Dissertation

Centromeric tRNA and Dnmt2-mediated Methylation in Mitotic Chromosome Segregation

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Centromeric tRNA and Dnmt2-mediated Methylation in Mitotic Chromosome Segregation

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Dedication

This work is dedicated to my beloved wife and son.

Zusammenfassung

Centromere sind die primäre Einschnürung auf mitotischen Chromosomen und eine Grundvoraussetzung für Chromosom-Segregation in der Mitose. Centromere dienen als Fundament für die Bildung des Kinetochors und die Verankerung des Spindel-Apparats, und sind epigenetisch reguliert. Spezifische Proteine und Protein-Modifikationen unterscheiden centromerisches vom umgebenden pericentromerischen Heterochromatin. Darüber hinaus sammeln sich die Anzeichen dafür, dass RNAs wichtige Faktoren für die centromerische Identität sind. Die Komposition und Regulation centromerischer Transkripte ist hingegen weitestgehend unbekannt. Überraschenderweise ist die Rolle von RNA-Modifikationen am Centromer komplett unbekannt. In dieser Doktorarbeit wurde ein Teil der zellulären Transfer-RNAs (tRNAs) auf mitotischen Centromeren lokalisiert, wo außerdem verschiedene RNA-prozessierende Enzyme detektierbar waren. Unter ihnen waren die Cytosin-5 Methyltransferasen Dnmt2 und NSun2. Die Depletion dieser Enzyme verursachte starke Chromosom-Segregations-Defekte, was eine Rolle der tRNA-Methylierung in der Mitose vermuten lässt. Bemerkenswerterweise zeigte die Untersuchung von enzymatisch inaktiviertem Dnmt2 eine direkte Rolle der Cytosin-5 RNA-Methylierung in der Centromer-Regulation. Die Depletion von Dnmt2 beeinflusste die Komposition von (peri-) centromerischem Chromatin, welches vermutlich zu den beobachteten Defekten führte. Die Detektion von Komponenten der RNA-Polymerase-III (RNAPIII) Transkriptionsmaschinerie ließ eine Rolle aktiver Transkription an mitotischen Centromeren vermuten. Tatsächlich führte die Inhibition von RNAPIII-abhängiger Transkription zu vergleichbaren Chromosom-Segregations-Defekten wie durch die Depletion der tRNA-Methyltransferasen. Bemerkenswerterweise war die centromerische Lokalisation der RNAPIII nicht nur abhängig von aktiver Transkription, sondern auch von centromerischem Dnmt2. Umgekehrt war Dnmt2 abhängig von centromerischer RNA und RNAPIII-Transkription, was einen voneinander abhängigen Zusammenhang von Transkription und Methylierung am Centromer vermuten lässt. In dieser Arbeit wurde eine bisher unbekannte Rolle der RNAPIII-Transkription und tRNA-Methylierung in der Mitose in Drosophila beschrieben, welches eine funktionelle Verbindung epitranskriptioneller Mechanismen und epigenomischer Regulation des Centromers darstellt. Darüber hinaus erscheint die mitotische Funktion von Dnmt2 als konserviert in Säugetier-Zellen, was wiederum eine Konservierung von RNA-Modifikation in der Regulation von Chromatin bedeuten könnte.

Summary

Centromeres are the primary constriction sites of mitotic chromosomes and a prerequisite for chromosome segregation during mitosis. Centromeres serve as a platform for kinetochore formation and spindle attachment and are epigenetically regulated. Specific proteins and protein modifications discriminate centromeric chromatin from the surrounding pericentromeric heterochromatin. Emerging evidence indicates that RNAs are important factors in centromere identity but the composition and regulation of centromeric transcripts are largely unknown. Surprisingly, the role of RNA modifications at centromeres is completely unknown. In this doctoral thesis, a subset of transfer RNAs (tRNAs) was found to localise to mitotic centromeres, as well as a number of different RNA processing enzymes. Among them were the cytosine-5 tRNA methyltransferases Dnmt2 and NSun2. Depletion of these enzymes caused severe chromosome segregation defects, suggesting a role of tRNA methylation in mitosis. Strikingly, analysis of enzymatically inactivated Dnmt2 indicated a direct role of cytosine-5 RNA methylation in the regulation of centromeres. Depletion of Dnmt2 affected (peri-) centromeric chromatin compositions, which presumably lead to the observed mitotic defects. The detection of components of the RNA polymerase III (RNAPIII) transcription machinery suggested a role of active transcription at centromeres during mitosis. Indeed, inhibition of RNAPIII-mediated transcription caused comparable chromosome segregation defects as observed in tRNA methyltransferase mutant backgrounds. Strikingly, the centromeric localisation of RNAPIII appeared sensitive not only to transcriptional inhibition but also to centromeric levels of Dnmt2. Vice versa, Dnmt2 was dependent on centromeric RNA and RNAPIII transcription, which suggests an interdependent role of RNAPIII transcription and tRNA methylation at centromeres. This thesis describes a novel role of RNAPIII transcription and tRNA methylation during mitosis in Drosophila, which functionally connects an epitranscriptomic mechanism and the epigenomic regulation of centromeres. Moreover, the mitotic function of Dnmt2 appeared to be conserved in mammalian cells, which suggests a conserved role of RNA modification in the regulation of centromeric chromatin.

Contents

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Introduction

In 1942, Conrad Waddington introduced the term 'epigenetics' to describe interactions between the environment and genes with phenotypic consequences [Waddington, 1942]. In modern life sciences, the classic definition of epigenetics can be paraphrased as "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in the sequence of DNA" [Armstrong, 2013]. Epigenetic mechanisms are reversible, which provides a high level of cellular plasticity and is a prerequisite for differentiation and development. However, disrupted epigenetic processes can also lead to malignant cellular transformation such as tumorigenesis [Feinberg, 2007, Sharma et al., 2009].

The interplay of multiple epigenetic marks and mechanisms composes a complex 'epigenetic code' that regulates genetic function 'on top' of the genetic information. The phenomenon of epigenetics can be subdivided into epigenomics, epitranscriptomics, and the epiproteome. Epigenomics summarises a variety of mechanisms that regulate structure and function of the genetic material into chromatin. The multitude of RNA processing events regulating transcriptomic function is called epitranscriptomics. The epiproteome as a third cellular regulatory layer summarises i.a. the set of post-translational modifications. The synergy of all of these mechanisms expands the classically unidirectional 'central dogma of molecular biology' to a complex and cross talking cellular regulatory network [Crick, 1958, Watson, 1965, Crick, 1970, Saletore et al., 2012].

This doctoral thesis demonstrates the first evidence to the best of my knowledge for a regulatory role of RNA modification on centromeric chromatin and thus the functional affiliation of the epitranscriptome and the epigenome.

1.1 Chromatin describes the functional structure of the genome

Chromatin, first described by Walther Flemming in 1878 [Flemming, 1878], constitutes the cellular foundation for epigenomic regulation. The discovery of the 'beads-on-a-string'-like structure of chromatin fibers displays the simplest level of chromatin organisation and broke the first ground for modern perspectives of chromatin biology [Kornberg, 1974, Olins and Olins, 1974]. Chromatin has classically been described as a nucleoprotein structure that provides structural stability and compaction to the genetic material [Schultz, 1941, Pierce, 2012]. Meanwhile, increasing evidence indicates that in addition to protein and DNA, RNA is an integral part of chromatin [Rodríguez-Campos and Azorín, 2007]. Furthermore, it is known that the function of chromatin goes far beyond structural stability [Olins and Olins, 2003, Margueron and Reinberg, 2010]. The structural organisation of chromatin is schematically summarised in Figure 1.1. The genetic information in form of DNA builds up a linear double helix as revealed by Watson, Crick and others [Watson and Crick, 1953, Wilkins et al., 1953, Franklin and Gosling, 1953]. The formation of nucleosomes stabilises this fragile structure and enables storage into cellular nuclei [Woodcock, 1973, Kornberg, 1974, Olins and Olins, 1974, Kornberg, 1974, Dekker and Oudet, 1975]. The nucleosome is the fundamental nucleoprotein subunit of chromatin. The 'core particle' comprises146 base pairs wrapped twice around an octameric histone core, which consists of four pairs of the histones H2A, H2B, H3, and H4, associated with histone H1. 'Linker DNA' connects the core particles as chromatin fibers [van Holde et al., 1974, Wolffe and Hayes, 1999, Richmond et al., 1984, Luger et al., 1997]. Supercoiling of these structures (30-nm-fibers) [Everid et al., 1970, Woodcock, 2005] and further looping into 250 nm thick fibers allows higher-order organisation of chromatin into evolutionarily conserved topologically associated domains (TADs) [Rabl, 1885, Boveri, 1909, Dixon et al., 2016]. The highest degree of chromatin com-

paction is achieved by further supercoiling into 700 nm-wide chromatids. This chromosomal organisation is essential for chromosome segregation during mitosis.

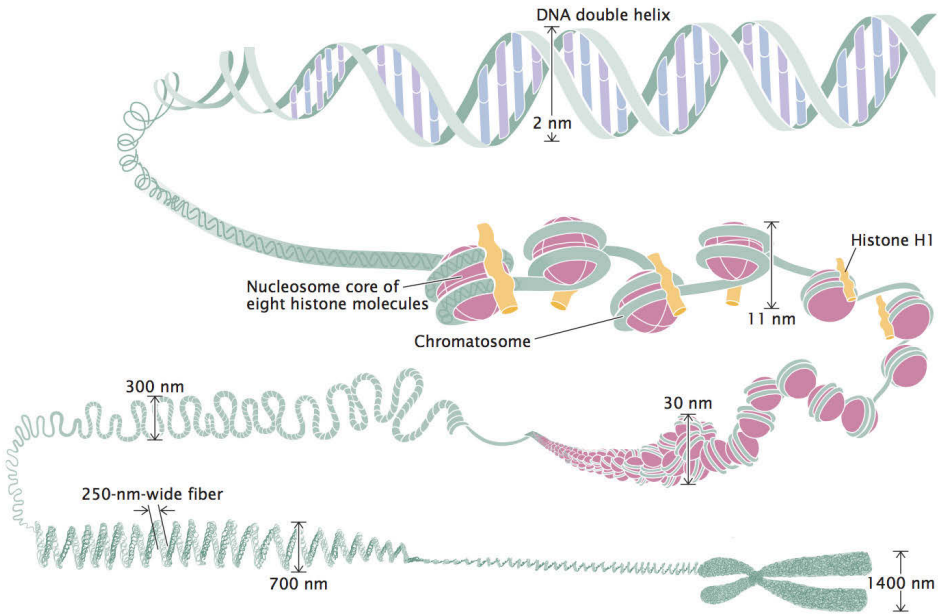


Figure 1.1: Chromatin is a highly complex structure of DNA, RNA and proteins organised in several levels of compaction. Double helical DNA is wrapped around octameric histones to form nucleosomes that associate with histone H1 as chromatosomes. This 'string of beads'-like nucleoprotein structure winds into a 30 nm chromatin fiber that is further looped (300 nm) and packed into 250 nm fibers. Tight chromatid structures arise from further supercoiling and folding, the maximum of chromatin compaction is observed in mitotic chromosomes. Modified from [Pierce, 2012].

For purposes of gene expression regulation, chromatin of the eukaryotic interphase nucleus exists in the forms of euchromatin and heterochromatin, which from a cytological point of view also describe two different nuclear compartments [Heitz, 1928, Grewal and Jia, 2007]. Simplified, the more diffuse and gene-rich euchromatin can by described as an open and generally transcriptionally accessible state or compartment, although it also includes a number of repressed genes [Hwang et al., 2001]. The highly condensed and gene-poor heterochromatin is generally transcriptionally repressed [Huisinga et al., 2006], however there is increasing evidence that some regions can be actively transcribed, as discussed below [Hall et al., 2012, Morris and Mattick, 2014]. The different levels of chromatin structure, including RNA, are mitotically heritable

and provide a complex network of functional regulation for purposes of storage, compaction, and gene regulation [Probst et al., 2009, Allis and Jenuwein, 2016]. Gene expression is regulated by a number of different interconnected mechanisms that include RNA as a functional chromatin component.

1.2 Epigenomics and the epigenetic inheritance of chromatin

In 1902, the Bovery-Sutton chromosome theory correctly proposed chromosomes as the carriers of genetic material [Sutton, 1902, Sutton, 1903, Boveri, 1904]. In 1993, chromatin was identified as a carrier of an additional, epigenetic, layer of information [Turner, 1993]. The development of genome-wide chromatin profiling technologies allowed the analysis of complex chromatin regulatory mechanisms that are recapitulated as epigenomics [Allis and Jenuwein, 2016]. The five key mechanisms reflecting the concept of epigenomics are summarised in Figure 1.2. Histone modifications, DNA methylation, histone variants, chromatin remodelling, and ncRNAs are interdependently regulated and alter chromatin structure and function. Epigenomic mechanisms directly affect processes such as imprinting, X-inactivation, enhancer and promoter interaction, heterochromatin formation and maintenance, repeat and mobile element silencing, DNA repair, chromatin remodelling, transcriptional regulation, and higher-order chromatin organisation [Allis and Jenuwein, 2016].

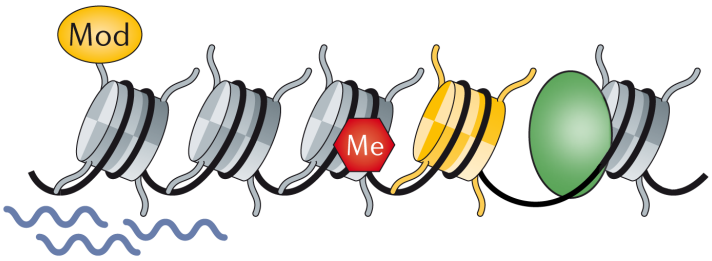


Figure 1.2: Epigenomic mechanisms regulate chromatin organisation. Schematic chromatin section consisting of five nucleosomes summarising epigenomic key mechanisms: Histone modifications (Mod), DNA methylation (Me), histone variants (yellow), histone remodelling (green), and chromatin-associated ncRNA (blue lines) are inter-dependently altering chromatin structure and function. Modified from [Allis and Jenuwein, 2016].

1.2.1 DNA methylation

DNA modifications were discovered in 1948 [Hotchkiss, 1948] and their regulatory roles in gene expression were proposed in 1975 [Holliday and Pugh, 1975]. The most prominent, reversible, and dynamic DNA modification, also known as the 'fifth base', is cytosine-5 methylation (5mC). 5mC can be oxidised to create further modifications, which have been suggested to lead to demethylation of the respective cytosines [Breiling and Lyko, 2015]. This covalent base modification of mostly (but not exclusively) CpG dinucleotides is associated with transcriptional regulation, and is critical for heterochromatin formation and genome stability [Razin and Riggs, 1980, Wigler et al., 1981, Bird et al., 1985, Goll and Bestor, 2005, Klose and Bird, 2006, Lister et al., 2009, Jones, 2012, Patel, 2016]. 5mC is catalysed by proteins of the highly conserved family of DNA methyltransferases (Dnmts) [Goll and Bestor, 2005] (Figure 1.10 a, b). The DNA methyltransferases DNMT3A and DNMT3B establish *de novo* methylation during embryonic development, which is partially regulated by DNMT3L. DNMT1 maintains this methylation pattern based on the methylated template strand during replication and thus beyond cell division [Goll and Bestor, 2005]. In contrast to these Dnmts functioning in DNA methylation, Dnmt2 has been described as a specific tRNA methyltransferase [Goll et al., 2006]. The importance of DNA methylation is confirmed by the essential roles of DNA methyltransferases in development of many organisms [Lei et al., 1996, Li et al., 1992, Okano et al., 1999]. However, DNA methyltransferases and hence 5mC DNA modification are not conserved throughout all species [Goll and Bestor, 2005, Raddatz et al., 2013] (Figure 1.10 a).

