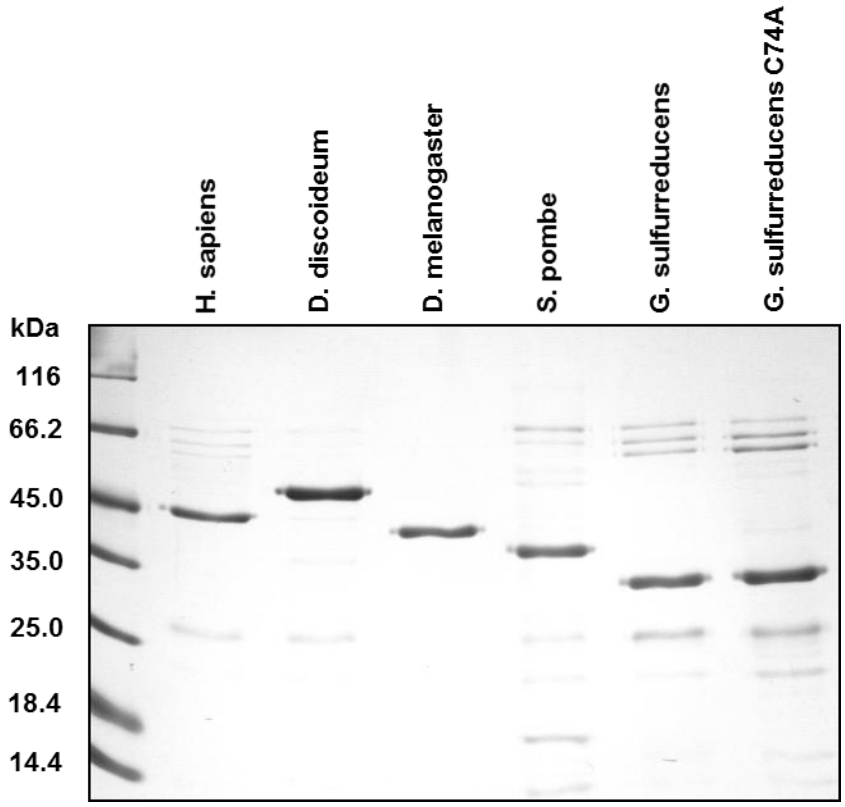


The putative Dnmt2 homolog from *Geobacter sulfurreducens* contains a CFT motif which is characteristic for Dnmt2 enzymes (highlighted), the putative Dnmt2 homology in *Holophaga foetida* contains a related CFI motif.

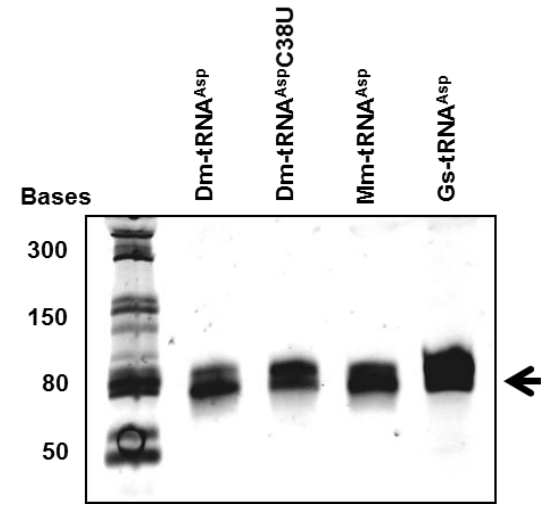
Suppl. Fig. 2: Alignment of the tRNA^{Asp} and tRNA^{Glu} from different species. The C38 position is highlighted with an arrow.

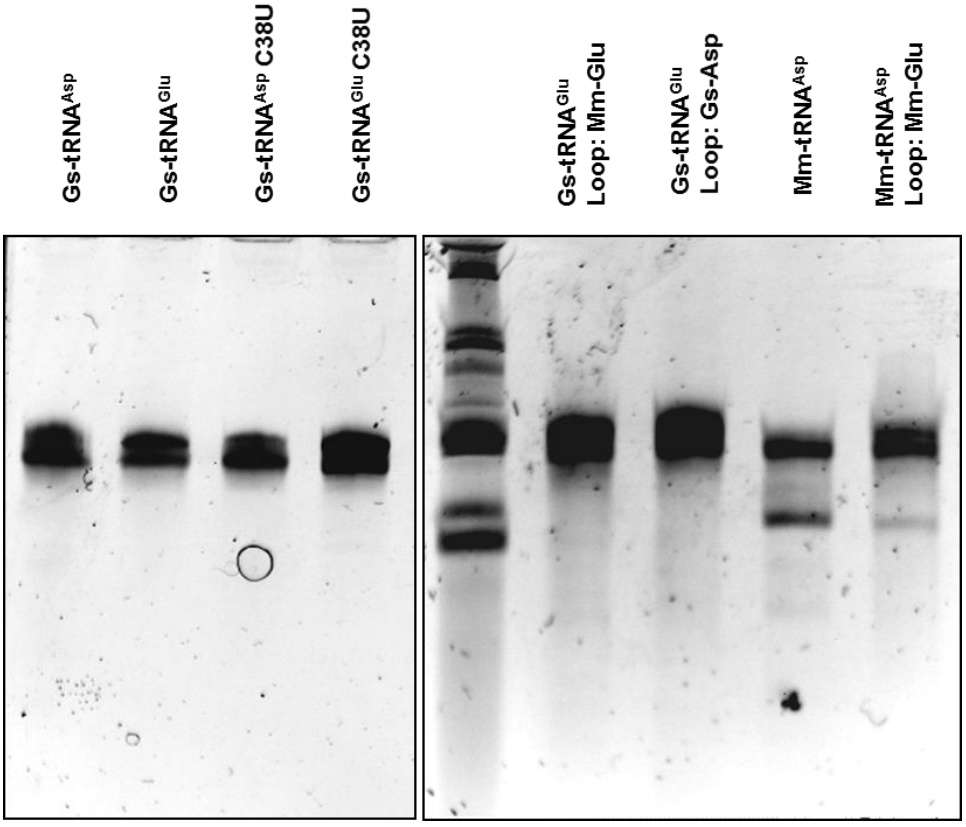


Suppl. Fig. 3: Coomassie-stained protein gel showing examples of the purification of the different Dnmt2 enyzmes used in the kinetic analysis.

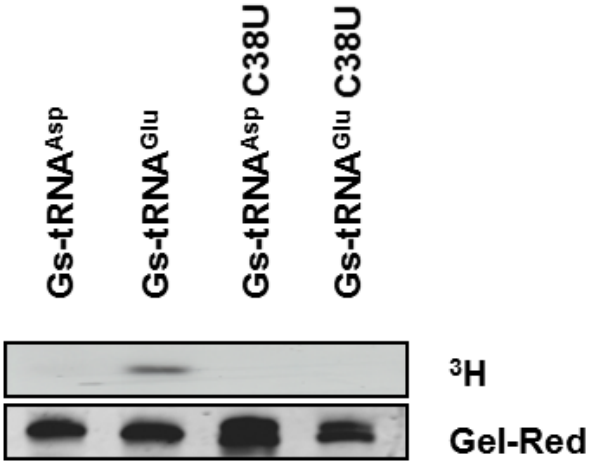


Suppl. Figure 4: Images of Gel Red-stained polyacrylamide gels showing examples of the substrate tRNAs used in this study.

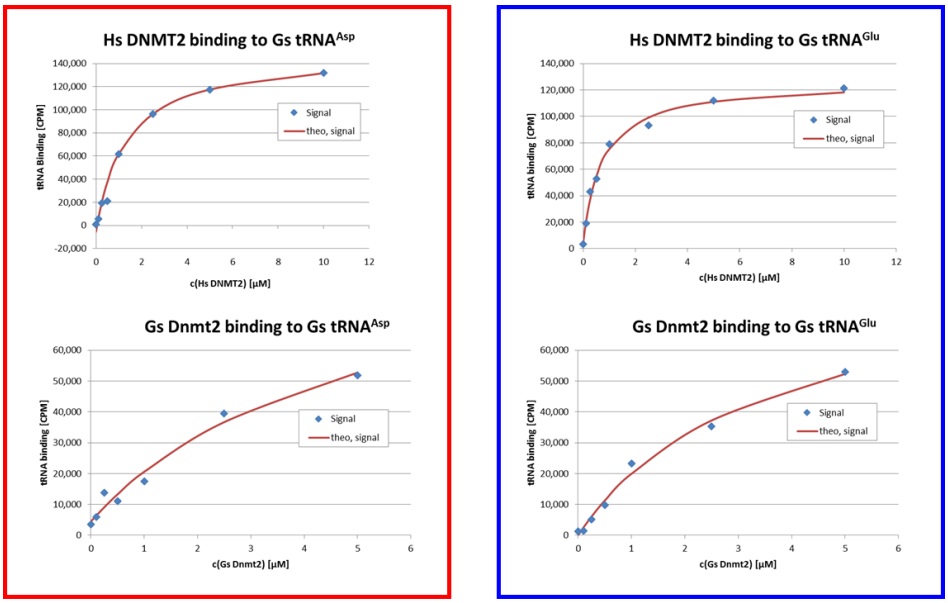


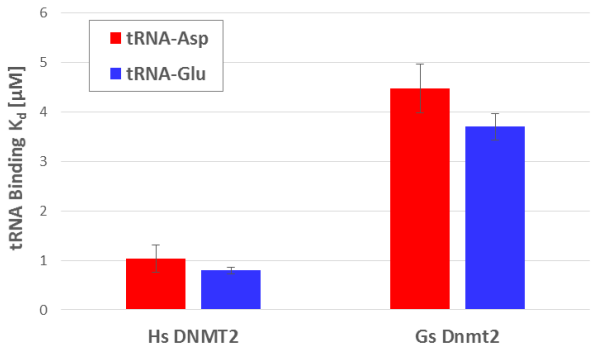


Suppl. Fig. 5: GsDnmt2 prefers methylation of *G. sulfurreducens* $tRNA^{Glu}$ over $tRNA^{Asp}$. *In vitro* methylation of Gs-$tRNA^{Asp}$ and Gs-$tRNA^{Glu}$ from were performed using 1 µM of *G. sulfurreducens* Dnmt2 and 0.5 µM of substrate tRNAs. The corresponding C38U tRNA mutants were used as controls. The upper gel picture shows the autoradiography image observed after 1 week of exposure. The lower part of the figures shows the corresponding tRNA bands stained with Gel-Red.