1.2.2 Post-translational histone modifications and histone variants

A multitude of covalent post-translational histone modifications such as methylation, acetylation, and phosphorylation provide a complex and interdependent regulation system for chromosomal function [Jenuwein and Allis, 2001, Probst et al., 2009]. The modification of histone tails is catalysed by a large number of enzymes that share evolutionarily conserved domains [Tschiersch et al., 1994, Brownell et al., 1996]. The variety of modifications and the discov-

ery of modification binding proteins led to the hypothesis of the 'histone code' [Turner, 1993, Dhalluin et al., 1999, Strahl and Allis, 2000]. The combinatorial patterns of histone modifications mediated by 'writers', 'readers', and 'erasers' that remove modifications provide an additional layer of gene regulation [Jenuwein and Allis, 2001].

The 'histone code' is a part of the 'epigenetic code', which summarises all kinds of epigenetic mechanisms, including histone variants and nucleosome repositioning [Allis and Jenuwein, 2016]. Histone variants often differ by only a small number of amino acids from the canonical major histones, but are differently regulated [Tagami et al., 2004, Smith and Stillman, 1989]. Non-canonical histones are thought to provide important variation to the chromatin fibre [Becker and Workman, 2013]. A highly conserved example is the histone H3 variant centromere protein A (Cenp-A), which is proposed to be the major factor that defines the epigenetic identity of centromeres [Henikoff and Smith, 2015].

1.2.3 Complexity of epigenomic regulation of chromatin

Analogous to euchromatin and heterochromatin, epigenetic modifications can be similarly categorised as 'on' or 'off'. For example, histone H3 lysine 4 di-methylation (H3K4me2) and and histone H3 lysine 9 di-methylation (H3K9me2) have been found anti-correlated in active and inactive chromatin, respectively [Noma et al., 2001, Litt et al., 2001]. The discovery of 'bivalent chromatin', which are domains containing both active and repressive marks, however, demonstrated a more complex and probably dynamic situation in chromatin regulation [Bernstein et al., 2006, Azuara et al., 2006]. Combined epigenomic profilings revealed distinct functional modification patterns of chromatin [Kundaje et al., 2015]. For example, at least nine chromatin states are differently distributed over the *Drosophila* genome [Kharchenko et al., 2011]. 'Writers', 'readers', and 'erasers' exist for both DNA and histone modifications, which are functionally connected via different kinds of effector proteins. This provides a complex regulatory network of chromatin function.

1.2.4 Chromatin-associated non-coding RNAs

A variety of long and small non-coding RNAs (ncRNAs) are functionally connected to chromatin [Morris and Mattick, 2014]. ncRNAs can function as epigenetic key players guiding the epigenetic machinery. RNAs, due to their nature as nucleic acids, provide the potential to serve as 'readers' that precisely recognise genomic loci. The key to this hypothesis was the discovery of RNAi-type small nuclear RNAs that direct heterochromatin formation, as described below [Hall et al., 2003, Volpe, 2002, Taverna et al., 2002, Mochizuki et al., 2002, Martienssen and Moazed, 2015]. RNA as a general chromatin regulator has been confirmed by the discovery of RNAi-independent ncRNAs that play a role in heterochromatin formation. transcription of such ncRNAs possibly serves as a sensor to sustain genome stability, since accumulation of ncRNAs induces nucleation of heterochromatin [Reyes-Turcu et al., 2011].

This introduces a putative sixth epigenomic mechanism: transcription itself. As reviewed below, indeed transcriptional activity can regulate chromatin regions such as centromeres. In conclusion, transcription and transcripts are fundamental aspects of epigenetic inheritance, since RNA provides a precise sequence-specific interplay with DNA [Liebers et al., 2014, Allis and Jenuwein, 2016].

1.3 Centromeres and pericentromeric heterochromatin

Walter Flemming first described centromeres as the primary constriction sites of mitotic chromosomes [Flemming, 1882]. Centromeres are the sites of kinetochore formation and spindle attachment and essential for chromosome segregation during mitosis (Figure 1.3). Centromeric chromatin is defined by repetitive DNA sequences and specific epigenetic factors, including the histone variant centromere protein A (Cenp-A). Increasing evidence emerges that centromeres are transcriptionally active sites and non-coding RNAs (ncRNA) physically and functionally interact with centromeres. The environment around centromeres is pericentromeric constitutive heterochromatin. In general, deregulation of such heterochromatin factors lead to chromosome segregation defects

and thus impaired centromeric function [Kellum and Alberts, 1995, Allshire et al., 1995, Dernburg and Sedat, 1996, Ekwall et al., 1997, Melcher et al., 2000].

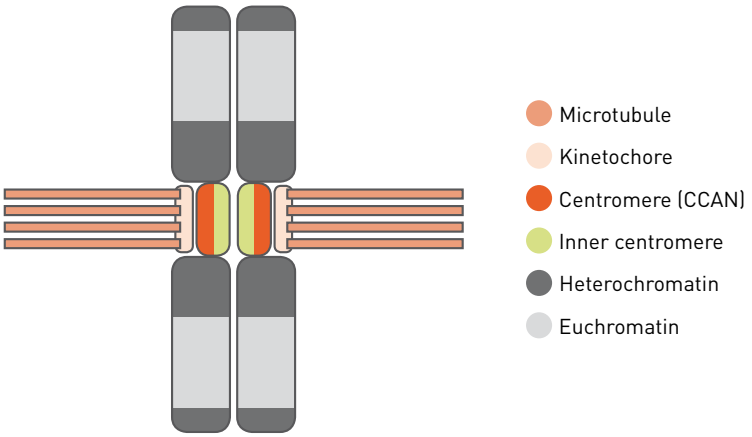


Figure 1.3: Centromeres are embedded in pericentromeric chromatin of mitotic chromosomes. Maximal condensed chromatin of the mitotic chromosome is organised in two chromatids. The chromosome arms are predominantly euchromatic (light grey), constitutive heterochromatin (dark grey) can be found at telomeres and pericentromeric chromatin. The latter embeds centromeric chromatin that is composed of alternating euchromatic H3K4me2 (green) and Cenp-A nucleosomes (orange). The centromere builds the basis for the kinetochore complex (rose) that is the attachment site for microtubules (brown) to separate chromosomes during mitosis.

1.3.1 Centromeres are embedded in tightly packed pericentromeric heterochromatin

Heterochromatin can be subdivided into the constantly condensed constitutive and the developmentally dynamic facultative heterochromatin, and various intermediates in between. The constitutive form is constantly in the heterochromatic state in all cell types of a given organism, whereas facultative heterochromatin is developmentally flexible to switch to euchromatic states in specific cell types [Grewal and Jia, 2007]. Constitutive heterochromatin is essential for genomic stability because it provides structural robustness needed in mitosis and meiosis, and it silences harmful mobile DNA elements [Sentmanat et al., 2013].

In *Drosophila*, large blocks of pericentromeric heterochromatin flank the centromeres of all chromosomes (Figure 1.3). Overall, the amount of constitutive heterochromatin is about 50% in flies, mice and humans [Hoskins et al., 2002, Perrod and Gasser, 2003]. Hallmarks of constitutive heterochromatin are the enrichment of histone lysine methyltransferases (e.g. Su(var)3-9 (suppressor of variegation 3-9) in *Drosophila*), the corresponding H3K9me2/3 marks, and the enrichment of the H3K9me2/3-binding protein HP1 [Rea et al., 2000, Tschiersch et al., 1994]. Additionally, DNA methylation is widely spread in heterochromatic domains in some species. In mammals and plants, pericentromeric heterochromatin is CpG-poor but hypermethylated [Patel, 2016].

Remarkably, the *Drosophila* genome has retained *Dnmt2* as its only Dnmt gene and lacks distinct 5mC patterns on DNA [Raddatz et al., 2013]. Thus, *Drosophila* does not rely on 5mC as an epigenetic inheritance mechanism.

1.3.2 Centromeric and pericentromeric DNA is diverse but highly repetitive

Centromeric DNA is generally highly repetitive and AT-rich but heterogenous [Biscotti et al., 2015, Aulner et al., 2002, Cortés and Azorín, 2000] (Figure 1.4). In *Drosophila*, the majority of pericentric and centromeric chromatin is constituted of large arrays of satellite repeats with low complexity interspersed with complex sequences such as transposable elements (TEs) [Le et al., 1995].

Large regions of centromeric and pericentromeric DNA sequences in *Drosophila* are not assembled yet due to their repetitive composition (D.melanogaster Release 6 reference genome sequence, [Hoskins et al., 2015]) (Figure 1.7). This is also true for the human (peri-) centromeric sequences [Aldrup-MacDonald and Sullivan, 2014], which leads to a lack of sequence information at and around centromeres in both organisms.

Repetitive sequences are a common feature of constitutive heterochromatin and centromeres in different organisms, however the sequences differ between species and even within a single organism. In *Drosophila*, specific repetitive sequences could be distinguished at different chromosomes [Abad and Villasante, 2001, Agudo et al., 1999, Lohe et al., 1993, Méndez-Lago et al., 2009, Sun et al., 1997, Abad et al., 1992]. For example, dodeca satellite consists of short 11/12 bp G- or C-rich tandem repeats encoded on the third chromosome [Abad

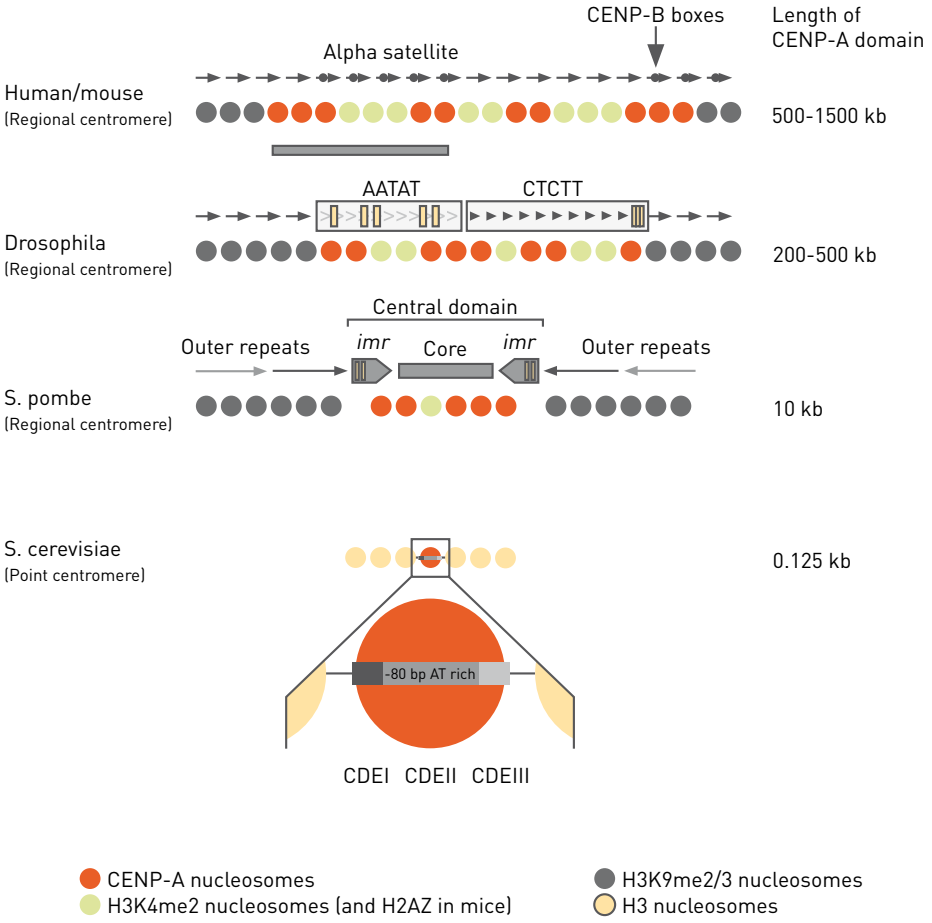


Figure 1.4: Centromeric DNA sequences are not evolutionary conserved. Schematics of regional centromeres of human/mouse, *Drosophila* and *S.pombe* are compared with each other and a point centromere of S.cerevisiae: The underlying DNA of centromeres is not conserved. It varies distinctly in size and sequence, but share a high degree of repetitive sequences (arrows and boxes mark the different sets of satellite repeats). In contrast, the general nucleosome competition is shared by centromeres of different species. In regional centromeres, Cenp-A containing nucleosomes (orange) are interspersed by euchromatic H3K4me2 nucleosomes (green). This centromeric chromatin is embedded in constitutive pericentromeric H3K9me2/3 containing chromatin (dark grey). The much smaller point centromere of S.cerevisiae consists of only one Cenp-A embedded in H3 nucleosomes. Sizes of the centromeres are labelled right next to the schematics. Modified from [Allshire and Karpen, 2008].

et al., 1992]. On the contrary, satellite-III repeats (SATIII) consist of 359 bp-long AT-rich single units that are encoded at the X chromosome [Lohe et al., 1993, Sun et al., 2003, Blattes et al., 2006].

The heterogeneous DNA sequences of centromeres contrast the high conservation of centromeric proteins [Henikoff, 2001]. A structural rather than a sequence-dependent motif might facilitate this co-evolution [Rošić and Erhardt, 2016, Schueler et al., 2001, Malik and Henikoff, 2001]. Indeed, non-canonical secondary structures have been detected within human and *Drosophila* centromeric repeats [Gallego et al., 1997, Garavís et al., 2015, Garavís et al., 2015].

1.3.3 Centromere identity is epigenetically regulated

An important aspect of centromere identity is that the function of centromeres in mitosis is conserved, whereas the DNA sequences are not. Thus genomic competency through repetitive sequences cannot be sufficient to accomplish centromere identity [du Sart et al., 1997, Williams et al., 1998, Rocchi et al., 2012, Han et al., 2009, Agudo et al., 2000, Sullivan and Willard, 1998, Fisher et al., 1997, Steiner and Clarke, 1994]. The majority of eukaryotic centromeres are complex regulated regional centromeres from *S.pombe* to humans as summarised in Figure 1.4 [Henikoff, 2001]. These centromeres require additional, epigenetic mechanisms to structural features of repetitive DNA sequences to provide the specificity of centromeric chromatin [Hayden et al., 2013, Rošić and Erhardt, 2016, ichirou Ohzeki et al., 2015]. An exception is the point centromere of the budding yeast S.cerevisiae that consists of only one Cenp-A nucleosome that is defined by the underlying DNA sequence.

A key factor in centromere identity is Cenp-A, a highly conserved histone H3 variant that distinctly marks centromeres during mitosis [Earnshaw et al., 2013, Talbert et al., 2012]. Depletion of Cenp-A leads to severe chromosome segregation defects, which is essential for development and disease [Allshire and Karpen, 2008, Carroll and Straight, 2006]. Cenp-A nucleosomes differ from canonical H3 nucleosomes. They bind less tightly to DNA but are stabilised by Cenp-C, another centromere-specific protein [Falk et al., 2015, Schuh et al., 2007, Foltz et al., 2006, Guse et al., 2011, Carroll et al., 2010] (Figure 1.5). At centromeres, blocks of H3 nucleosomes are interspersed with blocks of Cenp-

A nucleosomes [Blower et al., 2002], which provide the epigenetic competence to establish centromeric chromatin (Figure 1.4) [Allshire and Karpen, 2008, Black and Cleveland, 2011, Fachinetti et al., 2013], and to position Cenp-A nucleosomes at the opposing sides of sister chromatids (Figure 1.5).

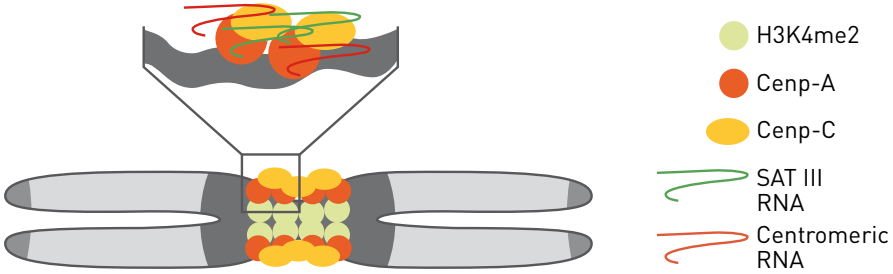


Figure 1.5: Repetitive non-coding transcripts associate with centromeres in *Drosophila*. Constitutive heterochromatin and mitotic chromosomes are not completely transcriptionally inactive. SATIII is a repetitive lncRNA transcribed by RNAPII from pericentromeric chromatin that associates to centromeres during mitosis. Besides SATIII another lncRNA called dodeca has been described in *Drosophila*. The exact molecular function of these transcripts, however, remains elusive. Likewise obscure is the existence and function of putative further centromere-associated transcripts.