Suppl. Fig. 6: Geobacter $tRNA^{Asp}$ (red) and $tRNA^{Glu}$ (blue) binding by human and *Geobacter* Dnmt2. The tRNA binding was analysed using radioactively labelled tRNAs that were incubated with increasing concentrations of Dnmt2. After filtration through a nitrocellulose membrane the bound radioactivity was quantified and the data fitted to an equilibrium binding model. In the upper part examples of the titration curves are shown. The lower part shows the averages of the K_d-values and corresponding SEM determined from two repeats of each experiment.





Supplemental table 1: Sequences of DNA (single stranded) used in the PCR to amplify the dsDNA template for *in vitro* transcription. The T7 promoter sequence is underlined. The hammer head ribozyme sequence of the murine $tRNA^{Asp}$ is shaded in grey and the sequence of the mature $tRNA^{Asp}$ in light blue. The red marks highlight the introduced mutations.

tRNA Name	Template DNA sequence
M. musculus tRNA Asp	AATACGACTCACTATAGGGAGATACTAACGAGGACTGATGAGTCCGTGAG GACGAAACGGTACCCGGTACCGTCTCCTCGTTAGTATAGTGGTGAGTATCC CCGCCTGTCACGCGGGAGACCGGGGTTCGATTCCCCGACGGGGAGCCA
M. musculus tRNA Asp mutant	AATACGACTCACTATAGGGAGATACTAACGAGGACTGATGAGTCCGTGAG GACGAAACGGTACCCGGTACCGTCTCCTCGTTAGTATAGTGGTGAGTATCC CCGCCTGTCACGCGGG**CGGC**CGGGGTTCGATTCCCCGACGGGGAGCCA
D. melanogaster tRNA Asp	TAATACGACTCACTATAGGCCTCGATAGTATAGTGGTTAGTATCCCCGCCT GTCACGCGGGAGACCGGGGTTCAATTCCCCGTCGGGGCGCCA
D. melanogaster tRNA Asp-C38U	TAATACGACTCACTATAGGCCTCGATAGTATAGTGGTTAGTATCCCCGCCT GTCA**T**GCGGGAGACCGGGGTTCAATTCCCCGTCGGGGCGCCA
Gs-tRNA Asp	TAATACGACTCACTATAGGGGTCGTAGTTAAGTTGGTTATAACGCCGGCCT GTCACGCCGGAGGCCGCGGGTTCGAGCCCCGTCGACCCCGCCA
Gs-tRNA Asp-C38U	TAATACGACTCACTATAGGGGTCGTAGTTAAGTTGGTTATAACGCCGGCCT GTCA**T**GCCGGAGGCCGCGGGTTCGAGCCCCGTCGACCCCGCCA
Gs-tRNA Glu (tRNA32)	TAATACGACTCACTATAGGTCCCATCGTCTAGTGGTTAGGACACCGGCCTT TCACGTCGGTAACAGGGGTTCAAGTCCCCTTGGGATCACCA
Gs-tRNA Glu-(32) C38U	TAATACGACTCACTATAGGTCCCATCGTCTAGTGGTTAGGACACCGGCCTT TCA**T**GTCGGTAACAGGGGTTCAAGTCCCCTTGGGATCACCA
Gs-tRNA Glu (tRNA20)	TAATACGACTCACTATAGTCCCCTTCGTCTAGCCCGGCCCAGGACACCGC CCTTTCACGGCGGCGACGGGGGTTCAAATCCCCCAGGGGACGCCA
Gs-tRNA Ala	TAATACGACTCACTATAGGGGGTGTAGCTCAGCTGGGAGAGCGCCTGCCT TGCACGCAGGAGGTCATCGGTTCGAACCCGTTCACCTCCACCA
Gs-tRNA His	TAATACGACTCACTATAGGGGCTATGGTGAAGGGGTCTAACACACATGACT GTGACTCATGCATTCGTGGGTTCAAATCCCACTAGCCACCCCA
Gs-tRNA Val	TAATACGACTCACTATAGGGCGCTTAGCTCAGCGGGAGAGCACTGCCTTC ACACGGCAGGGGTCACTGGTTCAATCCCAGTAGCGCCCACCA

Supplemental table 2: Sequences of forward and reverse primers used to amplify the dsDNA template for *in vitro* transcription.

Primer Name	**Primer sequence**
T7-Universal-Forward Primer	CGCGCGAAGCTTAATACGACTCACTATA
Mm-tRNA^{Asp} Rev Primer	TGGCTCCCCGTCGGGGAATCG
Dm-tRNA^{Asp} Rev primer	TGGCGCCCCGACGGGGAATTG
Gs-tRNA^{Asp} Rev primer	TGGCGGGGTCGACGGGGCTCG
Gs-tRNA^{Glu} Rev primer	TGGTGATCCCAAGGG
Gs-tRNA^{Ala} Rev primer	TGGTGGAGGTGAACGGGTTCG
Gs-tRNA^{His} Rev primer	TGGGGTGGCTAGTGGGATTTGAACC
Gs-tRNA^{Val} Rev Primer	TGGTGGGCGCTACTGGGATTGAACC

MANUSCRIPT 3

Cytosine methylation of tRNA-Asp by DNMT2 has a role in translation of proteins containing poly-Asp sequences

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Key words: Dnmt2, tRNA methylation, aminoacylation, regulation of translation, Asp-rich proteins

Running title: C38 methylation of tRNA-Asp has a role in translation

Abstract

The DNMT2 RNA methyltransferase catalyses the methylation of C38 in the anticodon loop of tRNA-Asp, but the molecular role of this methylation is unknown. Here, we report that mouse aspartyl-tRNA synthetase shows a 4-5 fold preference for C38 methylated tRNA-Asp. Consistently, the level of charged tRNA-Asp was found to be reduced in Dnmt2 knockout murine embryonic fibroblast cells. Gene expression analysis with fluorescent reporter proteins fused to an N-terminal poly-Asp sequence showed that protein synthesis of poly-Asp tagged reporter proteins was reduced in Dnmt2 KO cells as well. The same effect was observed with endogenous proteins containing poly-Asp sequences indicating that DNMT2 mediated C38 methylation of tRNA-Asp regulates the translation of proteins containing poly-Asp sequences. Gene ontology searches for proteins containing poly-Asp sequences in the human proteome showed that a significant number of these proteins have roles in transcriptional regulation and gene expression. Hence, the Dnmt2 mediated methylation of tRNA-Asp exhibits a post-transcriptional regulatory role by controlling the synthesis of a group of target proteins containing poly-Asp sequences.

Introduction

RNA methylation occurs in all types of RNA, including rRNA, tRNA, mRNA and small RNA ubiquitously in both prokaryotes and eukaryotes (Cantara et al, 2011; Czerwoniec et al, 2009; Khoddami & Cairns, 2013; Motorin & Helm, 2011; Motorin et al, 2010). In particular, tRNAs are extensively modified in all organisms and contain a variety of different modifications, including methylation, pseudouridylation, thiouridylation and addition of isopentenyl groups. Despite their systematic identification, the molecular function of most of these modifications is not well understood. Methylation of tRNAs is introduced by tRNA methyltransferases that are specific towards particular tRNAs and target sites. Different types of tRNA methyltransferases have been identified based on their fold and reaction mechanism (Motorin et al, 2010). DNMT2, also known as tRNA-aspartic acid methyltransferase 1 (Trdmt1), is a highly conserved cytosine-C5 methyltransferase that introduces the C38 methylation of $tRNA^{Asp}$ in many species, including lower eukaryotes, plants, insects and humans (Becker et al, 2012; Goll et al, 2006; Jeltsch et al, 2006;

Jurkowski et al, 2008; Muller et al, 2013; Schaefer et al, 2010; Tovy et al, 2010). Interestingly, DNMT2 resembles DNA methyltransferases in structure and catalytic mechanism (Jurkowski et al, 2008) but was shown in 2006 to function as an RNA methyltransferase (Goll et al, 2006). The strong conservation of Dnmt2 homologues in the eukaryotic kingdom (Jeltsch et al, 2006; Jurkowski & Jeltsch, 2011) suggests a very important function of these enzymes in maintaining cellular homeostasis. However, the molecular role of the methyl group at position C38 of $tRNA^{Asp}$ remains unknown. Although Dnmt2 knock-out mice lack strong phenotypes (Goll et al, 2006; Tang et al, 2003), Dnmt2 knockout flies have been shown to exhibit an increased sensitivity to oxidative stress and heat shock due to lack of the C38 methylation in $tRNA^{Asp}$ that lead to the fragmentation of this tRNA species (Schaefer et al, 2010). These fragments have been reported to influence the antiviral defence in Drosophila and to influence Dicer-2 processing of siRNAs (Durdevic et al, 2013a; Durdevic et al, 2013b). The only other cytosine tRNA methyltransferase in mammals is Nsun2, which methylates the C34 position in the anticodon loop of a $tRNA^{Leu}$ precursor (Blanco et al, 2011; Brzezicha et al, 2006) and some other sites in $tRNA^{Leu}$ (Tuorto et al, 2012). $Nsun2^{-/-}$ mice are viable under normal conditions but have reduced weight and male littermates are sterile (Blanco et al, 2011). Importantly, the combined knock out of both Dnmt2 and Nsun2 in mice led to reduced steady-state levels of tRNAs and reduced rates of overall protein synthesis (Tuorto et al, 2012).