Canonical H3 nucleosomes within centromeric chromatin are differently post-translationally modified than the surrounding pericentromeric nucleosomes. Specific and canonical, active and repressive marks modify centromeric H3 and Cenp-A nucleosomes [Sullivan and Karpen, 2004, Rošić and Erhardt, 2016]. This underlines the specific epigenetic nature of centromeres.

1.3.4 Centromeric function depends on flanking pericentromeric heterochromatin

The centromeric H3 variant Cenp-A has the capacity to generate functional ectopic centromeres upon Cenp-A overexpression [Heun et al., 2006]. Remarkably, heterochromatin boundaries are hotspots for Cenp-A islands and *de novo* kinetochore formation [Olszak et al., 2011], which demonstrates a functional interaction between the rather euchromatic centromeres and the heterochromatic pericentromeres.

In yeast, heterochromatic repeats are a prerequisite for centromere formation [Folco et al., 2008]. Heterochromatin factors like HP1 and Su(var)3-9 homologues as well as RNAi components are needed for the initial Cenp-A

deposition to establish centromeric chromatin [Folco et al., 2008]. This is also the case in *Drosophila*, where mutations of an Argonaute protein leads to disrupted heterochromatin silencing at pericentric sites, which was accompanied by Cenp-A mislocalisation [Deshpande et al., 2005]. Accordingly, mutations of general heterochromatic factors and RNAi components in particular lead to chromosome segregation defects in both yeast and *Drosophila* [Kellum and Alberts, 1995, Allshire et al., 1995, Dernburg and Sedat, 1996, Ekwall et al., 1997, Hall et al., 2003, Volpe et al., 2003, Deshpande et al., 2005, Deshpande et al., 2006].

1.3.5 Heterochromatin formation is a complex interplay of multiple mechanisms

Constitutive heterochromatin is described as a non-homogenous, mosaic-like structure [Huisinga and Elgin, 2009, Sentmanat et al., 2013]. In *Drosophila*, heterochromatin formation is a complex interplay of multiple mechanisms that are not entirely understood. Several studies indicate an important role of small RNA-dependent pathways that may be related to the more comprehensively examined mechanisms in yeast and plants. The concept of RNAi-mediated heterochromatin formation is hence described for *S.pombe* in the next section.

RNAi-mediated heterochromatin formation in yeast

RNA-directed transcriptional gene silencing (TGS) describes the repression of transcription of a particular gene or genomic region. In *S.pombe*, TGS is an important heterochromatin nucleation mechanism, leading to heterochromatin formation through recruitment of heterochromatin-related factors [Slotkin and Martienssen, 2007].

The RNA interference (RNAi) machinery processes heterochromatic transcripts into RNAs that guide the RNA induced transcriptional silencing complex (RITS) to transcription sites, which in turn leads to recruitment of epigenetic factors that modify the targeted chromatin domains [Volpe and Martienssen, 2011]. In *S.pombe*, centromeric sequences are transcribed by the RNA polymerase II (RNAPII). The RITS complex targets these centromeric transcripts via small-interfering RNAs (siRNAs) that are associated with the

RNAi component Argonaute-1 (Ago1) [Djupedal et al., 2005, Kato et al., 2005]. This recruits two further complexes: the multi-functional histone modification complex (CLRC) and the RNA-directed RNA polymerase complex (RDRC). The CLRC contains a histone methyltransferase (HMT) that methylates H3K9 residues [Irvine et al., 2006]. Subsequent binding of the HP1 homologue Swi6 mediates spreading of heterochromatin [Lachner et al., 2001]. The RDRC generates double-stranded RNA (dsRNA) from centromeric transcripts [Motamedi et al., 2004, Sugiyama et al., 2005], which is processed by the RNase Dicer (Dcr1) and Ago1, which in turn amplify siRNAs and the entire heterochromatin formation process [Colmenares et al., 2007]. In conclusion, the targeting signal principally originates from the target itself [Sentmanat et al., 2013].

piRNA-mediated heterochromatin formation in *Drosophila*

Heterochromatin assembly is also connected to RNAi-mediated TGS in *Drosophila* [Peng and Karpen, 2007, Huisinga and Elgin, 2009]. Argonaute proteins of the Piwi clade and piwi-interacting RNAs (piRNAs) conduct TGS in the germ line. Piwi-associated piRNAs transcriptionally repress transposons through heterochromatin assembly [Kuhfittig et al., 2001, Gunawardane et al., 2007, Brennecke et al., 2007, Klenov et al., 2011]. Therefore, establishment and spreading of H3K9me3 and HP1 are controlled by elements of the RNAi pathway, just as in yeast [Le Thomas et al., 2013, Sienski et al., 2012]. Importantly, disruption of the RNAi machinery deregulates histone marks, which leads to an accumulation of RNAPII-dependent pericentromeric transcripts [Usakin et al., 2007]. Also in mice, the depletion of Dicer caused increased levels of heterochromatin-originated major satellite transcripts [Kanellopoulou et al., 2005, Murchison et al., 2005], which indicates a conservation of RNA-mediated chromatin silencing in mammals.

In addition, not only piRNAs but also endogenous siRNAs have been shown to target heterochromatin [Wang and Elgin, 2011, Fagegaltier et al., 2009]. The RNAi-components Dicer-2 (Dcr2) and Argonaute-2 (Ago2) generate short-interfering RNA (siRNA) from endogenous or exogenous dsRNA [Förstemann et al., 2007, Kawamura et al., 2008, Ghildiyal et al., 2008]. siRNA-mediated post-transcriptional gene silencing (PTGS) in the cytosol is well established in

Drosophila, which is less clear for TGS in the nucleus. However, chromatin-associated functions at insulators or in transcriptional regulation have previously been demonstrated for Ago2 and Dcr2 [Moshkovich et al., 2011, Cernilogar et al., 2011]. Indeed, Ago2 mutations lead to defects in pericentric heterochromatin formation and hence to distinct mitotic defects [Deshpande et al., 2005]. Strikingly, disruption of the siRNA pathway confirmed the impact on heterochromatin regulation [Fagegaltier et al., 2009]. Although heterochromatin patterns are disturbed in RNAi mutants, pericentromeric heterochromatin is not completely depleted. This indicates the presence of multiple mechanisms for heterochromatin formation in the fly, which may be redundant to each other [Sentmanat et al., 2013].

Constitutive heterochromatin is established in early embryogenesis and maintained throughout development [Rudolph et al., 2007], since genomic stability and faithful chromosome segregation need to be guaranteed throughout development. At least a subset of heterochromatin marks may be established by the piRNA pathway in the embryo [Pal-Bhadra et al., 2004, Brower-Toland et al., 2007, Rouget et al., 2010, Foe and Alberts, 1983, Lu et al., 1998, Huisinga and Elgin, 2009], which is probably maintained by the siRNA- and other alternative or complementing pathways.

RNAi-independent mechanisms of heterochromatin regulation

Constitutive heterochromatin is heterogeneous and regulated in a complex fashion. TEs are widespread targeted by the RNAi-pathway, as described above. Alternatively to RNA-directed silencing mechanisms, particular proteins can recognise specific, complex and repetitive DNA sequences and participate in heterochromatin regulation in *Drosophila* [Aravind, 2000, Aulner et al., 2002, Filion et al., 2010, Smith and Weiler, 2010]. Also the transcriptional activity of pericentric major satellite repeats has been connected to HP1-dependent but RNAi-independent heterochromatin formation [Maison et al., 2011], which is supported by the role of major satellite transcripts in the formation of mouse embryonic chromocentres [Probst et al., 2010].

Besides *trans*-acting RNAs and proteins, *cis*-acting genomic elements have been demonstrated to be able to induce HP1-dependent heterochromatin assembly [Haynes et al., 2006, Sentmanat and Elgin, 2012]. Genomic elements,

RNA-directed pathways, and protein effectors presumably regulate heterochromatin formation and maintenance in an interdependent fashion.

1.3.6 Heterochromatin boundaries protect centromeres from spreading pericentromeres

The functional dependency of centromeres on pericentrodmeric chromatin is reviewed above. Further, the ability of heterochromatic marks to spread into euchromatin can compromise centromeric stability. Chromatin boundaries that prevent spreading can be DNA sequence elements that act as higher-order insulators or *cis*-acting barriers [Sun and Elgin, 1999]. In yeast, boundary elements have been found to separate centromeric from pericentromeric chromatin [Donze, 2012], and ectopic centromeres can emerge at heterochromatin boundaries in *Drosophila* [Olszak et al., 2011]. These findings demonstrate important roles of structural chromatin elements for centromere integrity.

In *S.pombe*, tRNA genes (tDNA) are encoded at the junctions between centromeric and non-centromeric chromatin (Takahashi et al. 1991, Kuhn et al. 1991) and bind components of the RNAi machinery [Cam et al., 2005]. These genes appear in clusters and function as *cis*-acting chromatin barriers to protect centromeric chromatin [Partridge et al., 2000, ichi Noma et al., 2006, Scott et al., 2006]. Importantly, centromeric tDNA is actively transcribed [Bernard et al., 2001] and the act of transcription is probably necessary for the barrier function [Scott et al., 2006] (Figure 1.6). Interestingly, also the association of tRNA genes with centromeres in *trans* is transcriptionally regulated [Iwasaki et al., 2010]. Non-tDNA chromatin boundaries also rely on active transcription and on the interplay of boundary-encoded lncRNA with HP1 and H3K9me2 [Keller et al., 2013, Stunnenberg et al., 2015].

Similarly in human cells, RNAPIII-related elements participate in insulation and barrier function [Willoughby et al., 2000, Raab et al., 2011]. The degree of functionality of tDNA as insulators correlates with the number of tRNA genes in an array [Ebersole et al., 2011].

In the fly, a multitude of different non-tDNA insulating elements have been discovered [Gaszner and Felsenfeld, 2006]. However, the highly repetitive pericentric and centromeric DNA sequences are not entirely assembled, which leads to a lack of sequence information as displayed in Figure 1.7 (supplements B.1).

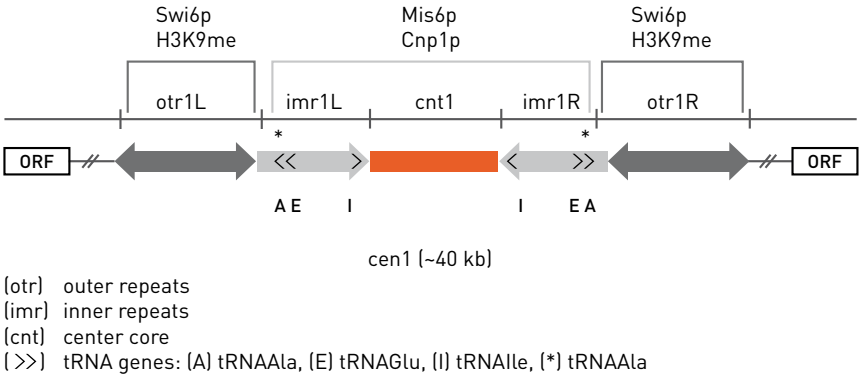


Figure 1.6: Actively transcribed tRNA genes function as chromatin barriers in yeast. Schematic of *S.pombe* centromere and neighboring chromatin elements: The Cenp-A (Cnp1p) and Mis6p containing center core (cnt, orange) is flanked by inner repeats (imr, grey) that contain tRNA genes (arrowheads) encoding tRNAAla (A), tRNAGlu (E) and tRNAIle (I). The outer repeats (otr) are represented by bidirectional arrows (dark grey) that consist of H3K9me nucleosomes and associated HP1 (Swi6p). (Modified from [Haldar and Kamakaka, 2006].)

Nevertheless, single tRNA genes and large tRNA gene clusters are encoded within repetitive pericentric chromatin. Similarly to yeast and human, these tRNA gene clusters are distributed at the borders of physical domains and probably competent as chromatin insulators [Kuhn et al., 1991, Raab et al., 2011, Sexton et al., 2012, Bortle and Corces, 2012]. Remarkably, the centromeres of both major autosomes (chromosomes 2 and 3) are asymmetrically flanked by tRNA gene clusters (Figure 1.7, supplements B.1), which approve the presence of complement boundary mechanisms as demonstrated for yeast [Keller et al., 2013, Stunnenberg et al., 2015].

1.3.7 Centromeric and pericentromeric chromatin are transcriptionally active

In contrast to the classical picture of silenced chromatin, transcription of centromeric and pericentromeric chromatin is well documented [Hall et al., 2012]. Indeed, heterochromatin of multiple different species is extensively transcribed. Well-documented examples are the above mentioned tDNA barrier elements in yeast and a variety of repetitive lncRNAs [Bernard et al., 2001, Azzalin et al., 2007, Blasco and Schoeftner, 2008, Värv et al., 2010, Hall et al., 2012]. In

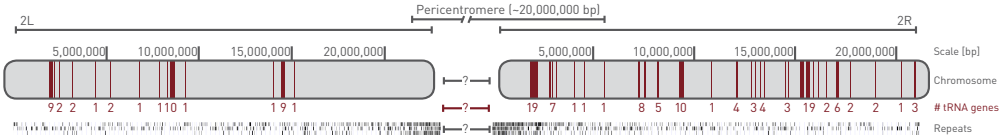


Figure 1.7: tRNA genes in *Drosophila* are spread all over the genome including pericentric chromatin domains. Schematic representation of *Drosophila* chromosome arms 2L and 2R and the predicted constitutive pericentromeric chromatin domain, scale in bp. The position of annotated tRNA genes is represented as red lines with corresponding gene numbers underneath (red). At the bottom repetitive DNA sequences are summarised by black lines. Question marks (?) represent the lack of DNA sequence information of wide peri-/centromeric chromatin regions. Genome loci are taken from GtRNAdb [Chan and Lowe, 2009].

Drosophila, at least 230 protein-coding genes, 32 pseudogenes and 13 ncRNAs are pericentromerically encoded [Smith et al., 2007].

The concept 'pervasive transcription' proposes that the majority of eukaryotic genomes are transcribed [Birney et al., 2007, Clark et al., 2011, Morris and Mattick, 2014]. However, these studies are controversial and a specific role for pervasive transcription needs to be established to distinguish it from transcriptional noise [van Bakel et al., 2010, Palazzo and Lee, 2015]. In this regard, the question whether these transcripts are functionally active is crucial.

Heterochromatic transcripts are functionally active

Heterochromatic transcription is not only possible but also required for a variety of cellular processes. transcription of heterochromatic domains has been linked to chromosomal integrity and transcriptional silencing [Haag and Pikaard, 2011, Berretta and Morillon, 2009, Jacquier, 2009]. Furthermore, dynamic pericentric transcription is connected to stress and a variety of cancers [Jolly et al., 2004, Ugarkovic, 2005, Valgardsdottir et al., 2008, Carone et al., 2009, Eymery et al., 2009, Ting et al., 2011]. The most prominent form of heterochromatic transcription is the generation of siRNAs for RNAi-mediated heterochromatin formation in yeast and other organisms, as described above [Volpe, 2002, Huisinga and Elgin, 2009, Sentmanat et al., 2013]. Moreover, a number of RNAs transcribed from repetitive domains have recently been connected to HP1 [Alekseyenko et al., 2014]. Accordingly, centric or

pericentric transcripts are required for faithful chromosome segregation and connected to global genomic and particularly centromeric stability and hence also essential in mitosis and meiosis [Guenatri et al., 2004, Bouzinba-Segard et al., 2006, Sentmanat et al., 2013, Deshpande et al., 2005].