Here, we aimed to characterise the physiological role of the Dnmt2 mediated C38 methylation of $tRNA^{Asp}$. Given the essential role of tRNAs in protein synthesis, we investigated the influence of Dnmt2 catalysed $tRNA^{Asp}$ methylation on this process. As the addition of methyl groups often has a role in modulating the specificity of protein-nucleic acid interactions, we investigated the role of C38 methylation in the charging of $tRNA^{Asp}$ by aspartyl-tRNA-synthetase (AspRS). We show that AspRS activity is stimulated by C38 methylation and that loss of this mark decreases the level of $tRNA^{Asp}$ charging. Using a fluorescent reporter system and endogenous proteins containing poly-Asp sequences, we show that the loss of Dnmt2 leads to reduced synthesis of poly-Asp containing proteins. We show enrichment of poly-Asp proteins in genes with regulatory roles and based on our findings we propose that Dnmt2 has a post-transcriptional regulatory role in global

modulation of the expression of these proteins. This novel translational regulation pathway could participate in the Dnmt2 mediated stress response.

Results

Cytosine-38 methylation of $tRNA^{Asp}$ increases the rate of its aminoacylation

Despite strong conservation and ubiquitous presence of the Dnmt2 tRNA methyltransferase, the molecular function of the C38 methylation of $tRNA^{Asp}$ introduced by Dnmt2 remains unknown. Searching for a direct molecular function of this tRNA methylation, we have focused on its potential role in modulating the interaction with the aspartyl-tRNA synthetase (AspRS), because modifications in the anticodon loop of tRNAs have been shown to affect their charging in other cases (Martin et al, 2004; Rudinger et al, 1992; Ruff et al, 1991) and C38 modification is present in the anticodon loop. Unfortunately, there is no crystal structure of an AspRS available from species containing a Dnmt2 homologue in their genome. In *S. cerevisiae*, the specificity determinants for the AspRS include the anticodon, the ψ=G base pair at the basis of the D stem (G10, U25), and G73 (Putz et al, 1991; Rudinger et al, 1992), which are all fully conserved among $tRNA^{Asp}$ also in species containing Dnmt2 homologues. The amino acid residues interacting with the conserved nucleotides in the yeast AspRS-$tRNA^{Asp}$ complex (Ruff et al, 1991) are widely conserved, suggesting a similar mode of $tRNA^{Asp}$ recognition by the mouse and human AspRS enzymes. This structure suggests that methylation of C38 might stabilize the tRNA bound to the AspRS, because the methyl group would point towards the first base pair in the anticodon stem and stack on the π-electron system (Figure 1). Supporting this notion, C38 has been mapped as a direct identity contact of yeast AspRS (Giege et al, 1996). To investigate the role of C38 methylation of $tRNA^{Asp}$ in tRNA charging, we have cloned mouse AspRS from cDNA and purified the recombinant enzyme from *E. coli* (Escalante & Yang, 1993; Reed et al, 1994) (Suppl. Figure 1a). Next, we have determined the rates of aminoacylation of methylated and unmethylyated $tRNA^{Asp}$ using an in vitro aminoacylation assay (Cavarelli et al, 1994; Ryckelynck et al, 2003). For this, we synthesised the mouse $tRNA^{Asp}$ in C38 methylated and unmethylated form by splint ligation (Suppl. Figure 1b) and used it as a substrate for aminoacylation reactions in the

presence of ^{3}H-labelled aspartate. We observed that the aspartylation efficiency was significantly higher with methylated tRNA^{Asp} than with unmethylated tRNA^{Asp} (Figure 2a). Experiments using the tRNA at different concentrations in the range of 100-1000 nM showed a 5.5 fold increase in the V_{max}/K_m for the C38 methylated tRNA substrate compared to the unmethylated tRNA (Figure 2b). These results suggest that C38 methylation is needed for an efficient charging of tRNA^{Asp} by AspRS.

The charging level of tRNA^{Asp} is reduced in DNMT2 KO cells

Next, we studied whether the reduced aminoacylation rate of unmethylated tRNA^{Asp} leads to reduced charging levels of tRNA^{Asp} in Dnmt2 knock out cells. To this end, an incorporation assay was employed. Total RNA containing tRNA^{Asp} was isolated from murine embryonic fibroblast (MEF) cells under mild acidic condition where the charging of tRNA is preserved (Varshney et al, 1991; Zaborske & Pan, 2010). Then, the RNA was incubated with recombinant AspRS and ^{3}H-labelled aspartate and the transferred radioactivity was quantified. Since aminoacylated tRNA is refractory to the in vitro aminoacylation, a higher incorporation of radioactively labelled asparate in this assay is indicative of a lower aminoacylation level of the specific tRNA^{Asp}. A portion of each RNA preparation was deacylated by incubation at alkaline pH (Goss & Parkhurst, 1978) and treated identically to serve as input correction of the amount of tRNA^{Asp} in the RNA preparations. We observed that the aminoacylation level of tRNA^{Asp} isolated from the Dnmt2 KO cells was approximately 30% lower when compared to tRNA^{Asp} isolated from wild type cells (Figure 2c). This result indicates that the loss of C38 methylation in cells leads to reduced availability of charged tRNA^{Asp}.

DNMT2 KO cells show a reduced expression of Asp-tagged fluorescent reporter proteins

Next, we wanted to investigate if the observed reduction in the charging levels of tRNA^{Asp} leads to a decreased translational efficiency in Dnmt2 KO cells. We hypothesized that a consecutive stretch of aspartate residues present at the N-termini of a reporter protein would allow us to challenge the efficiency of Asp incorporation in the cell. We have,

therefore, constructed reporter plasmids expressing the YFP and CFP fluorescent proteins fused with an N-terminal Asp_6-tag (6DYFP and 6DCFP) (Figure 3a). Wild type and Dnmt2 KO MEF cells were co-transfected with two reporter constructs one having an Asp-tag and the other one without. The construct without a tag served as an internal control. 48 hours after transfection the cells were fixed and the fluorescence intensity of YFP and CFP was measured. As shown in Figure 3b (and Suppl. Figure 2), the relative fluorescence intensity of Asp-tagged proteins was higher in wild type cells when compared to Dnmt2 KO cells. Swapping of the reporters indicated that this effect was not due to technical artifacts. Control experiments with single transfections indicated absence of crosstalk between the YFP and CFP channels with our settings (Suppl. Figure 3). For quantitative analysis, we determined the fluorescence intensities of both reporter proteins from more than 150 individual cells for each experiment (Suppl. Figure 4). The intensity averages of the expression levels showed that the expression of the Asp-tagged reporters was lower in both cotransfection experiments (Figure 4a). However, this effect was more pronounced with Dnmt2 KO cells than with wild type cells. Next, we calculated the ratio of 6DYFP/CFP or 6DCFP/YFP signals for individual cells such that the individual fluctuations of reporter gene expression between cells could be compensated. As shown in Figure 4b the relative expression of the Asp-tagged proteins was lower in Dnmt2 KO cells than in wild type cells and this effect was highly significant. These data show that that Dnmt2 KO cells have difficulties in synthesising the Asp-tagged proteins.

Synthesis of endogenous proteins with poly-Asp sequences is reduced in DNMT2 KO cells

After showing the reduction in the synthesis of Asp-tagged reporter proteins in Dnmt2 KO cells, we investigated whether expression of cellular proteins which naturally contain stretches of aspartate residues is also influenced by lack of C38 methylation in $tRNA^{Asp}$. Using the Scansite 2.0 web server (Obenauer et al, 2003) we identified a total of 49 endogenous candidate proteins in the murine proteome, which contain stretches of 6 or more Asp residues in their sequence. From them, we have selected 7 proteins that have been previously reported to be expressed in mouse embryonic fibroblast cells (Suppl.

Table 1). Cellular levels of these proteins were analysed in the total protein extract prepared from wild type and Dnmt2 KO MEF cells by western blots. We observed reduced levels of Protein-SET, TFDP-1, TAF9 and Ezh2 proteins in Dnmt2 KO cells compared to wild type MEF cells (Figure 5a). No differences were observed with DAXX and NPM; the FGFR1 antibody failed (data not shown). The quantitative analysis showed that the levels of the Transcription activation factor 9 (TAF9) protein, which contains an Asp_{13} sequence in its C-terminal region were affected the most (Figure 5b) supporting the hypothesis that the synthesis of proteins with long Asp runs is strongly reduced in Dnmt2 KO cells. The mRNA expression levels of the candidate proteins were determined previously for the same cells as used here, showing that the transcript levels for the selected candidate proteins were identical in wild type and Dnmt2 KO MEFs (Suppl. Figure 5). This result implicates that the reduced levels of these proteins are due to their reduced translation in Dnmt2 KO cells, which can be explained by the reduced level of charged $tRNA^{Asp}$ in these cells.