Centromeric transcripts are needed for chromosome segregation

The euchromatic nature of centromeres suggests active centromeric transcription [Sullivan and Karpen, 2004], which is indeed a common feature of centromeres of many different species [Rošić and Erhardt, 2016] (Figure 1.5). In maize, fly, mouse, and human, repetitive centromeric transcripts are required for centromere functions as Cenp-A loading, kinetochore formation and consequently chromosome segregation, cell cycle regulation, and stress response [Topp et al., 2004, Du et al., 2010, Rošić et al., 2014, Ideue et al., 2014, Bouzinba-Segard et al., 2006, Carmena et al., 2012, Ferri et al., 2009, Wong et al., 2007, Quénet and Dalal, 2014]. A recent report demonstrated a functional connection between replication, chromatin regulation, α-satellite transcription, and centromeric function in mitosis of human cells [Huang et al., 2016]. Therefore, repetitive transcripts are functionally required components of centromeric identity. These transcripts presumably function as chromatin boundaries, molecular chaperones, or factors required for higher-order chromatin structures [Rošić and Erhardt, 2016].

The act of transcription as a regulatory mechanism

On the one hand, centromeric chromatin silences incorporated transgenes, suggesting a repressive chromatin state [Castillo et al., 2007]. On the other hand, centromeric transcription occurs naturally and disruption of this affects the centromeric chromatin composition [Choi et al., 2011, Chen et al., 2008]. Transcription of centromeres and pericentromeres respond differently do different kinds of stress [Hédouin et al., 2017, Jolly et al., 2004, Rizzi et al., 2004, Valgardsdottir et al., 2008, Morozov et al., 2012], which demonstrates specific regulatory mechanisms of repetitive sequences. Indeed, transcription of centromeric sequences is sensitive to different histone modifications that affect Cenp-A loading [Bergmann et al., 2012, Bergmann et al., 2011]. Vice versa, ectopically tethered Cenp-A loading factor CAL1 can recruit RNAPII and induce transcription in *cis* [Chen et al., 2015]. The connection of active transcrip-

tion and centromere function is confirmed by the presence of the active form of RNAPII and transcriptional activators and inhibitors at centromeres [Rošić et al., 2014, Ohkuni and Kitagawa, 2011].

1.3.8 Mitotic chromosomes are actively transcribed

RNA synthesis is cell cycle-dependently regulated and lowest during M-phase [Gottesfeld and Forbes, 1997]. From the 1960s on, transcription was believed to be globally repressed during mitosis [Prescott and Bender, 1962, Taylor, 1960, Terasima and Tolmach, 1963, Davidson, 1964, Fink and Turnock, 1977, Edgar and Schubiger, 1986]. Recent publications, however, indicate that total repression of transcription might not be entirely correct [Chueh et al., 2009, Sciortino et al., 2001]. Chan et al. observed active RNAPII at human mitotic centromeres and described altered α-satellite transcription and decreased centromeric Cenp-C levels upon RNAPII inhibition, which led to chromosome segregation defects [Chan et al., 2012]. Also in *Drosophila*, the detection of the active form of RNAPII at centromeres suggests transcription during mitosis [Rošić et al., 2014]. Furthermore, the chromatin remodelling factor FACT, which facilitates RNAPII transcription, is connected to Cenp-A deposition in yeast and flies, which might also be the case in human cells [Belotserkovskaya, 2003, Chen et al., 2015, Choi et al., 2012, Deyter and Biggins, 2014, Izuta et al., 2006, Quénet and Dalal, 2014].

It is hypothesised that active transcription is generally correlated to the active phase of the respective chromatin domain [Rošić and Erhardt, 2016]. This suggests that active transcription and centromeric transcripts, respectively, are part of a regulatory mechanism of mitotic centromeres. The phenomenon of transcription during mitosis underlines the specific nature of centromeres and suggests unique regulatory mechanisms. Strinkingly, recent reports demonstrated indeed direct roles of RNAPII transcription as a centromeric transport mechanism that recruits cohesin-related factors to the inner centromere, to release paused RNAPII from centromeres, and in Aurora B recruitment to centromeres [Liang et al., 2015, Liu et al., 2015, Blower, 2016].

In summary, these findings demonstrate that active transcription and (peri-) centromeric transcripts themselves are required for centromeric function [Rošić and Erhardt, 2016] (Figure 1.5). Of note, a direct connection of centromeric

RNA and RNA modification in the regulation of centromeres has not been shown. This is surprising because of the highly modified nature of RNAs in general.

1.4 Epitranscriptomics and the regulation of RNA function

In 1869, Friedrich Miescher discovered nucleic acids [Miescher, 1869, Dahm, 2005] and it took nearly a century to phrase the 'central dogma of molecular biology', which excluded nucleic acid modifications as a transmitting mechanism of information [Crick, 1958, Watson, 1965]. RNA was for a long time seen as a simple intermediate between DNA and protein. The discovery of catalytically active and non-coding RNA (ncRNA) changed this view [Fedor and Williamson, 2005, Eddy, 2001].

RNA is a highly modified biological macromolecule. Over 100 RNA modifications have been described so far, many of them discovered decades ago [Limbach et al., 1994, Rozenski et al., 1999, Machnicka et al., 2013]. Epitranscriptomics as a scientific concept, however, started to expand only recently. A milestone in the field was the discovery of an N6-methyladenosine (6mA) demethylase [Jia et al., 2011]. 6mA is the most abundant RNA base modification [Dominissini et al., 2012, Meyer et al., 2012, Niu et al., 2013] and was discovered decades before [Perry et al., 1975, Krug et al., 1976, Wei et al., 1975]. The identification of the core components of the methyltransferase complex and the discovery that 6mA-metylation is negatively correlated with gene expression indicated the epigenetic function of RNA modification [Bokar et al., 1997, Liu et al., 2014]. The recent finding of the dynamic nature of this modification and the identification of 6mA-binding proteins gave rise to the term epitranscriptomics [Zheng et al., 2013b, Zheng et al., 2013b, Schwartz et al., 2013, Wang et al., 2014]. Until now, the definition of epitranscriptomics is less precise than for epigenomics, as the reversibility of RNA modifications and definition of complete sets of 'writers', 'readers', and 'erasers' for a given modification are less well defined. Another point of discussion is the change of sequence: RNA editing can change the base sequence of the RNA, however, does not change the underlying DNA sequence.

In this regard, editing may be categorised as an epigenetic mechanism in the classical sense, changing the phenotype without changing the underlying DNA sequence.

Li and Mason categorise RNA modifications into known reversible and non-reversible modifications and exclude none of them for a possible crosstalk between the epitranscriptome and the epigenome. Both epitranscriptomic and epigenomic processes use similar substrates and mechanisms, and function along common pathways [Li and Mason, 2014]. Publications around the 'epitranscriptome' mostly describe 6mA methylation; however, the entire set of RNA modifications or processing events in a broader sense is to be considered as the epitranscriptome [Meyer and Jaffrey, 2014, Saletore et al., 2013, Saletore et al., 2012, Hussain et al., 2013a, Hussain and Bashir, 2015, Rose et al., 2015, Delatte et al., 2016, Dominissini et al., 2016, Frye et al., 2016, Licht and Jantsch, 2016, Manning and Cooper, 2016]. Of note, a recent study demonstrated the demethylation of tRNAs [Liu et al., 2016], which supports the hypothesis of a general reversibility of RNA modifications [Wang et al., 2014].

Interestingly, key epigenetic regulators such as Dnmt1 are differentially expressed in cells that are impaired for 6mA demethylation [Zheng et al., 2013a]. This indicates a regulatory network between the epigenome and the epitranscriptome as inter-dependent epigenetic processes, which is confirmed by the direct impact of RNA processing via the RNAi-pathway on heterochromatin formation (Section 1.3.5).

1.4.1 tRNAs are highly modified non-coding transcripts

The first ncRNA to be structurally characterised was a transfer RNA (tRNA) in 1965 [Holley et al., 1965]. The canonical function of tRNAs is the translation of the genetic information during protein synthesis. Messenger RNAs (mRNAs) transfer the genetic information of DNA to the cytosolic translation machinery, where ribosomal RNAs (rRNA) and proteins catalyse the tRNA-mediated translation of the genetic code into an amino acid sequence. The typical 'clover leaf' secondary structure was solved by X-ray crystallography [Ladner et al., 1975, Kim et al., 1973] (Figure 1.8), that folds to an 'L-shaped' tertiary structure [Itoh et al., 2013].

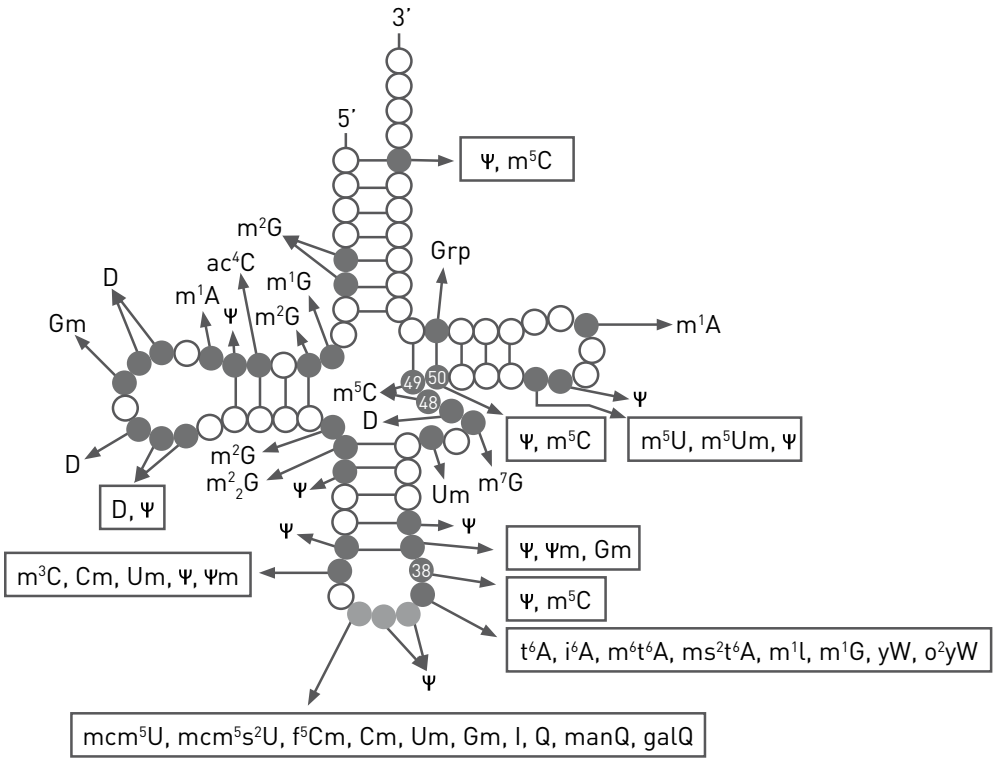


Figure 1.8: tRNAs are highly modified transcripts. Schematic representation of the 'clover leave' structure of tRNAs and summary of known tRNA modifications in eukaryotes. The acceptor stem is formed by base pairing of the 5'- and 3'-termini, and the 3'-CCA tail is required for covalent amino acid loading. The D-arm is a stem loop that probably acts in aminoacyl-tRNA synthetase recognition, whereas the T-arm is required for ribosome recognition. The anticodon stem loop contains the anticodon triplet that decodes the genetic information of an mRNA. Between the anticodon loop and the T-arm is a variable loop that differs in length and composition between tRNAs and species. Circles represent nucleotides in the 'clover leaf' structure of tRNAs. Grey circles mark nucleotides modified in some or all tRNA species, arrows point to the respective kind of modification. Light grey circles represent the nucleotides of the anticodon. Numbers indicate known methylation sites of tRNA methyltransferases Dnmt2 (C38) and NSun2 (C48-50). Modified from [Motorin and Grosjean, 2005].

tRNA biosynthesis

The biosynthesis of tRNAs takes place in the nucleus and cytosol, and is tightly connected to RNA processing and controlled degradation, processes which are well studied in yeast [Wichtowska et al., 2013]. tRNA precursors (pre-tRNA) are transcribed by the DNA-dependent RNA polymerase III (RNAPIII), which is a large complex (0.7 MDa) that consists of nine core and eight regulatory subunits in the periphery. Only five subunits are unique in RNAPIII when compared to RNAPI and II. The RNAPIII machinery consists of three essential complexes: the general transcription factors TFIIIB and TFIIIC that are required for promoter recognition and transcriptional initiation, and the RNAPIII complex itself. RNAPIII-mediated transcription occurs in multiple transcription cycles of initiation, elongation, termination, and re-initiation, which is probably mediated by assembly and disassembly of different subunits. Termination is mediated by oligo(A) stretches that cause polymerase pausing and transcript clearance with a crucial role for nascent RNA [Arimbasseri and Maraia, 2015, Arimbasseri et al., 2013, Braglia et al., 2005]. Following transcription, multiple stable and unstable intermediate pre-tRNAs are produced during the process of tRNA maturation in different sub-cellular locations.

Chemical tRNA modifications are introduced throughout the maturation process in the nucleus and cytoplasm, which is reflected by the different subcellular localisation of RNA-modifying enzymes. Nucleotide modifications impact stability and function and contribute to the surveillance against exosomal degradation in the nucleus. Hypomodified or mutated mature tRNAs are exonucleolytically degraded 5'-to-3' through rapid tRNA decay (RTD) [Alexandrov et al., 2006, Kadaba et al., 2004]. Under stress conditions, tRNA halves are generated by endonucleolytic cleavage, which can be further processed to functionally active tRNA fragments that do not significantly change the amount of mature tRNAs [Thompson et al., 2008, Thompson and Parker, 2009a]. In summary, tRNA biosynthesis, quality control, and turnover are highly regulated throughout the maturation process [Wichtowska et al., 2013].

tRNA modification

The majority of known RNA modifications are described for tRNAs. Out of over 100 different RNA modifications [Machnicka et al., 2013], seven to seven-

teen modified nucleotides have been observed simultaneously on single tRNA transcripts [Phizicky and Alfonzo, 2010] (Figure 1.8). Post-transcriptional modifications are a hallmark of all known tRNAs in all species. Almost all types of RNA modifications, which have been reported so far, can be found on tRNAs [Phizicky and Alfonzo, 2010].

The functions of single non-essential modifications often remain elusive, which may be caused by the concerted or redundant function of several modifications on a single transcript [Phizicky and Alfonzo, 2010], or by highly specialised functions that require sensitive methods and accurate analyses as recently shown for the translational role of 5mC [Tuorto et al., 2015]. In addition, tRNA modifications can regulate the aminoacylation of tRNAs or modulate the decoding capability of the anticodon [Nameki et al., 1995, Johansson et al., 2008, Begley et al., 2007, Tuorto and Lyko, 2016]. The overall tRNA 'clover leaf' structure is not disrupted by the lack of tRNA modifications, instead, modifications are thought to fine-tune and protect the established structure of tRNAs [Motorin et al., 2010].

tRNA methylation

About two thirds of all known RNA modifications are methylations. These can occur at different atoms and positions of the ribose or the base, respectively. A variety of RNA methyltransferases have been identified, of which the vast majority use S-adenosylmethionine (SAM) as a methyl group donor [Motorin and Helm, 2011].

5mC is well known for its widespread appearance on DNA and its function in epigenetic regulation. On RNA, 5mC is phylogenetically distributed among all domains of life (archaea, bacteria, and eukaryotes). It is most prominently present on tRNA and rRNA, whereas the appearance on mRNAs and other ncRNAs and the biological relevance are under debate [Motorin et al., 2010, Blanco and Frye, 2014]. At least three conserved cytosine-5 methyltransferases in higher eukaryotes are known to modify conserved positions of tRNAs. NSun2 (NOP2/Sun domain protein 2) methylates the majority of tRNAs at several positions in yeast and mammals [Motorin Y, 1999, Blanco et al., 2014], whereas a comprehensive substrate analysis in *Drosophila* is lacking. The human NSun6 has recently been shown to methylate tRNAs at position C72 of two tRNAs

[Haag et al., 2015]. Similarly, Dnmt2 has a smaller set of substrates than NSun2 as it methylates C38 of three tRNAs, which is conserved from yeast to human [Müller et al., 2015, Schaefer et al., 2010, Goll et al., 2006].

Loss of single RNA modifications often lacks strong phenotypic consequences. Nevertheless, a variety of molecular functions have been described that reflect the diversity of RNA modifications. For example, hypomodified tRNAs can rapidly be degraded or endonucleolytically cleaved to produce tRNA fragments, as observed upon depletion of the tRNA methyltransferase Dnmt2 [Schaefer et al., 2010, Tuorto et al., 2012].

1.5 Dnmt2 is an evolutionarily conserved tRNA methyltransferase

Dnmt2 is evolutionarily highly conserved and orthologs have been found in 65 species [Schaefer and Lyko, 2010b] (Figure 1.9). Indeed, Dnmt2 is the most conserved member of the Dnmt family of proteins (Figure 1.10 a). Dnmt2 lacks an N-terminal regulatory domain but shares the catalytic domain of Dnmts with Dnmt1, Dnmt3A and B (Figure 1.10 b). Remarkably, a number of species contain solely Dnmt2 and no other Dnmts (Figure 1.10 a). Phylogenetic studies indicate an evolution of Dnmt2 from bacterial DNA methyltransferases and a switch of substrates from DNA to RNA [Jurkowski and Jeltsch, 2011]. Remarkably, Dnmt2 uses a DNA methyltransferase mechanism to methylate RNA [Jurkowski et al., 2008], which is different to other RNA methyltransferases like NSun2 [Liu and Santi, 2000]. This may also be the reason for the ability of Dnmt2 to bind DNA [Dong et al., 2001], however the DNA methylation activity is only weak and distributive [Fisher et al., 2004, Hermann et al., 2003]. Recently, Dnmt2 has been shown to be able to methylate DNA in a tRNA structural context *in vitro*, although the natural relevance remains elusive [Kaiser et al., 2016].

The controversially discussed substrate-specificity of Dnmt2 [Schaefer and Lyko, 2010a] has previously been examined in organisms that lack other Dnmts than Dnmt2. Strikingly, the DNA of 'Dnmt2-only' organisms Schistosoma and *Drosophila* did not show any specific methylation, which was confirmed by mouse embryonic stem cells depleted for Dnmt1 and Dnmt3 (thus only left

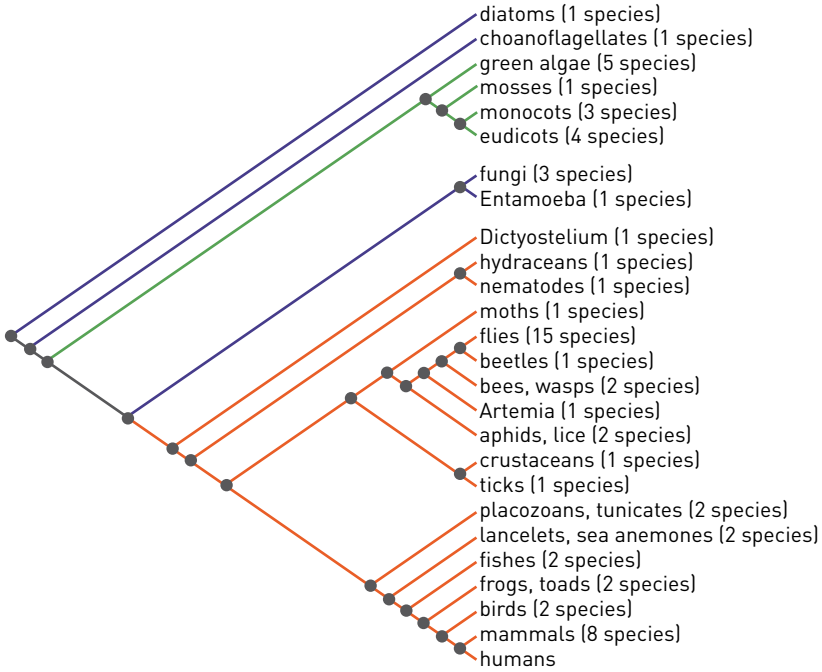


Figure 1.9: Dnmt2 is a highly conserved cytosine-5 RNA methyltransferase. Orthologs of the human Dnmt2 have been found by BLAST (Basic Local Alignment Search Tool) analysis in 65 different species among protists, plants, fungi, and animals . Modified from [Schaefer and Lyko, 2010b].

with Dnmt2) that lost their natural DNA methylation [Raddatz et al., 2013]. Importantly, tRNA methylation activity on C38 in the anticodon loop is robust for $tRNA^{Asp(GTC)}$, $tRNA^{Gly(GCC)}$, and $tRNA^{Val(AAC)}$ [Goll et al., 2006, Schaefer et al., 2010, Khoddami and Cairns, 2013].

1.5.1 Dnmt2-mediated methylation and stress-induced tRNA fragmentation

Hypomodified tRNAs are generally more sensitive to cleavage and degradation [Wichtowska et al., 2013]. Stress appears as an important aspect of tRNA fragmentation since conditions like starvation, heat, cold, oxidative stress, UV, and hypoxia affect tRNA stability [Fu et al., 2009, Hsieh et al., 2009, Lee and Collins, 2005, Schaefer et al., 2010, Thompson et al., 2008, Yamasaki et al., 2009, Zhang et al., 2009].

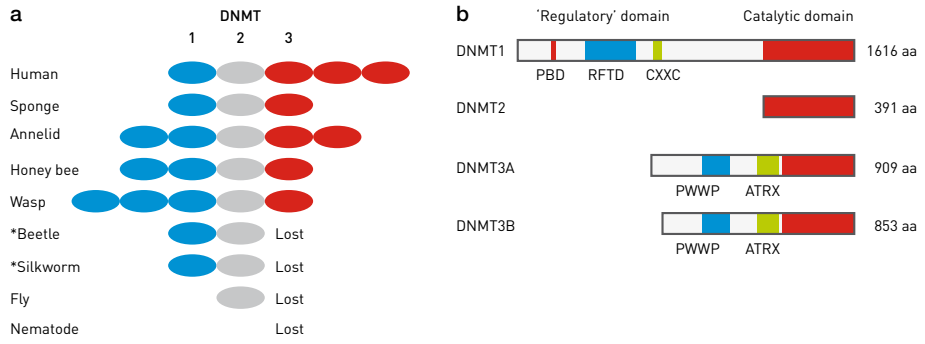


Figure 1.10: Dnmt2 is the evolutionary most conserved member of the Dnmt family of proteins. **a**, Table summarising the distribution of Dnmt proteins in human (typical for mammals) and selected invertebrates. The number of circles represents the number of Dnmt genes per species. *Drosophila* is a Dnmt2-only organism, lacking Dnmt1/2 proteins. Asterisks indicate DNA methylation although Dnmt3 is missing. Modified from [Lyko and Maleszka, 2011]. **b**, Dnmt2 proteins consist solely of the catalytic domain of Dnmt proteins containing ten typical mitofs in canonical order. Modified from [Goll and Bestor, 2005]

tRNA modifications in general and Dnmt2 in particular had both been proposed to be fundamentally connected to the cellular stress response [Durdevic and Schaefer, 2013b, Durdevic and Schaefer, 2013a]. Non-standard laboratory conditions revealed a connection of Dnmt2 to biotic and abiotic stress [Becker et al., 2012, Schaefer et al., 2010, Thiagarajan et al., 2011, Durdevic, 2013]. Strikingly, Dnmt2-mediated C38-methylation protects tRNAs against stress-induced cleavage [Schaefer et al., 2010]. In this way, Dnmt2 regulates the generation of tRNA fragments, which have functionally been connected to small RNA mediated processes [Haussecker et al., 2010]. Correspondingly, Dnmt2 has been shown to affect the siRNA pathway in *Drosophila* [Durdevic et al., 2013b, Durdevic, 2013].

The discovery of and research on defined tissue- and cell-type-specific tRNA-derived fragments proved biological relevance of tRNA fragments beyond tRNA degradation. Remarkably, tRNA fragments are already present in steady-state conditions. The variety of tRNA fragments have repeatedly been categorised, on the basis of their sequence, origin, and function, respectively [Haussecker et al., 2010, Lee et al., 2009, Kumar et al., 2014, Heyer et al., 2012, Li et al., 2012, Li et al., 2008, Yamasaki et al., 2009]. The functional relevance of tRNA fragments is confirmed by the non-random length distribution, the de-

velopmental control of fragmentation, and defined associations to specific proteins [Kumar et al., 2014, Haiser et al., 2008, Lee and Collins, 2005, Li et al., 2008, Durdevic, 2013, Durdevic et al., 2013b, Burroughs et al., 2011, Cole et al., 2009, Haussecker et al., 2010].

1.5.2 Dnmt2-mediated tRNA methylation affects diverse cellular functions

In contrast to its high conservation, Dnmt2 mutations are mostly not accompanied by severe phenotypes such as sterility or lethality under laboratory conditions [Goll et al., 2006, Kunert et al., 2003, Wilkinson et al., 1995]. The conserved molecular function of Dnmt2 is the methylation of tRNAs. The cellular functions, however, are diverse and ambiguous.

Dnmt2 has been connected to protein synthesis regulation through its role in discrimination of near-cognate codons during translation [Tuorto et al., 2015], and its role in post-transcriptional regulation of poly-Asp-containing transcripts via charging rate control of $tRNA^{Asp}$ [Shanmugam et al., 2015]. Both of these mechanisms have been shown to affect particular subsets of proteins. Global effects on translation rates, however, have only been observed upon double mutations of *Dnmt2* and *NSun2*, which causes reduced steady-state levels of tRNAs and synthetic lethality [Tuorto et al., 2012].

Interestingly, Dnmt2 has been connected to paramutations in mice [Kiani et al., 2013], a phenomenon of uncoupling genotype from phenotype, which is transmitted by RNA [Rassoulzadegan et al., 2006]. Although the role of tRNA methylation in this process remains elusive, tRNA stability in mouse sperm is affected by Dnmt2 depletion [Liebers, 2015].

In *Dictyostelium* and *Drosophila*, Dnmt2 controls transposable elements and overall genomic stability [Kuhlmann et al., 2005, Phalke et al., 2009]. Of note, Dnmt2 is a dominant modifier of heterochromatin states in *Drosophila* [Phalke et al., 2009], whereas the exact molecular mechanism is elusive considering the controversially discussed substrate specificity of Dnmt2 [Schaefer and Lyko, 2010b].

1.5.3 tRNA methyltransferases in mitosis

Not much is known about the role of RNA methyltransferases in mitosis. Dnmt2 has been described as a predominantly cytosolic protein that methylates mature tRNAs. Interestingly, a small but distinct fraction of Dnmt2 is associated to the nuclear matrix, which may imply roles in higher order chromatin organisation or compaction. Although Dnmt2 has been reported to have some affinity to chromatin and to the mitotic spindle apparatus during mitosis [Schaefer et al., 2008], a molecular function of Dnmt2 in mitosis remained elusive.

A direct role of Dnmt2 in meiosis has been shown in *Drosophila* male germline stem cells [Yadlapalli and Yamashita, 2013a]. The sister chromatids of the sex chromosomes X and Y naturally segregate non-randomly. This asymmetry is randomised upon Dnmt2 depletion, demonstrating a determining role of Dnmt2 in asymmetric cell division. DNA-independent information is transgenerationally transmitted from parental flies to the zygote, maintained in embryogenesis and all developmental stages, and transferred back to the adult fly germline. The inherited information is proposed to be primed in gametogenesis by Dnmt2, although the molecular mechanism remains elusive. A role in chromatin regulation is confirmed by the observation that Dnmt2 regulates heterochromatin states in *Drosophila* [Phalke et al., 2009].

Of note, internal data of the Erhardt-laboratory found Dnmt2 to be centromere-associated [Rosic, 2013], providing an appealing locus for a functional examination of Dnmt2 in chromosome segregation and the starting hypothesis of this doctoral work.

For another cytosine-5 tRNA methyltransferase, NSun2, more detailed functions in mitosis have been described. Interestingly, NSun2 is Myc-controlled and required for cell-cycle progression [Frye and Watt, 2006]. Moreover, Aurora B regulates the enzymatic activity of NSun2 in a cell cycle-dependent manner [Sakita-Suto et al., 2007]. Similar to Dnmt2, overexpressed NSun2 shows spindle-like structures during mitosis [Schaefer et al., 2008, Frye and Watt, 2006, Hussain et al., 2009]. In contrast to Dnmt2, however, distinct mitotic defects have previously been described in NSun2 depleted cells [Hussain et al., 2009].

For *Drosophila*, a detailed substrate analysis of NSun2 is missing. In yeast, mouse, and human, however, NSun2 methylates cytosines at multiple positions (34, 40, 48 to 50, and 61 to 63) of the majority of tRNA isoacceptor families [Motorin Y, 1999, Brzezicha et al., 2006, Auxilien et al., 2012, Becker et al., 2012, Blanco et al., 2014]. Besides tRNAs, some mRNAs and ncRNAs appeared to display NSun2-dependent methylation patterns [Squires et al., 2012, Hussain et al., 2013b, Khoddami and Cairns, 2013].

Remarkably, the observed mitotic defects appeared to be independent of its methylation activity [Hussain et al., 2009]. Interestingly, NSun2 has functionally been connected to Dnmt2 and tRNA stability [Tuorto et al., 2012], although a common function in mitosis remains elusive.

2

Aims of the Thesis

Centromeric chromatin is epigenetically defined. Non-coding RNAs and active transcription appear as important regulators of centromeric identity and function. However, how centromeric RNAs are regulated remains elusive. The main aim of this doctoral thesis was the identification and characterisation of centromeric RNAs and their regulators. The two key aspects of this thesis were:

1. Identification and characterisation of RNAPIII-dependent transcripts at centromeres.

2. Characterisation of the role of centromeric Dnmt2 and Dnmt2-mediated tRNA methylation during mitosis.

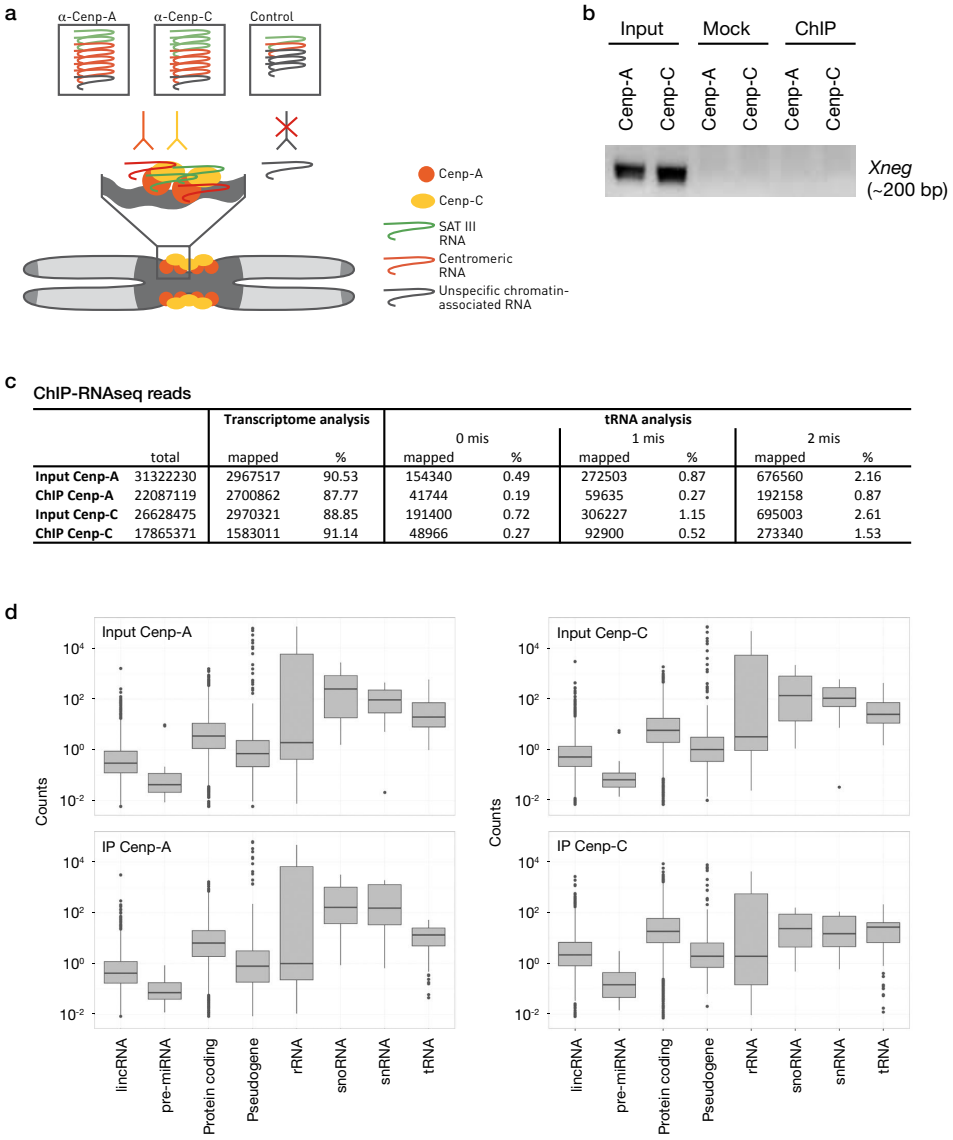
3
Results

3.1 tRNA$^{Gly(GCC)}$ specifically localises to mitotic centromeres in *Drosophila*

Increasing evidence that RNA participates in chromatin regulation emerged in recent years [Rodríguez-Campos and Azorín, 2007, Hall et al., 2012]. The exact role of various chromatin-associated transcripts, however, appears to be diverse and rarely unambiguous. This is particularly true for centromere-associated RNA (cenRNA). Only few publications demonstrate important roles of cenRNA in centromere identity and function [Wong et al., 2007, Chan et al., 2012, Carone et al., 2009, Quénet and Dalal, 2014, Rošić et al., 2014]. However, a general set of centromere-associated RNAs has not been clearly defined and functional mechanisms of identified transcripts remain elusive in most cases.