Protein degradation does not cause reduction of protein level in Dnmt2 KO cells

To test if the reduced levels of Asp-tagged protein were due to their increased degradation in Dnmt2 KO cells, the rate of protein degradation in wild type and Dnmt2 KO MEF cells was determined by cycloheximide chase. The wild type and Dnmt2 KO cells were grown and treated with cycloheximide which blocks protein synthesis. Cells were harvested at specific time points between 0-6 hours. After cell lysis, the amounts of Taf9 and Ezh2 proteins were compared by western blot (Figure 5c), because these proteins showed largest changes in expression levels at high level of expression. The results indicated that both proteins were degraded with almost the same rates in wild type and Dnmt2 KO cells indicating that the differences in steady-state levels determined here directly reflect the reduced synthesis of Asp-tagged proteins in Dnmt2 KO cells.

Discussion

Methylation of tRNA has been documented to have regulatory roles in translation. For example, the U34 modification of $tRNA^{Glu}$ in *E. coli* has an important role in

aminoacylation (Madore et al, 1999) and absence of the 7-methylguanine and 5-methylcytosine modifications in $tRNA^{Val\ (AAC)}$ leads to its rapid deacylation at high temperature {Alexandrov, 2006}. Furthermore, the methylation of the uridine wobble base of $tRNA^{Arg}$ (UCU) and $tRNA^{Glu}$ (UUC) by *Saccharomyces cerevisiae* tRNA methyltransferase 9 (Trm9) changes the preferences of the tRNA in reading different codons and thereby prevents cell death via translational enhancement of DNA damage response proteins (Begley et al, 2007). Moreover, the Trm4 catalysed modification of C34 in $tRNA^{Leu}$ has been shown to influence the codon preferences of the tRNA (Chan et al, 2012) which was connected to the translational enrichment of survival proteins like Rpl22A under stress condition in yeast. The formation of a modified 1-methylguanosine at position 37 (adjacent to and 3' of the anticodon) in several tRNAs has been shown to prevent frame shifting (Bjork et al, 2001). Moreover, methylation of G37 in $tRNA^{Asp}$ has been shown to prevent mischarging by the arginyl-tRNA synthetase (Putz et al, 1994). Interestingly, G37 is located next to the target position of Dnmt2 (C38) but it is present only in $tRNA^{Asp}$ of *S. cerevisiae*, which does not possess a Dnmt2 homolog. These examples emphasize the role of tRNA modification in modulating the translational efficiency and fine tuning the cellular response to various stimuli. In accordance to that, we show here that C38 methylation of $tRNA^{Asp}$ by Dnmt2 has a role in the recognition of the tRNA by AspRS. Reduced Dnmt2 activity leads to lower charging levels of $tRNA^{Asp}$ in cells and less efficient translation of poly-Asp containing proteins which represents a novel mechanism of post-transcriptional regulation.

In agreement with our findings, a reduced synthesis of proteins has also been found in the Dnmt2 and Nsun2 double knockout mice (Tuorto et al, 2012). Strikingly, a classification of gene ontology gene functions of human proteins containing Asp-runs (Table 1) revealed that a significant proportion of these proteins have nuclear localisation and they have roles in gene expression and transcription regulation. Hence, the change in Dnmt2 activity under cellular stress conditions could trigger a signalling cascade in which reduced methylation of $tRNA^{Asp}$ leads to its reduced charging, which in turn causes a reduced synthesis of proteins with poly-Asp runs, which then can change the expression of further target genes. The reduced synthesis of “standard” gene regulatory proteins,

thereby, could support synthesis of specific factors needed during response to cellular stress. In accordance with the mild phenotypes of Dnmt2 knock out animals, the role of Dnmt2 is in the modulation of the transcriptional profile of cells is leading to a fine-tuning of the cellular properties to better adapt to conditions of cellular stress response. Our data identify a molecular function of the C38 methylation introduced by Dnmt2 that can be directly connected to a physiological function which lies in a novel post-transcriptional regulation mechanism. Homopolymeric stretches of amino acids other than poly-Asp are widespread in the human proteome, but their function often is not clear. Based on our findings, they may affect protein biosynthesis using a similar mechanism regulated by a modification of the corresponding tRNA.

Materials and Methods

Cloning, protein expression and purification

The gene coding for mouse aspartyl-tRNA synthetase (AspRS) (UniProt ID: Q922B2) was amplified from cDNA prepared from mouse embryonic fibroblast (MEF) cells using specific primers (forward 5'-GGC TAG CAT GCC CAG CGC CAA CGC-3' and reverse 5'-GTG CTC GAG TTA AGG CGT GAG TCG TTT GGG-3') and cloned into pET28a+ using NheI and XhoI sites. The protein was expressed in *E. coli* BL21 (DE3) Rosetta2 cells as outlined below. Protein expression was induced with 1 mM IPTG at OD_{600} of 0.6 and conducted at 22° C overnight in shaking culture. After harvesting the cells were disrupted by sonication of the cell pellet in wash buffer (50 mM HEPES pH 7.2, 500 mM NaCl, 10% glycerol, 0.1 mM DTT, 0.5 mM EDTA, 10 mM Imidazole). The cleared lysate was applied onto Ni-NTA beads followed by washing steps and elution in buffer (50 mM HEPES pH 7.2, 500 mM NaCl, 10% glycerol, 0.1 mM DTT, 0.5 mM EDTA, 200 mM Imidazole). The purified protein was dialysed against dialysis buffer I for 2 hours (50 mM HEPES pH 7.2, 250 mM NaCl, 20% glycerol, 0.1 mM DTT) and dialysis buffer II (50 mM HEPES pH 7.2, 150 mM NaCl, 50% glycerol, 0.1 mM DTT) overnight. The cloning of 6X aspartate leader sequence into pEYFP-N1 and pECFP-N1 was achieved through ExSite PCR using the forward primer 5' CCA CCG GTC GCC ACC ATG 3' and reverse primer 5' GCT CCT CGC CCT TGC TCA CGT CAT CAT CGT CAT CGT C 3'.

Substrate tRNA preparation

Unmethylated and C38 methylated mouse $tRNA^{Asp}$ substrates were created by splint ligation (Hengesbach et al, 2008; Kurschat et al, 2005). The ligation reaction was performed by annealing two synthetic RNA fragments (IBA Göttingen, Germany) (Suppl. Table 2) corresponding in sequence to mouse-$tRNA^{Asp}$, onto a 52 nt long complementary oligodeoxynucleotide. Unmethylated or methylated fragments (4 nmol) were 5'-phosphorylated by incubating in KL buffer (50 mM Tris-HCL pH 7.4, 10 mM $MgCl_2$) supplemented with 5 mM ATP, 5 mM DTT and 0.75 u/µl T4 polynucleotide kinase (PNK, Fermentas, Germany) in a final volume of 150 µl in a thermomixer at 37°C for 1 hour. Afterwards, an equimolar amount of the 5'-fragment and the DNA splint dissolved in KL buffer containing 5 mM ATP and 5 mM DTT were added, leading to a final volume of 500 µl and a concentration of 8 µM of each fragment. The RNA fragments were hybridized to the DNA splint by heating to 75 °C in a thermomixer for 4 min and slowly cooling down to room temperature within 15 min. Then T4 DNA ligase (1.5 u/µl; Fermentas) and T4 RNA ligase 2 (22 ng/µl) were added and the ligation was performed in the thermomixer at 16°C overnight. Template DNA was removed by the addition of 1.5 u/µL DNase I (Fermentas), followed by 1 hour of incubation at 37°C. The tRNAs were purified from ligation mixtures by denaturing PAGE, excised and eluted from the gel, and precipitated with ethanol. Concentrations were calculated from absorption at 254 nm, determined using a Nanodrop ND-1000 spectrometer.

In vitro aminoacylation reactions

In vitro aminoacylation was performed on the synthesized murine-$tRNA^{Asp}$, which was either methylated or unmethylated at the C38 position. For the in vitro aminoacylation, 100 nM to 1 µM tRNA substrate (as indicated) was incubated with 50 nM of the recombinant AspRS in aminoacylation buffer (50 mM PIPES pH 7.0, 30 mM KCl, 10 mM $MgCl_2$, 1 mM DTT and 2 mM ATP) containing 1 µM [^{3}H]-aspartate (MP Biomedicals, Germany; 9.25 MBq) at 37 °C. The reaction progress was monitored by removing 5 µl

aliquots of the reaction mixture at specific time points and precipitating them in 500 µL of 5% TCA. Then, the samples were spotted on a DE81 filter paper, washed with 5% TCA followed by ethanol wash and the radioactivity was counted in a Hidex 300 SL liquid scintillation counter (Hidex, Finland) using Rotiszint eco plus scintillation liquid (Roth, Germany). The reaction progress curves were fitted by linear regression analysis and the reaction rates were used to calculate the V_{max}/ K_m. by least-squares fitting of the data to the Michaelis-Menten equation.

Cell lines, culture conditions and transfection

Wild type and Dnmt2 KO mouse embryonic fibroblast cells (MEF) were prepared as described (Tuorto et al, 2012) and were grown in standard DMEM media containing 10% FBS and supplemented with 2 mM Glutamate in a 5% CO_2 incubator. The cells were grown to 90% confluence and split into a 6-well plate prepared with cover slides at a seeding density of $1X10^6$ cells per well. Afterwards, the cells were grown for another 24 hours in DMEM medium to reach approximately 50% confluence. Then, the MEF cells were transfected with fluorescent reporter construct (YFP and CFP) with and without poly-Asp_6 coding sequence at their N terminal side using Xfect™ mESC Transfection Reagent (Clontech, Germany) following the instructions of the supplier. The medium was exchanged 3 hours after transfection.