In need of a method that reliably identifies cenRNA, a centromeric-ChIP-RNAseq protocol was established (in collaboration with Merrit Romeike). Antibodies targeting endogenous Cenp-A in *Drosophila* S2 cells or His-tagged Cenp-C in pMT-Cenp-C-V5-His transfected S2 cells were used as specific factors to pull down centromeric chromatin in two independent setups. The extracted RNA from centromeric ChIP was subsequently analysed by qPCR or RNAseq. The known centromere-associated lncRNA satellite-III (SATIII) served as a positive and beads as a negative control for every qPCR analysis carried out on ChIP experiments (Figure 3.1.a).

As a general negative control and to prove the specificity of the chromatin IP we examined the known Cenp-A-devoid chromatin locus '*Xneg*' (Olszak et



		Transcriptome analysis		tRNA analysis					
				0 mis		1 mis		2 mis	
	total	mapped	%	mapped	%	mapped	%	mapped	%
Input Cenp-A	31322230	2967517	90.53	154340	0.49	272503	0.87	676560	2.16
ChIP Cenp-A	22087119	2700862	87.77	41744	0.19	59635	0.27	192158	0.87
Input Cenp-C	26628475	2970321	88.85	191400	0.72	306227	1.15	695003	2.61
ChIP Cenp-C	17865371	1583011	91.14	48966	0.27	92900	0.52	273340	1.53

Figure 3.1: ChIP-RNAseq analysis reveals centromere-associated RNA in S2 cells. **a**, Schematic illustration of the principle of ChIP-RNAseq of Drosophila S2 cells: Chromatin immunoprecipitation of two different centromere-specific factors (Cenp-A, Cenp-C) or control (beads only) is followed by either deep sequencing or qPCR analysis of centromere-associated transcripts. Centromeric lncRNA SatIII was used as a positive control and (b) *Xneg* DNA as a negative control. (All ChIP experiments were performed together with Merrit Romeike. Bioinformatic analysis of ChIP-RNAseq was done by Chunxuan Shao.) **b**, PCR-analysis of Cenp-A negative DNA locus *Xneg* (by Merrit Romeike). **c**, Table summarising total and mapped read counts and rates (%) of transcriptome-wide and tRNA-specific (Figure 3.2.) analysis of ChIP-RNAseq. Allowed number of alignment mismatches (mis) are indicated. **d**, Transcriptome analysis: Read counts distribution of different RNA types within the indicated libraries (by Chunxuan Shao).

al. 2011). This locus was detected using PCR in input but not in control, Cenp-A, and Cenp-C IPs, respectively (Figure 3.1.b). Input samples represent nuclear transcripts. Library size distributions and per base sequence quality from Illumina sequencing are presented in the supplements (Figure B.2-3).

ChIP-RNAseq provided high read numbers (17 to 13 M reads) for all libraries. The analysis was carried out employing two separate approaches – a transcriptome-wide and a tRNA-specific mapping (in collaboration with Chunxuan Shao of the Thomas Höfer laboratory). The transcriptome analysis revealed 9-12% unmapped reads both in input and ChIP sequencing libraries, likely reflecting non-annotated transcripts (Figure 3.1.c). Classification of mapped reads into different RNA types revealed that all analysed types are present in input and IP libraries and that the global distribution does not differ distinctly between input and ChIP libraries (Figure 3.1.d).

To compare the data at single transcript resolution, tRNAs, representing an independent class of RNAs with a reasonable number of individual genes, were further analysed. tRNAs are highly modified, which needs to be considered for mapping parameters. For example, RNA editing can lead to changes from the annotated reference sequence. Detailed analysis of $tRNA^{Val(AAC)}$ testing various numbers of mapping mismatches indeed revealed substantial effects on mapping efficiencies (Figure 3.2.a), and as a result also on mapping rates (Figure 3.1.c). Coverage for the anticodon loop was completely lost when no mapping mismatches were allowed. Setting the mapping parameters to zero mismatches [Fuchs et al., 2015], and thus disregarding mappable reads, may have led to a loss of information in previous transcriptome methylation studies. However, applying one or two mismatches and thus accounting for the known Adenosin-to-Inosin editing enabled mapping and analysis of the entire Val transcript (Figure 3.2.a).

The optima of allowed mismatches vary for the individual transcripts as displayed by the different correlations in the two IPs (Figure 3.2.b). To account for known and unknown modifications while retaining sufficient mapping specificity, the data was analysed allowing zero, one, or two mismatches and compared. In order to determine differentially abundant transcripts, ChIP-RNAseq data were normalized to input by calculating the proportion of ChIP over input reads. In contrast to the detected similar global read distribution, differences of distinct tRNAs at centromeres were found and comparable for

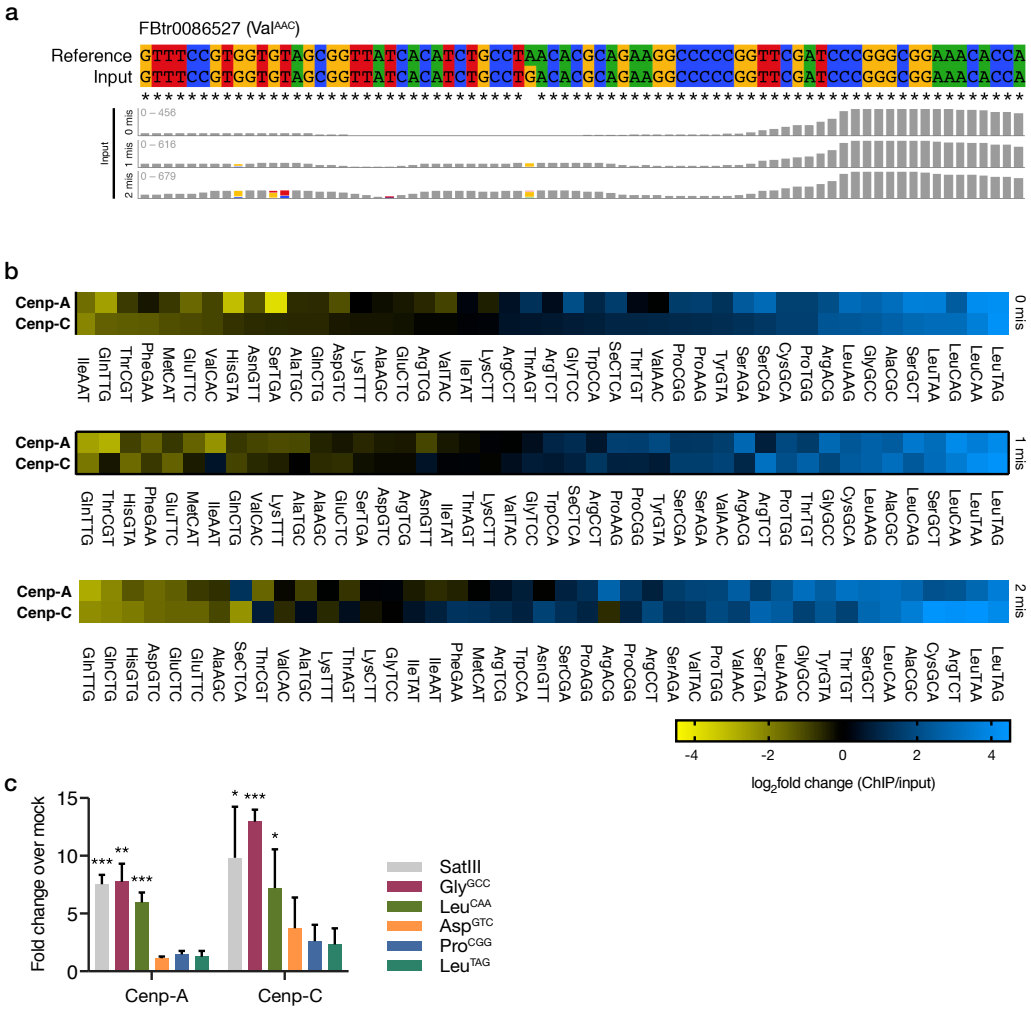


Figure 3.2: Centromere-associated tRNA distribution is distinct from nuclear tRNA in S2 cells. **a**, ChIP-RNAseq: Alignment of tRNA$^{Val(AAC)}$ consensus sequence from input library (2 mis) to the reference sequence. Bars show read distributions allowing zero, one, or two alignment mismatches (mis). Grey bars indicate matched alignments, mismatches are coloured as in the alignment above. **b**, ChIP-RNAseq tRNA analysis: Differential distribution of tRNA read counts (RPM) in ChIP over input libraries ($\log_2$ fold change) allowing zero, one, or two alignment mismatches (mis). tRNA isoacceptor families are ordered by the sum of reads of both IPs. **c**, ChIP-RNA qPCR: Enrichment analysis of selected tRNAs in ChIP over control. Centromeric lncRNA SatIII served as a positive control. (n=3, mean±SD, p<0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001 (****), Student's t-test).

all three mapping settings. Out of 44 different tRNA isoacceptor families, the majority (26) did not change distinctly or were only slightly depleted, 18 families were enriched more than 2-fold ($\log_2$>1) and 10 were enriched over 4-fold ($\log_2$>2) (allowing zero mismatches) (Figure 3.2.b). The differences between ChIP and input libraries and the similarity between the ChIP libraries confirmed the technical suitability of the method. ChIP-RNAseq thus provided a subset of candidates of centromere-enriched tRNAs with respect to the nuclear input of *Drosophila* S2 cells. Of note, sequencing reads were obtained for all tRNAs in the ChIP libraries including centromere IPs. Positive fold-changes ($\log_2$) reflect an enrichment of a given tRNA at centromeres (IP) with respect to overall nuclear levels (input); vice versa, a negative value reflects relative depletion. Values close to zero do not necessarily mean the absence of the respective transcript rather than similar levels compared to the input.

To test the presence and to quantify the amount of particular tRNAs at centromeres, qPCR analysis on ChIP-RNA over control was performed. As a positive control for centromeric enrichment, SATIII was used. Two of the top ten transcripts of the ChIP-RNAseq analysis, namely $tRNA^{Gly(GCC)}$ and $tRNA^{Leu(CAA)}$, were highly enriched in qPCR analysis of centromere IP samples. $tRNA^{Asp(GTC)}$ and $tRNA^{Pro(TAG)}$ were distinctly less enriched but not absent, confirming the ChIP-RNAseq results. Although $tRNA^{Leu(TAG)}$ was the top hit for all three mapping settings, this could not be confirmed for either of the IPs. This discrepancy is likely caused by the high sequence similarity of the different isoacceptors of the $tRNA^{Leu}$ isoform class.

As the gold standard method for cellular RNA localisation is fluorescence in situ hybridisation (FISH), a FISH protocol for locked nucleic acid (LNA) probes on mitotic chromosome spreads was established and analysed (in collaboration with Sarah Doppler). A specific LNA probe targeting the 5' half of $tRNA^{Gly(GCC)}$ was used to confirm this highest confidence tRNA as determined by ChIP-RNAseq and -qPCR. Unspecific scrambled probe and no probe were used to control for the specificity of the $tRNA^{Gly(GCC)}$ probe on mitotic chromosome spreads (Figure 3.3.a-c). The advantages of chromosome spreads over whole cells are higher resolution and better signal-to-noise ratios on isolated chromosomes. Both controls showed a slight level of background staining predominantly caused by the anti-DIG antibody that was used to detect the double-DIG-labelled LNA probes. The specific probe targeting $tRNA^{Gly(GCC)}$,

however, showed distinct centromeric stainings (Figure 3.3.a), which was confirmed by quantification of the mean fluorescence intensities at centromeres (Figure 3.3.b). Noteworthy, unambiguous signals were detected at approximately one third of all centromeres (Figure 3.3.c).

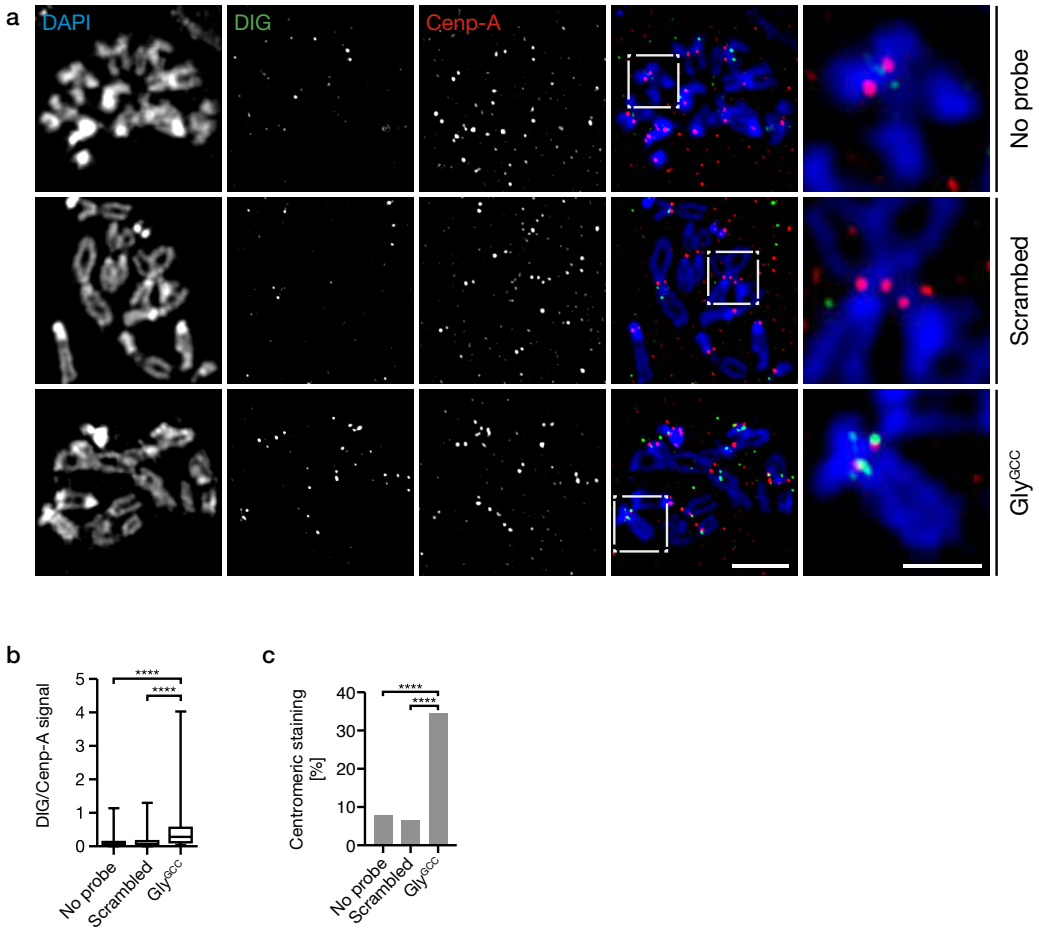


Figure 3.3: tRNA$^{Gly(GCC)}$ associates with centromeres in S2 cells. a, Combined RNA-FISH-IF on mitotic chromosome spreads of S2 cells applying a no probe control, scrambled, or tRNA$^{Gly(GCC)}$ DIG-labeled LNA probes, stained with DAPI (blue), anti-DIG (green), and anti-Cenp-A (red). Coloured images generally show merged channels. Scale bars, 5 µm and 2 µm (zoom). **b**, Quantification of anti-DIG mean fluorescence intensities of all centromeres normalised to corresponding centromeric Cenp-A signal (no probe n=132, scrambled n=84, tRNA$^{Gly(GCC)}$ n=40 ($p<0.0001$), Student's t-test). **c**, Quantification of centromeric presence or absence of tRNAGly(GCC) by counting co-localising (yellow signal) anti-DIG with anti-Cenp-A signals (no probe n=126, scrambled n=167, tRNA$^{Gly(GCC)}$ n=52 ($p<0.0001$), chi-square test).(tRNA FISH experiments were performed together with Sarah Doppler.)