Fluorescence microscopy

After 48 hours the cells transfected with reporter constructs were washed 3 times with 2 mL of PBS, fixed using 3.7% formaldehyde solution for 10 minutes and washed 3 more time with PBS. After this, the cover slides were carefully removed from the plate and mounted on a base slide spotted with 50 µl of Mowiol (48 mg/ml Mowiol dissolved in 0.2 M Tris/HCl pH 8.5 containing 0.12 g/ml glycerol). The slides were kept in the dark for 2-3 h and subsequently sealed to avoid drying. Fluorescent images were taken using a Zeiss Laser Scanning Microscope 510 (Carl Zeiss, Germany) with 20x magnification using specific filters for CFP (430 nm excitation and 475 nm emission readout) and YFP (510 nm

excitation and 530 nm emission readout). The fluorescent intensities from more than 150 individual cells were quantified using the ImageJ analysis tool (rsb.info.nih.gov/ij/).

Determination of cellular aminoacylation levels of $tRNA^{Asp}$

For the measurement of aminoacylation level of $tRNA^{Asp}$ in cells, the tRNA was isolated from early passage (P10) wild type and Dnmt2 KO MEF cells under mild acidic condition which preserve the aminoacylation (Varshney et al, 1991; Zaborske & Pan, 2010). For this, the cells were grown in a T75 flask until they reached 90% confluence, trypsinized and collected in 500 µL of 0.3 M sodium acetate pH 4.5 and 10 mM EDTA followed by addition of an equal volume of sodium acetate-saturated phenol/chloroform pH 4.5. The cell pellet was vortexed 3 times for 30 s with 30 s pauses on ice. The supernatant was collected by centrifugation at 18600 g for 30 min at 4°C. The RNA was then precipitated by addition of 2.5 volumes of ethanol and resuspended in 10 mM sodium acetate pH 4.5 and 1 mM EDTA. The isolated RNA containing tRNA was then divided into two fractions, one of these parts was treated with alkaline buffer (100 mM Tris-Cl pH 8.0 and 100 mM NaOH) at 37°C to deacylate the tRNAs (Goss & Parkhurst, 1978). The tRNA was precipitated and resuspended in RNase-free water. The total concentration of the alkaline treated and untreated tRNA was calculated from absorbance at 254 nm as determined with a Nanodrop ND-1000 spectrometer and confirmed by gel analysis. To determine the aminoacylation level of the tRNA, an incorporation assay was used. For the assay, equal amounts of the tRNA fractions were incubated with 50 nM AspRS in the presence of 1 µM [^{3}H]-aspartate and aminoacylation buffer at 37°C. The reaction was stopped after 90 min by TCA precipitation. The samples were spotted on DE81 filter discs and washed with 5% TCA and absolute ethanol. Following this the DE81 discs were air dried and the incorporated radioactivity was counted using Hidex 300 SL liquid scintillation counter (Hidex, Finland) using Rotiszint eco plus (Roth, Germany). The aminoacylation of the $tRNA^{Asp}$ was quantified from the ratio of [^{3}H]-labelled $tRNA^{Asp}$ in the samples isolated from wild type and Dnmt2 knock out cells. Counts from the corresponding deacylated samples were used to normalize the input of $tRNA^{Asp}$ in both samples. In this acceptor

assay method, the counts obtained from the acylated samples are inversely proportional to their charging levels.

Western blotting

Early passage (P10) wild type and Dnmt2 KO MEF cells were grown till 90% confluence and the cell pellet was collected after trypsination. Protein extracts were prepared by cell lysis using RIPA buffer. The protein amount was determined by absorbance at 280 nm using a NanoDrop ND-1000 spectrometer and equal amounts were separated on a 15% SDS gel. After transferring the proteins to a nitrocellulose membrane specific proteins were detected with antibodies against poly-aspartate containing proteins. The candidate proteins and the antibodies used against them are listed in Suppl. Table 3. Antibodies were used at dilutions specified by the supplier for Western Blotting. After washing three times with TTBS , the blots for Taf9, protein-SET, Daxx and NPM were incubated with anti-rabbit (GE Healthcare NA934 in 1:5000) secondary antibody while blots for FGFR1, Ezh2 and TFDP-1 were incubated with anti-mouse (GE Healthcare NA931 in 1:5000) secondary antibody for 2 hours at room temperature and developed using enhanced chemiluminescence Western blotting solution (Thermo Scientific). Images were captured on X-ray film, scanned and analysed using ImageJ (rsb.info.nih.gov/ij/). For loading control either Ponceau stain or rabbit anti-β-actin IgG (AbCam ab8227 in 1:5000) followed by goat anti-rabbit IgG conjugated to horseradish peroxidase (GE Healthcare) was used.

In vivo protein degradation analysis

To study protein degradation in wild type and Dnmt2 knock-out MEFs, cells were grown in a 6 well plate in DMEM medium to 90% confluence and cycloheximide was added to a final concentration of 50 µg/mL. This stops ongoing protein synthesis such that protein degradation rates can be observed. The cells were incubated for 6 more hours after cycloheximide addition. At specific time points cells from a single well for each wild type and Dnmt2 KO were trypsinized and lysed in RIPA buffer (50 mM Tris pH 8.0 150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate, and 1 mM PMSF). Equal amounts of protein were

separated on a 15% SDS-PAGE gel and transferred to a nitrocellulose membrane. The blots were blocked in 5% milk overnight at 4°C and incubated with corresponding primary antibodies as specified above or rabbit anti-β-actin IgG (AbCam ab8227 in 1:5000) antibodies followed by goat anti-rabbit IgG conjugated to horseradish peroxidase (GE Healthcare). The blots were developed using enhanced chemiluminescence Western blotting solution (Thermo Scientific). Images were captured on X-ray film, scanned and analysed using ImageJ.

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Author Contribution

TPJ and AJ designed the study. RS and JF conducted the experiments. RS, JF, TPJ and AJ analyzed and interpreted the data. SK, FT and MH provided reagents and materials. RS, TPJ and AJ wrote the manuscript draft. All authors have seen and approved the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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Figures and figure legends

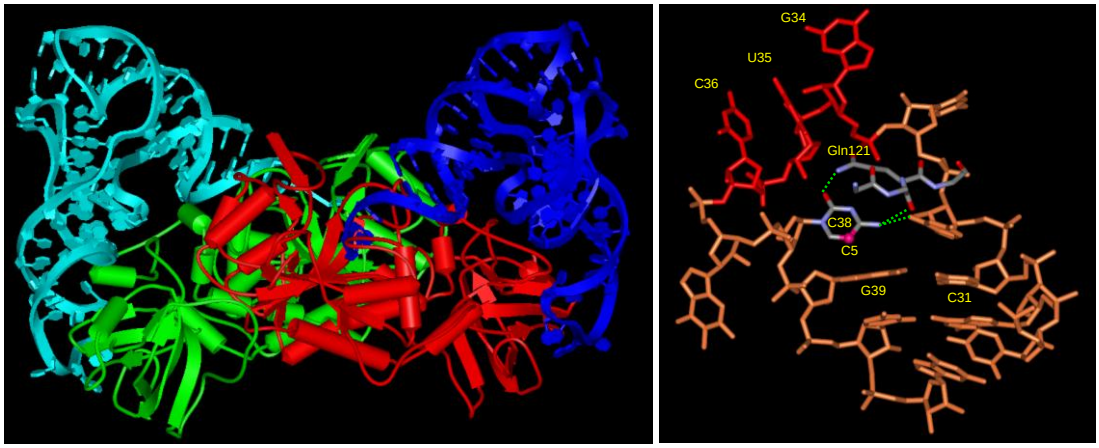


Figure 1: Structure of the yeast AspRS homodimer (colored in red and green) in complex with two tRNA-Asp (colored in blue and cyan) (Ruff et al, 1991) (PDB 1ASZ). The enzyme mainly contacts the anticodon loop and CCA end of the tRNA. The right panel shows details of the structure including the anticodon loop of the tRNA (with the anticodon residues colored in red). C38 is contacted by the side chain and main chain carbonyl of Gln121 and the O4 of ψ32. The C5 atom of C38 (colored in purple) stacks on G39 and its methylation might stabilize the fold of the tRNA.

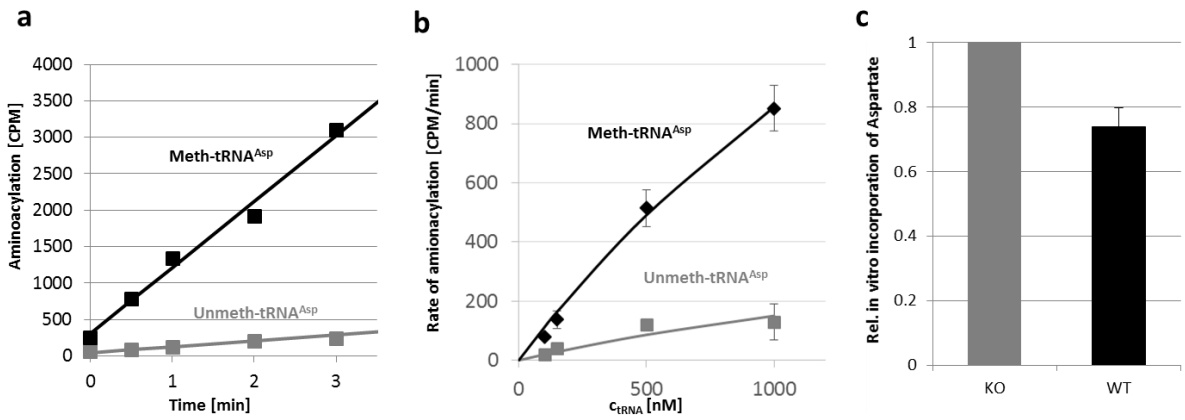


Figure 2: Aminoacylation of C38 methylated and unmethylated $tRNA^{Asp}$. a) Example kinetics of aminoacylation of C38-methylated mouse-$tRNA^{Asp}$ (black) and unmethylated mouse-$tRNA^{Asp}$ (grey) with the AspRS enzyme using 1 µM of tRNA. b) Aminoacylation rates of C38-methylated (black) or unmethylated (grey) mouse-$tRNA^{Asp}$ determined at different concentrations of tRNA. The line shows a fit of the rates to a Michaelis-Menten model indicating a 5.5 fold increase in k_{cat}/K_m. c) Aminoacylation level of $tRNA^{Asp}$ from wild type and Dnmt2 KO cells. The incorporation of labelled aspartate in the in vitro aminoacylation reactions was higher with the tRNA isolated from Dnmt2 KO MEF cells as compared with corresponding wild type cells. This indicates that the charging level $tRNA^{Asp}$ isolated from Dnm2 knock out cells is about 30% lower. All error bar indicate the standard errors.

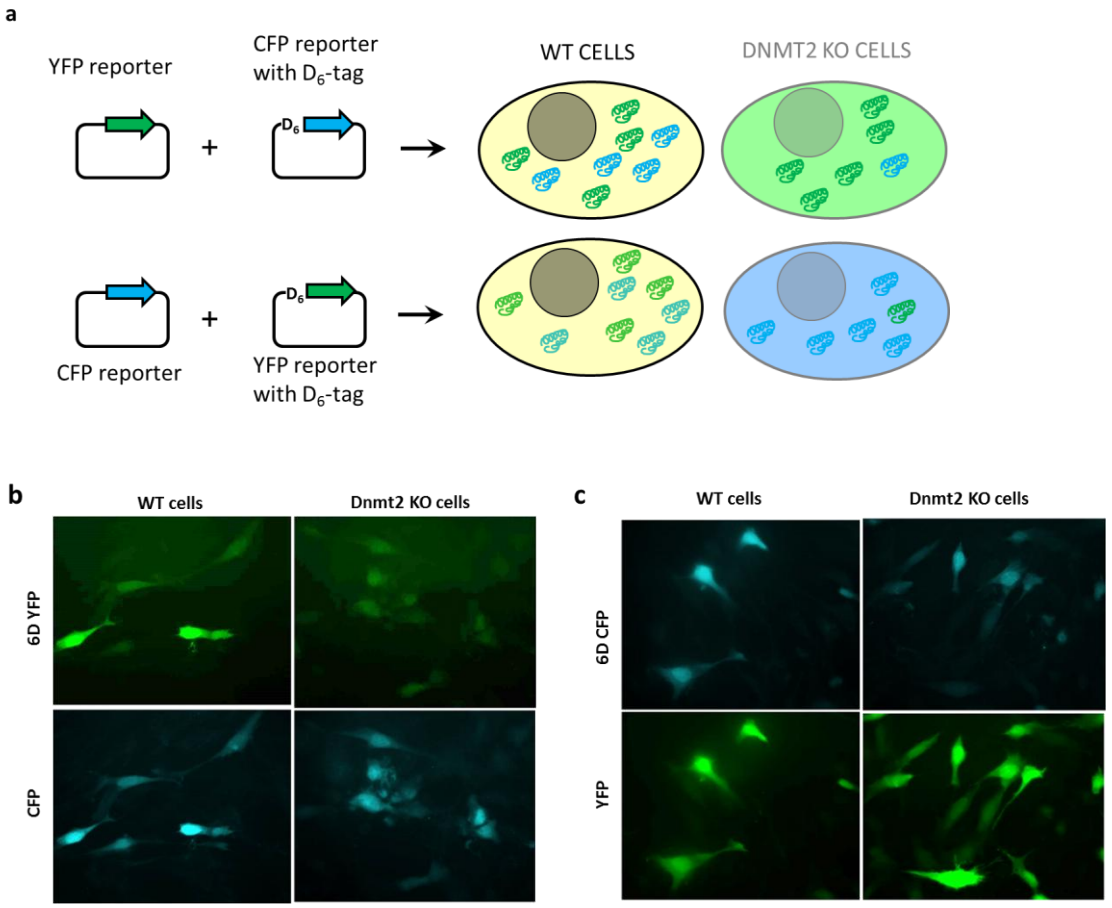


Figure 3: Double reporter analysis for in vivo synthesis of poly-Asp tagged proteins. a) Schematic drawing of the experimental design. Wild type and Dnmt2 KO MEF cells were cotransfected with a normal YFP and Asp_6-tagged CFP or vice versa, and the expression of both proteins quantified in individual cells. In Dnmt2 KO cells the expression of Asp_6-tagged protein was decreased due to reduced efficiency of translational. b) Example pictures of wild type and Dnmt2 KO cells co-transfected with 6D-YFP and CFP. In Dnmt2 KO cells the 6D-YFP proteins showed a reduced synthesis. b) Example pictures of wild type and Dnmt2 KO cells co-transfected with 6D-CFP and YFP. In Dnmt2 KO cells the 6D-CFP proteins showed a reduced synthesis. The images were taken 48 hours after transfection and the cells were fixed by formaldehyde. See also Suppl. Figures 2-4.

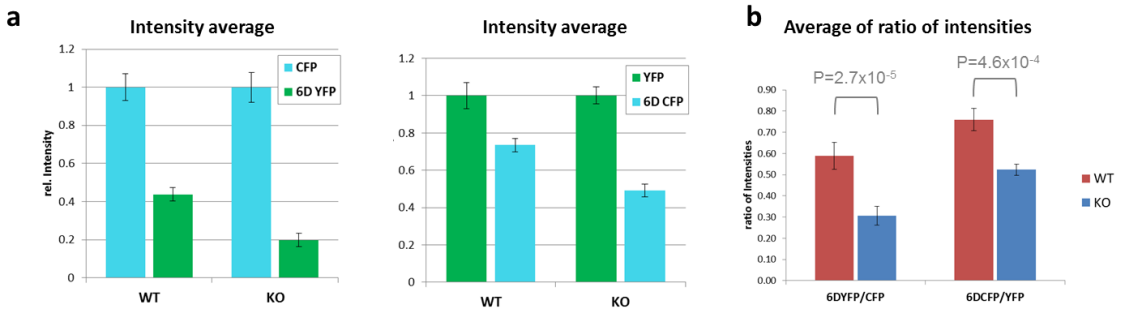


Figure 4: Quantitative analysis of reporter gene expression in wild type or Dnmt2 KO cells after cotransfection of CFP and 6D-YFP or YFP and 6D-CFP. Expression was analysed in approximately 150 cells for each experiment (Suppl. Fig. 3). a) Intensity averages of the YFP, 6D-YFP, CFP and 6D-CFP expression in individual wild type or Dnmt2 KO cells in the two cotransfection experiments. The synthesis of Asp_6-tagged proteins was lower in all experiments, but the reduction was stronger in Dnmt2 KO cells as compared to wild type cells. Intensities were normalized to the value of the untagged reporter. The error bars indicate the standard error of the mean. b) Averages of the ratios of 6DYFP and CFP or 6DCFP and YFP expression levels in individual wild type and Dnmt2 KO cells. In both experiments the KO cells showed a reduced relative expression of the Asp-tagged proteins, when compared to wild type cells. The error bars indicate the standard error of the mean. The p-values were derived from a two sided Ttest assuming equal variance of the data sets.