Taken together, a method to identify centromere-associated RNA was established, which revealed a specific subset of tRNAs as potentially centromeric. $tRNA^{Gly(GCC)}$ was confirmed to localise to centromeres during mitosis, which suggests a mitotic function of tRNAs themselves or of functionally associated factors.

3.2 Mitotic centromere-associated tRNAs are methylated.

Out of over 100 known RNA modifications, more than 80 have been described to be present on tRNAs [Motorin and Grosjean, 2005]. Structure and function are thought to be closely related to the composition of tRNA modifications [Motorin and Helm, 2011, Jackman and Alfonzo, 2013, Paris et al., 2012]. ChIP-RNAseq analysis revealed editing on nuclear tRNAs (Figure 3.6.a). Following this observation, a detailed analysis of $tRNA^{Gly(GCC)}$ reads was done. Allowing two mapping mismatches, both centromere IPs showed at least two sites of sequence variances (Figure 3.4.a). These sites did not overlap between Cenp-A and Cenp-C IPs, meaning they may either be technical alignment issues (decreased specificity caused by allowed mismatches) or real biological differences of the pull-downs of Cenp-C and the more widespread Cenp-A (Bodor et al. 2014). An explanation for a real biological variance could be single-nucleotide polymorphism (SNP) of centromere-encoded, thus not annotated tRNA genes. Another explanation could be single-nucleotide variants (SNV) as a result of RNA editing at these sites. Because PCR analyses of SNPs on DNA and SNVs on cDNA from centromeric ChIP samples failed, neither of the two hypotheses can be excluded. Strikingly, the four most confident variance sites in the ChIP consensus sequences reflect the four most frequent editing events in human – A-to-G(Inosine), T-to-C, G-to-A, and C-to-T [Peng et al., 2012]. Moreover, 8 of 9 variance sites are found at described modification sites of tRNAs. Together, this indicated that centromeric $tRNA^{Gly(GCC)}$ may be modified, however more specified analyses such as NGS-based profiling of the RNA-editome are needed to answer this question [Peng et al., 2012].

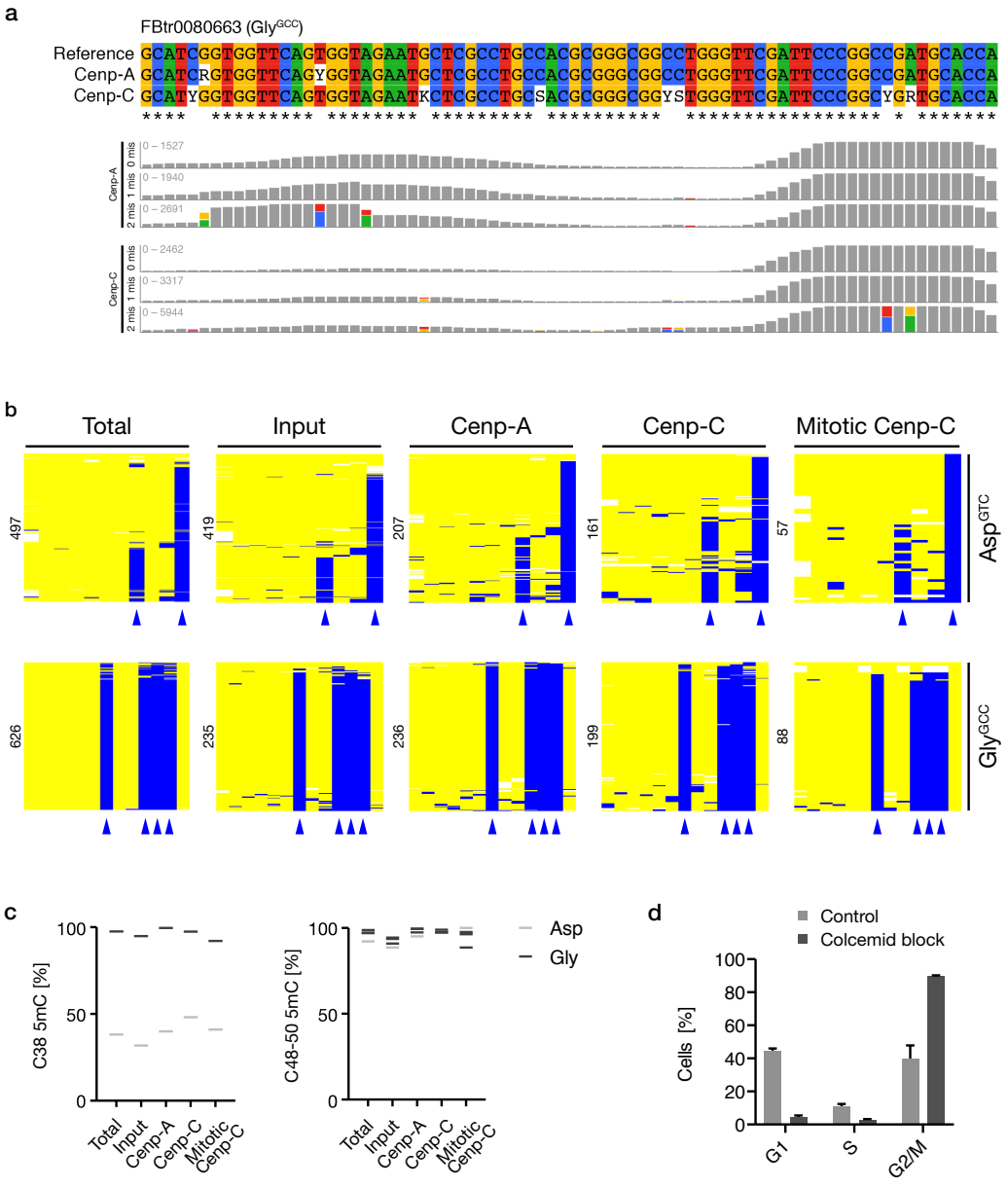


Figure 3.4: Methylated tRNAs localise to centromeres during mitosis in S2 cells. **a**, ChIP-RNAseq: Alignments of $tRNA^{Gly(GCC)}$ consensus sequences from Cenp-A and Cenp-C libraries to reference sequences. Bars show read distributions allowing zero, one or two alignment mismatches (mis). Grey bars show matched alignments, mismatches are coloured. Base code: K, G/T; R, A/G; S, G/C; Y, C/T. **b-c**, ChIP-RNA 454 bisulfite sequencing of $tRNA^{Asp(GTC)}$ and $tRNA^{Gly(GCC)}$ in total RNA, input, and centromere-associated RNAs of non-synchronised (Cenp-A, Cenp-C) and mitotically enriched S2 cells (Mitotic Cenp-C). **b**, 5mC heatmaps: Columns indicate cytosine residues and rows single sequencing reads. Numbers represent the coverage. Converted cytosines are shown in yellow and unconverted cytosines in blue. Arrowheads mark known 5mC sites (C38, C48-50). **c**, Quantification of unconverted cytosines at marked sites (b) in percentage. **d**, FACS analysis of mitotically enriched S2 cells blocked by colcemid (10 hours) for ChIP analysis (mean±SD, n=3).

Cytosine-5 methylation (5mC) is a well-studied modification present on $tRNA^{Gly(GCC)}$ as well as other tRNAs promoting tRNA stability and function that is intensively studied in the Lyko laboratory using RNA bisulfite (BS) sequencing [Schaefer et al., 2009, Schaefer et al., 2010, Tuorto et al., 2012]. To quantify the methylation level of centromeric tRNAs, ChIP was used to enrich for cenRNA, followed by targeted RNA BS sequencing of $tRNA^{Asp(GTC)}$ and $tRNA^{Gly(GCC)}$. Both tRNAs are known to contain Dnmt2-mediated 5mC sites at position C38, whereas 5mC at the positions C48-50 are catalysed by NSun2, the only other known tRNA methyltransferase in *Drosophila* (Figure 3.7.c). Strikingly, all analysed samples showed typical methylation patterns of both MTases (Figure 3.4.b). Nuclear (input) and centromeric tRNAs displayed a comparable degree of methylation to total RNA. The same was true for centromeric tRNA from mitotically enriched S2 cells (Figure 3.4.c-d). In conclusion, ChIP-BS-RNAseq revealed that centromeric RNA is methylated and that cytosine-5 methylation is also present in mitosis.

3.3 tRNA methyltransferases regulate centromeric function in mitosis

The detection of methylated tRNAs at centromeres during mitosis raised the question whether RNA modification regulates centromeric function. A prerequisite for methylation at centromeres is the presence of the responsible tRNA methyltransferases (MTases) at the same place and time. The subcellular localisation of Dnmt2 and NSun2 was examined using immunofluorescence on interphase and mitotic S2 cells, and on mitotic chromosome spreads (Figure 3.5.a-c). As previously described for Dnmt2 in *Drosophila* and NSun2 in human cells [Schaefer et al., 2008, Hussain et al., 2009], the bulk of cellular signals were cytosolic for Dnmt2 and nuclear for NSun2 (Figure 3.5.a). During mitosis, both proteins were distributed within the entire cell excluding most of the mitotic chromatin (Figure 3.5.b). Inspecting mitotic chromosome spreads confirmed that most of the chromatin was free of MTases. The only clear chromatin-associated signals for both enzymes were at mitotic centromeres, where the enzymes co-localised with the kinetochore-specific protein Cenp-C (Figure 3.5.c).

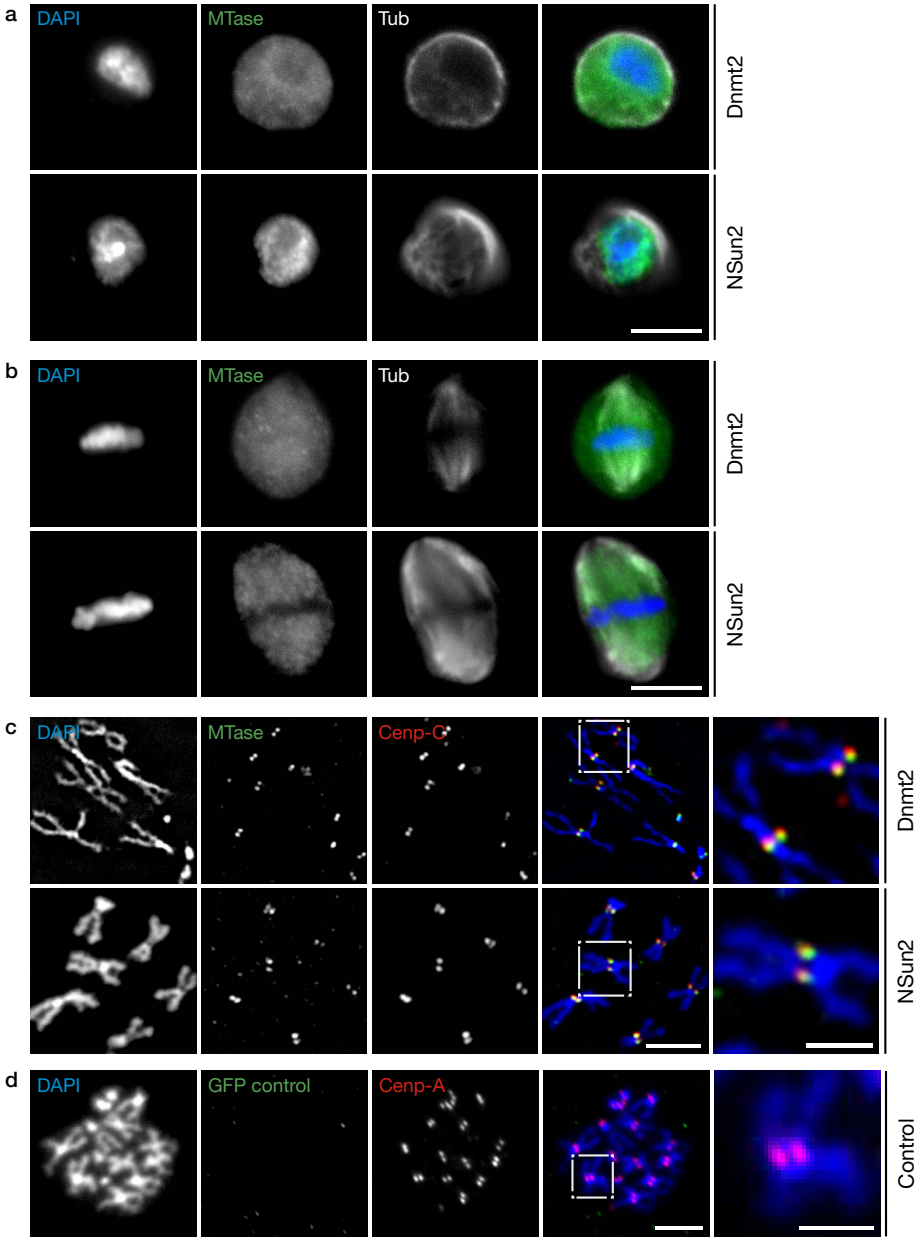


Figure 3.5: RNA methyltransferases Dnmt2 and NSun2 associate with centromeres during mitosis. **a**, Immunofluorescence on GFP-tagged MTase (green) Dnmt2 or NSun2 expressing S2 cells in (**a**) interphase and (**b**) metaphase, stained with DAPI (blue), and anti-tubulin (grey). Scale bar, 5 µm. **c**, Immunofluorescence on mitotic chromosome spreads of S2 cells expressing GFP-tagged (green) Dnmt2 or NSun2, stained with DAPI (blue), anti-GFP (green), and anti-Cenp-C (red). Scale bars, 5 µm and 2 µm (zoom). **d**, Immunofluorescence on mitotic chromosome spreads of S2 cells expressing GFP (green), stained with DAPI (blue), anti-GFP (green), and anti-Cenp-A (red). Scale bars, 5 µm and 2 µm (zoom).