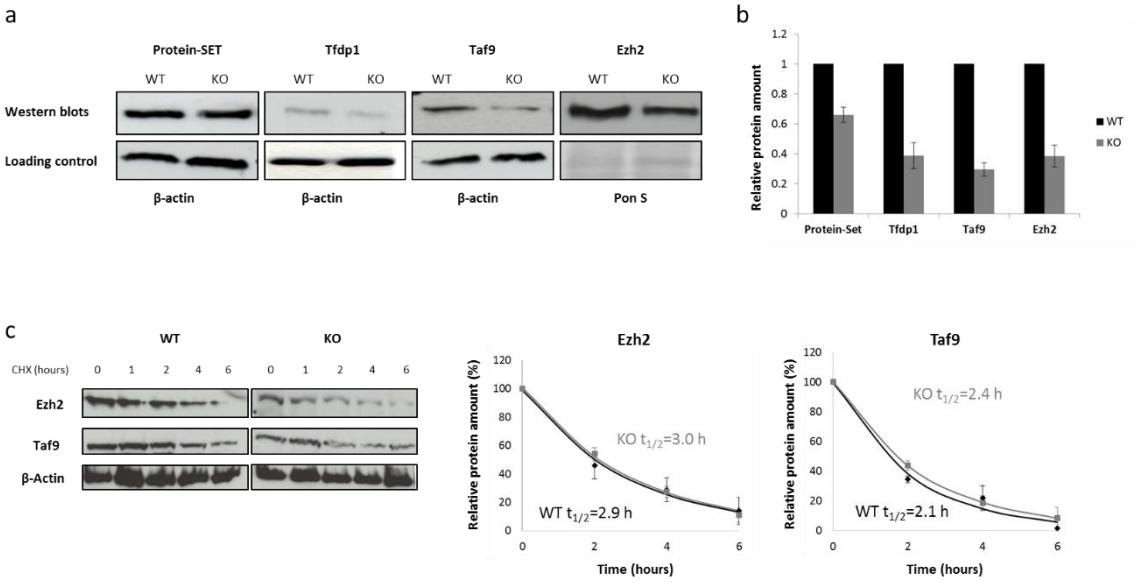


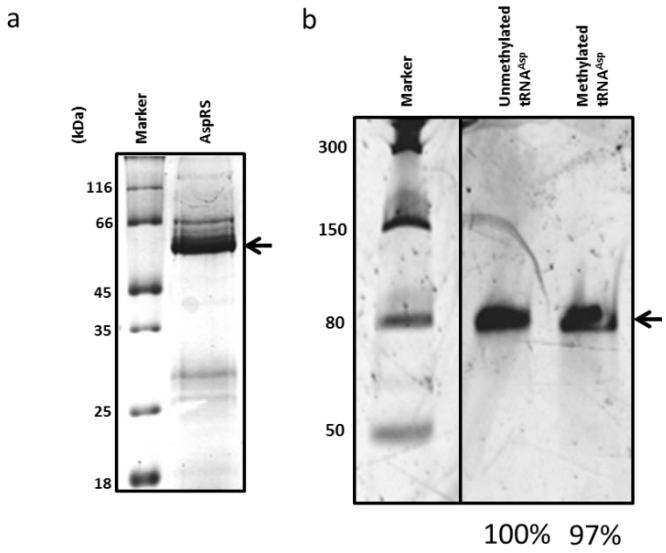
Figure 5: Levels of endogenous proteins with poly-Asp sequences are reduced in Dnmt2 KO cells. a) Images of western blots for specific proteins showing a difference in the amount of poly-Asp tagged protein in Dnmt2 KO and wild type cells (upper panel). The lower panel represents the corresponding loading controls. b) Quantified data showing relative levels of specific proteins in wild type (black) and Dnmt2 KO (grey) cells. The error bars show the standard errors of the mean from three experiments. c) Degradation of the Ehz2 and Taf9 proteins after Cycloheximide treatment in wild type and Dnmt2 KO cells. The errors bar shows the standard error of two repeats. The lines show a fit of the data to a single exponential decay curve. The experimental half-lives of the proteins ($t_{1/2}$) as derived from these fits are indicated. The protein levels were normalized to the initial amount and background was subtracted.

Tables

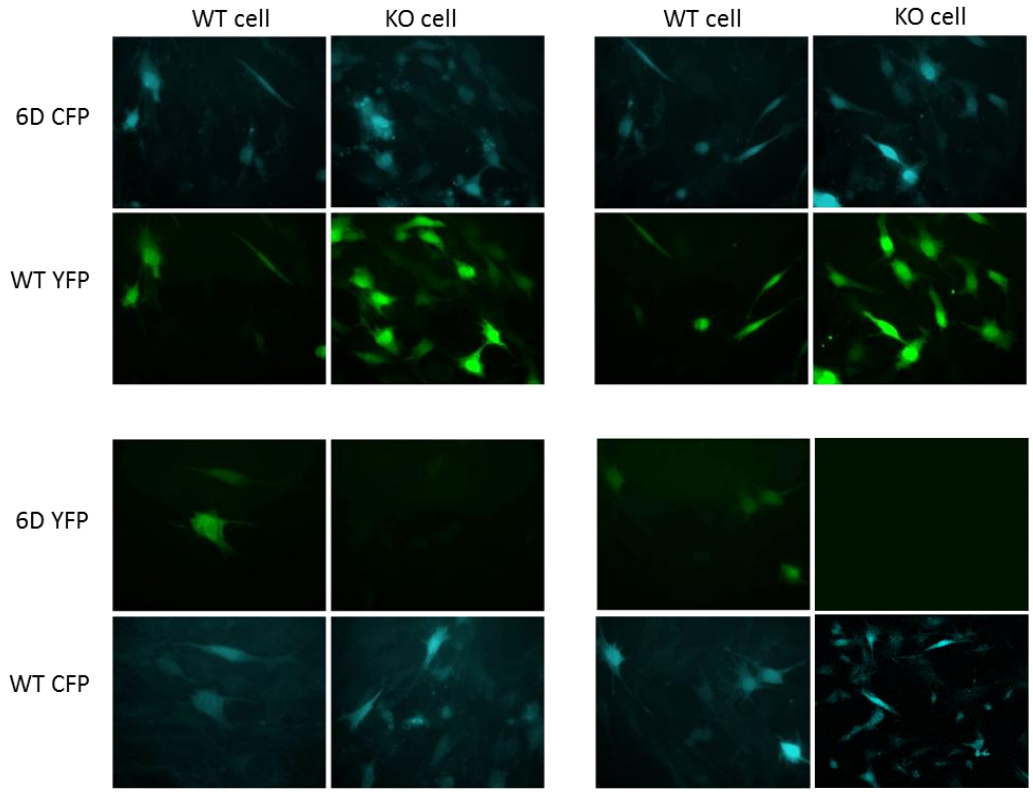
	Description	No. of proteins	P-value
Proteins containing a D4 sequence	Total No. of proteins	306	
	Mapped with GO classification	185	
	Nuclear proteins	86	6.76×10^{-10}
	Regulation of gene expression	51	6.71×10^{-5}
	Nucleic acid binding	52	1.80×10^{-3}
Proteins containing a D5 sequence	Total No. of proteins	118	
	Mapped with GO classification	68	
	Nuclear proteins	34	4.66×10^{-5}
	Nucleic acid binding	22	2.86×10^{-2}
	Chromosome organization	9	3.50×10^{-3}
Proteins containing a D6 sequence	Total No. of proteins	50	
	Mapped with GO classification	29	
	Nuclear proteins	16	2.00×10^{-3}
	Transcription regulator	9	8.80×10^{-3}
	Gene expression	13	1.54×10^{-2}

Table 1: Gene ontology (GO) classification of human proteins with poly-Asp sequence motifs. A major proportion of proteins are associated with nuclear function and gene expression (prepared using Gene set analysis toolkit V2 http://bioinfo.vanderbilt.edu/webgestalt/).

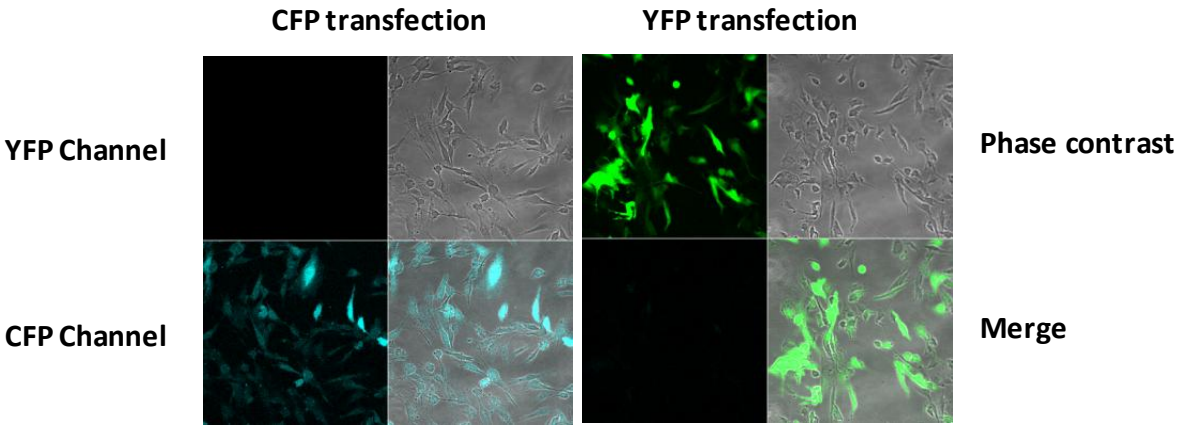
Supplemental information



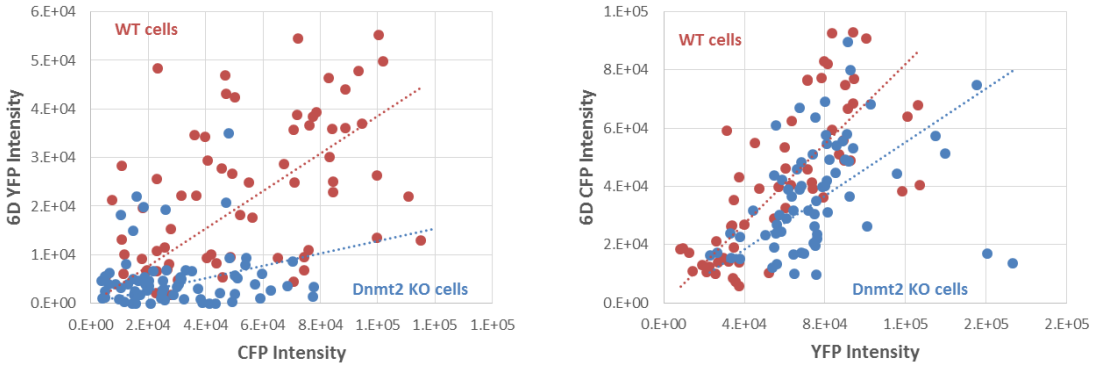
Suppl. Figure 1: Exemplary gels showing the tRNA substrates and AspRS enzymes used in this study. a) Coomassie-stained protein gel showing the purification of the AspRS enzyme used in the study. b) Image of a Gel Red-stained polyacrylamide gel showing the corresponding amounts of substrate tRNAs used in the kinetic analysis.



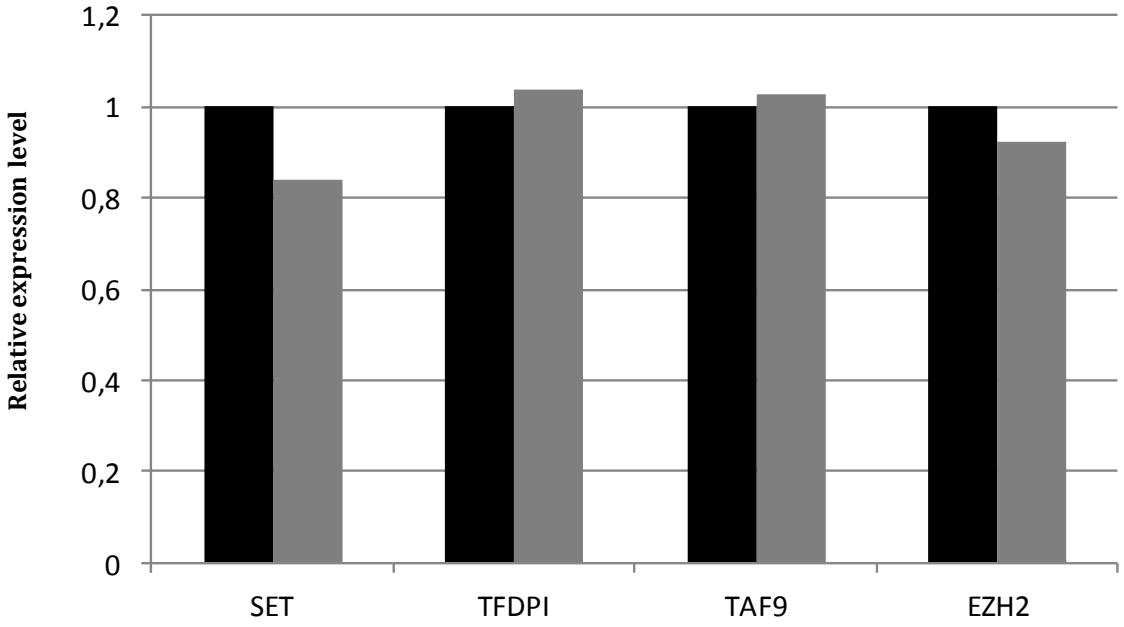
Suppl. Figure 2: Additional example pictures of wild type and Dnmt2 KO cells co-transfected with 6D YFP/CFP or vice versa (cf. Figure 3). In Dnmt2 KO cells the 6D YFP/6D CFP proteins showed a reduced synthesis compared to the CFP/YFP protein respectively. The images were taken 48 hours after transfection and the cells were fixed by formaldehyde.



Suppl. Figure 3: Images of cells transfected only with YFP or CFP in the different fluorescent channels showing the absence of crosstalk between the fluorophores (cf. Fig. 3).



Suppl. Figure 4: Quantitation of the YFP and CFP fluorescence in many individual wild type and Dnmt2 KO cells co-transfected with 6D YFP/CFP or vice versa. Approximately 150 cells were analysed for each experiment. The lines show linear regressions and are only shown for comparison. Compilations of these data are shown in Figure 4.



Suppl. Figure 5: Gene expression of the proteins containing poly Asp-runs analysed in this study. The mRNA levels of the candidate genes was compared between the MEFs wild type (GSM906315) and MEFs Dnmt2 KO (GSM906316) cells based on the reported data from the whole transcriptome analysis (GEO accession: GSE36918) {Tuorto, 2012 #3}. The expression of candidate gene was unaffected for all the proteins except protein-SET that showed a moderate reduction in the Dnmt2 KO cells.

Protein	Amino acid sequence
Ezh2	MGQTGKKSEKGPVCWRKRVKSEYMRLRQLKRFRRADEVKTMFSSNRQKILERTETLNQEW KQRRIQPVHIMTSVSSLRGTRECSVTSDLDFPAQVIPLKTLNAVASVPIMYSWSPLQQNFMV EDETVLHNIPYMGDEVLDQDGTFIEELIKNYDGKVHGDRECGFINDEIFVELVNALGQYNDD DDDDDGDDPDEREEKQKDLEDNRDDKETCPPRKFPADKIFEAISSMFPDKGTAEELKEKYKE LTEQQLPGALPPECTPNIDGPNAKSVQREQSLHSFHTLFCRRCFKYDCFLHPFHATPNTYKRK NTETALDNKPCGPQCYQHLEGAKEFAAALTAERIKTPPKRPGGRRRGRLPNNSSRPSTPTISV LESKDTDSDREAGTETGGENNDKEEEEKKDETSSSSEANSRCQTPIKMKPNIEPPENVEWSG AEASMFRVLIGTYYDNFCAIARLIGTKTCRQVYEFRVKESSIIAPVPTEDVDTPPRKKKRKHRL WAAHCRKIQLKKDGSSNHVYNYQPCDHPRQPCDSSCPCVIAQNFCEKFCQCSSECQNRFPG CRCKAQCNTKQCPCYLAVRECDPDLCLTCGAADHWDSKNVSCKNCSIQRGSKKHLLLAPSD VAGWGIFIKDPVQKNEFISEYCGEIISQDEADRRGKVYDKYMCSFLFNLNNDFVVDATRKGN KIRFANHSVNPNCYAKVMMVNGDHRIGIFAKRAIQTGEELFFDYRYSQADALKYVGIEREME IP
Protein- SET	MAPKRQSAILPQPKKPRPAAAPKLEDKSASPGLPKGEKEQQEAIEHIDEVQNEIDRLNEQASE EILKVEQKYNKLRQPFFQKRSELIAKIPNFWVTTFVNHPQVSALLGEEDEEALHYLTRVEVTEF EDIKSGYRIDFYFDENPYFENKVLSKEFHLNESGDPSSKSTEIKWKSGKDLTKRSSQTQNKASR KRQHEEPESFFTWFTDHSDAGADELGEVIKDDIWPNPLQYYLVPDMDDEEGEAEDDDDDD EEEEGLEDIDEEGDEDEGEEDDDEDEGEEGEEDEGEDD
Taf9	MESGKMASPKSMPKDAQMMAQILKDMGITEYEPRVINQMLEFAFRYVTTILDDAKIYSSHA KKATVDADDVRLAIQCRADQSFTSPPPRDFLLDIARQRNQTPLPLIKPYSGPRLPPDRYCLTAP NYRLKSLQKKAPAPAGRITVPRLSVGSVSSRPSTPTLGTPTPQTMSVSTKVGTPMSLTGQRFT VQMPASQSPAVKASIPATSTVQNVLINPSLIGSKNILITTNMVSQNTAESANALKRKREDDDD DDDDDDDDDYDNM
Tfdp1	MAKDASLIEANGELKVFIDQNLSPGKGVVSLVAVHPSTVNTLGKQLLPKTFGQSNVNITQQV VIGTPQRPAASNTIVVGSPHTPNTHFVSQNQTSDSSPWSAGKRNRKGEKNGKGLRHFSMK VCEKVQRKGTTSYNEVADELVAEFSAADNHILPNESAYDQKNIRRRVYDALNVLMAMNIISK EKKEIKWIGLPTNSAQECQNLEVERQRRLERIKQKQSQLQELILQQIAFKNLVQRNRQAEQQ ARRPPPPNSVIHLPFIIVNTSRKTVIDCSISNDKFEYLFNFDNTFEIHDDIEVLKRMGMACGLES GNCSAEDLKVARSLVPKALEPYVTEMAQGSIGGVFVTTTGSTSNGTRLSASDLSNGADGMLA TSSNGSQYSGSRVETPVSYVGEDDDDDDDFNENDEED

Suppl. Table 1: Sequences of the poly-Asp stretch containing proteins investigated in this work.

Construct name	5‘ fragment		3‘ fragment	
tRNA^{Asp}	MH 565	UCCUCGUUAGUAUAGUGGUGAGUAUCCCCGCCU	MH 566	GUCACGCGGGAGACCGGGGUUCGAUUCCCCGACGGGGAGCCA
tRNA^{Asp} m^{5}C38	MH 565	UCCUCGUUAGUAUAGUGGUGAGUAUCCCCGCCU	MH 606	GUCAm^{5}CGCGGGAGACCGGGGUUCGAUUCCCCGACGGGGAGCCA
Splint	MH 570	dGdGdAdAdTdCdGdAdAdCdCdCdCdGdGdTdCdTdCdCdCdGdCdGdTdGdAdCdAdGdGdCdG dGdGdGdAdTdAdCdTdCdAdCdCdAdCdTdAdTdAdC		

Suppl. Table 2: Sequences of mouse tRNA^{Asp} fragments and the DNA splint used in the synthesis of unmethylated and C38 methylated tRNA^{Asp} (methyl cytosine is indicated in red)

Protein	Short name	SwissProt ID	Antibody Cat.No
Phosphatase 2A inhibitor I2PP2A	Protein SET	Q9EQU5	PA5-21756 (Thermo scientific)
Transcription factor Dp-1	TFDP1	Q08639	MA5-11268 (Thermo scientific)
Transcription initiation factor TFIID subunit 9	TAF9	Q8VI33	10544-1-AP (protein tech)
Histone-lysine N-methyltransferase	EZH2	Q61188	MA5-15101 (Thermo scientific)
Death domain-associated protein 6	DAXX	O35613	PA5-19885 (Thermo scientific)
Nucleophosmin	NPM	Q61937	10306-1-AP (protein tech)
Basic fibroblast growth factor receptor 1	FGFR1	P16092	MA1-26256 (Thermo scientific)

Suppl. Table 3: Compilation of the candidate proteins selected for our analysis and the respective antibodies used.