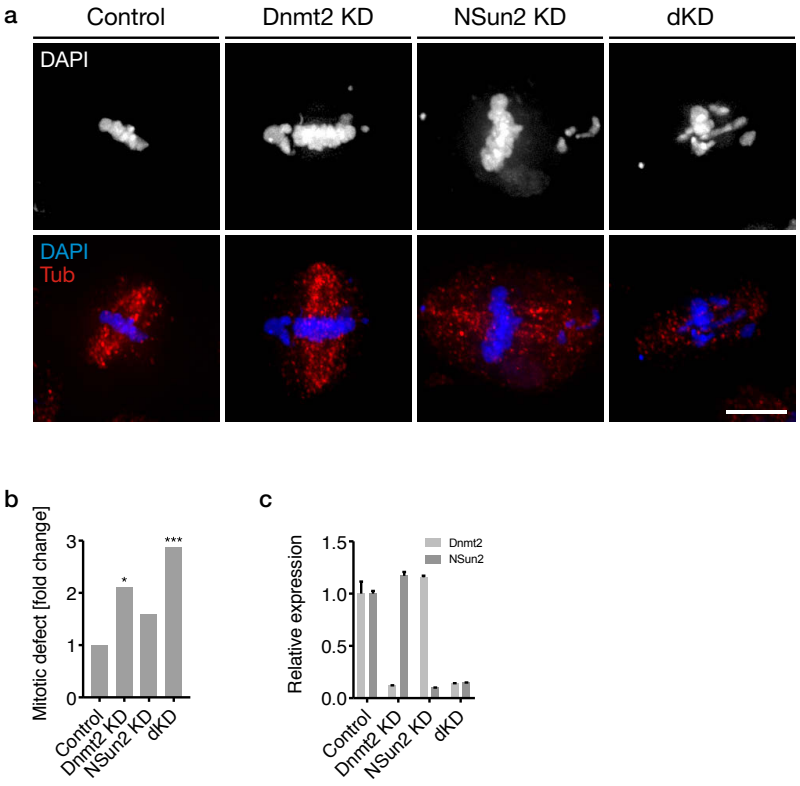


Figure 3.6: Depletion of Dnmt2 and NSun2 lead to mitotic chromosome segregation defects in S2 cells. a-b, Immunofluorescence on fixed S2 cells, stained with anti-tubulin (red), and DAPI (blue). **a**, Representative images of anaphase cells from control, single (Dnmt2 KD, NSun2 KD), or double knock downs (dKD). **b**, Anaphase cells were categorised either as intact or impaired chromosome segregation (control: n=40, Dnmt2 KD: n=42 (p=0.0174), NSun2 KD: n=39 (p=0.1900), dKD: n=37 (p=0.0002), chi-square test). Quantified defects are represented as fold change over control. **c**, Relative expression of MTases by qPCR: Knock downs (KD) in S2 cells were performed for 4 days using dsRNA against Brown (Control), Dnmt2, NSun2, or both MTases (dKD) (mean±SD, n=3).

Although mitotic chromatin is accessible to chromatin-associated factors [Chen et al., 2005], the bulk of protein-DNA and RNA-DNA interactions are significantly reduced during mitosis [Black et al., 2016]. In contrast, centromeric components that form the kinetochore are especially recruited for mitotic chromosome segregation. Therefore, the centromeric presence of Dnmt2 and NSun2 during mitosis suggests a role of tRNA-MTases in chromosome segregation. To review this hypothesis we examined S2 cells assessing the degree of mitotic defects in cells depleted for one or both MTases (Figure 3.6.a-b). The

efficiency of knock downs in S2 cells was controlled using qPCR analysis (Figure 3.6.c). Depletion of both Dnmt2 and NSun2 (10% relative expression) led to increased mitotic defects with regard to control (2.1-fold for Dnmt2, 1.6-fold for NSun2) in form of lagging chromosomes, chromosome bridges, and chromosome fragments. The number of defects was highest in the double knock down (2.9-fold) but did not result in the quantitative sum of both single knock downs (Figure 3.6.b). This may indicate that both proteins function sequentially or redundantly in the same pathway. In conclusion, these data demonstrate that tRNA methyltransferases not only associate with centromeres but also regulate their function.

3.4 tRNA methylome analysis confirms Dnmt2 as a highly specific tRNA-methyltransferase

ChIP-RNAseq and ChIP-qPCR analyses revealed centromere-associated tRNAs and bisulfite sequencing confirmed centromeric $tRNA^{Gly(GCC)}$ to be methylated. For Dnmt2, only three tRNAs have been described as substrates in *Drosophila* [Schaefer et al., 2010]. A methylation analysis for NSun2 targets remained to be done in the fly. To get a comprehensive picture of tRNA 5mC in *Drosophila*, a tRNA methylome analysis was performed on third instar larval brain tissue, which undergoes a classical mitotic cell cycle. Moreover, by using *Dnmt2* and *NSun2* null mutant flies, all tRNA substrates of the currently known tRNA 5mC-MTases were taken into account. The experiment achieved a deamination efficiency of about 98.4% and the three known Dnmt2 substrates were used as controls (Figure 3.7.c). Library size distributions and per base sequence quality from Illumina sequencing are presented in the supplements (Figure B.4-5). The base distribution plots display the reduction of cytosines upon bisulfite treatment with remaining peaks in the small RNA fractions corresponding to Dnmt2 and NSun2 methylation sites on tRNAs. This demonstrates the suitability of bisulfite treatment on large-scale RNA analysis (Figure B.6).

To compare methylation data of different species (Figure 3.7.b), a tRNA methylome analysis for *S.pombe* was performed in a side project of this doctoral thesis, which was published as part of Müller et al. 2015 (Figure B.7).

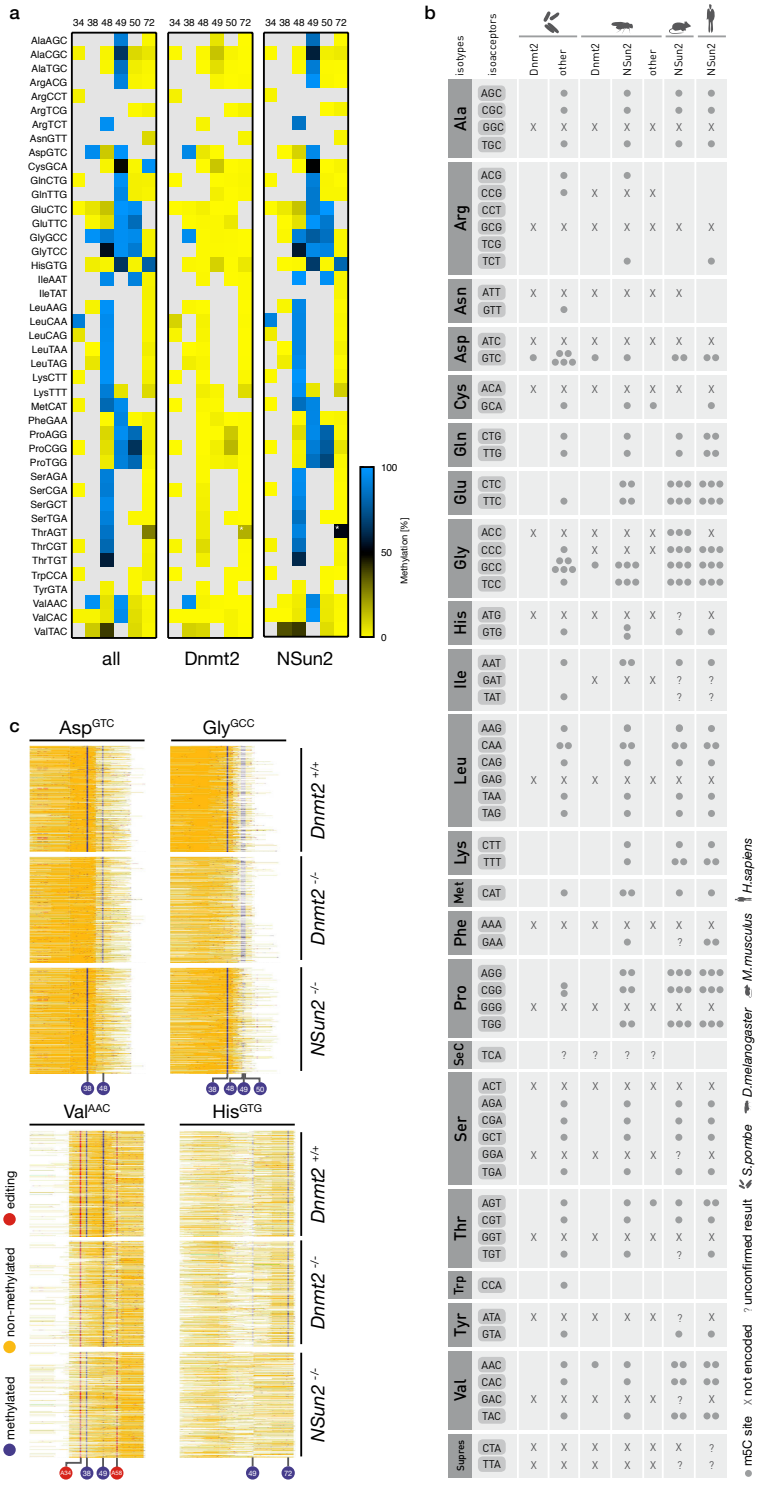


Figure 3.7: tRNA methylome analysis reveals a comprehensive map of tRNA methylation and substrate-specificity of Dnmt2 and NSun2.

Figure 3.7: tRNA methylome analysis reveals a comprehensive map of tRNA methylation and substrate-specificity of Dnmt2 and NSun2. a, Genome-wide tRNA methylome analysis of third instar larval brains. All detected tRNA 5mC sites are shown in the left heatmap. Dnmt2- and NSun2-dependent methylation sites are shown in the middle and right heatmaps. The colour gradient displays the amount of unconverted cytosines at the indicated position. *Asterisks marks a negative value representing an increase of methylation in the mutant. **b**, Table comparing tRNA methylome analyses of published yeast and mammalian with unpublished Drosophila data examined in this study. S.pombe data was generated in the course of this PhD thesis in collaboration with the Ehrenhofer-Murray laboratory and published by Müller et al, 2015. Mammalian data from *M.musculus* and *H.sapiens* was generated by the Frye laboratory and published by Blanco et al., 2014. Grey circles mark 5mC sites, 'X' marks isoacceptors not encoded in the respective species, and '?' marks unconfirmed results. A lack of any of these symbols indicates positions without 5mC. **c**, Genome-wide tRNA methylome analysis: Heatmaps of selected tRNAs ($tRNAs^{Asp(GTC),\ Gly(GCC),\ Val(AAC),\ His(GTG)}$) from wild type, *Dnmt2*$^{-/-}$, and *NSun2*$^{-/-}$ third instar larval brains. Columns indicate base positions and rows single sequencing reads. Correct alignments after bisulfite treatment are shown in yellow, editing sites in red, and unconverted cytosines in blue. Blue circles mark positions of known (C38, C48-50) or novel (C72) Dnmt2- or NSun2-dependent methylation sites, red circles mark known editing sites.

In addition, mammalian data was obtained from [Blanco et al., 2014] (Frye laboratory). For *Drosophila*, the left heatmap in Figure 3.7.a summarises all tRNA positions of all tRNA isoacceptor families that show robust methylation levels (>50%) in wild type tissue. 81% (17 of 21) of all isotype classes (76% in human, 76% in mouse, 90% in yeast) and 84% (37 of 44) of all isoacceptor families (79% in human, 79% in mouse, 78% in yeast) were methylated on at least one cytosine in *Drosophila*. All of the 44 *Drosophila* isoacceptor families are also encoded in mammals. Both in *Drosophila* and mammals, 34 of the 44 isoacceptors were found to be methylated and four were not, reflecting a high degree of conservation of methylation sites in tRNAs. Only $tRNA^{Tyr(GTA)}$ was not methylated in Drosphila but in mammals, and vice versa for $tRNA^{Arg(ACG)}$. Two Isoacceptors are not encoded both in *Drosophila* and mammals, and the remaining two isoacceptors could not be confidently analysed due to low coverage. In contrast to fly, mouse, and human samples, in *S.pombe* even $tRNA^{Asn(GTT)}$ and $tRNA^{Trp(CCA)}$ were found to be methylated, demonstrating that probably all isotype classes may principally be methylated on cytosine-5 in eukaryotes (Figure 3.7.b).

The heatmaps for Dnmt2- and NSun2-dependent methylation sites were generated by subtraction of the mutant from the wild type values (Figure 3.7.a). The heatmaps display a clear difference in the number of substrates between

the two MTases. Dnmt2 appears as a three-tRNA specific methyltransferase, in contrast to NSun2, which shows a much broader substrate specificity. C38 was confirmed as the only Dnmt2 target site, whereas C34 as well as C48, C49, and C50 are all targeted by NSun2. Remarkably, a novel NSun2-dependent 5mC site (C72) may have been found in $tRNA^{His(GTG)}$ (Figure 3.7.a + c). Furthermore, $tRNA^{Cys(GCA)}$ and $tRNA^{Thr(AGT)}$ were found to be methylated at position C72, independently of Dnmt2 or NSun2. In human, NSun6 has been shown to methylate this position of exactly these tRNA isotypes [Haag et al., 2015], which is possibly an ortholog of *Drosophila* CG11109. Amplicon-based sequencing is necessary to confirm these observations in fly. As in principal non-tRNA transcripts that function in mitosis could be regulated by cytosine-5 methylation, amplicon-based bisulfite sequencing was used to assay methylation of centromeric SATIII. The sequencing results did not show any cytosine-5 methylation (data not shown).

Additionally to the tRNA methylome analysis, a screen for 5mC sites within the entire larval brain transcriptome was performed (see supplements B.5). Only a single methylation site (other than the tRNA sites described above) was confirmed. This site carries an NSun2-dependent methylation mark within the coding region of an mRNA (Figure B.9). Although controversially discussed, few non-tRNA targets have been described for NSun2 in mammals [Squires et al., 2012, Hussain et al., 2013b, Khoddami and Cairns, 2013, David et al., 2017]. At this stage, non-tRNA NSun2 substrates regulating centromeric function cannot be excluded, since a comprehensive screen requires a replicate-based whole-transcriptome bisulfite sequencing analysis [Legrand et al.,] (submitted).

For Dnmt2 however, non-tRNA substrates could neither be detected here (*Drosophila*) nor in a whole-transcriptome-bisulfite-sequencing approach in mouse [Legrand et al.,], strongly suggesting that Dnmt2 is a conserved tRNA-specific methyltransferase that is supported by further publications [Goll et al., 2006, Khoddami and Cairns, 2013, Schaefer et al., 2010]. Due to these results, the ambiguous substrate-specificity of NSun2 and the focus of this work on tRNA methylation, further studies focused on Dnmt2 only.

3.5 Dnmt2-mediated tRNA methylation is required for mitotic chromosome segregation

The RNA methyltransferase Dnmt2 was confirmed to be highly specific for tRNA, and Dnmt2 and its substrate $tRNA^{Gly(GCC)}$ were found to be centromere-associated in mitosis. This raised the question, whether tRNA methylation is necessary for centromeric function.

To gain a more detailed picture of the effects of a dysregulated Dnmt2 on chromosome segregation, an S2 cell culture system was set up to compare controls with Dnmt2 knock down (KD) cells and with cells overexpressing (OE) either recombinant wild type Dnmt2 or recombinant dominant-negative ΔcatDnmt2 (Figure 3.8.a). The ΔcatDnmt2 construct was generated by site-directed mutagenesis changing the catalytically essential PCQ-motif to PAQ that inhibits the methylation activity (data not shown). $tRNA^{Asp(GTC)}$ with a C38 methylation level of about 40% in control cells was used as a reporter for methylation changes. The OE of wild type Dnmt2 led to an increase of C38 methylation, whereas the KD of endogenous or OE of ΔcatDnmt2 both led to a decrease (Figure 3.8.c). These conditions did not change cell cycle progression (Figure 3.8.b). This system was used to analyse live cell imaging for anaphase defects, namely chromosome fragments, lagging chromosomes and anaphase bridges (Figure 3.8.d). Quantification confirmed increases in defects upon either Dnmt2 depletion, OE of wild type, as well as OE of ΔcatDnmt2 (Figure 3.8.e). These results indicated a methylation-dependent role of Dnmt2 in chromosome segregation.

However, it cannot be excluded that the effects observed upon altered protein levels (KD, wt OE, deltaCat OE) are caused by a catalytically-independent mechanisms. To answer this question, *Drosophila* third instar larval neuroblasts (which have a classical mitotic cell cycle) from various mutants were examined. w^{1118} was used as a control and compared with null [Schaefer et al., 2010] and $Dnmt2^{\Delta cat}$ mutants (Matthias Schaefer, unpublished). In the $Dnmt2^{\Delta cat}$ mutant fly, the C38 methylation of $tRNA^{Asp(GTC)}$ was lost, while the NSun2-mediated C48 methylation was retained (Figure 3.9.c). Therefore, the

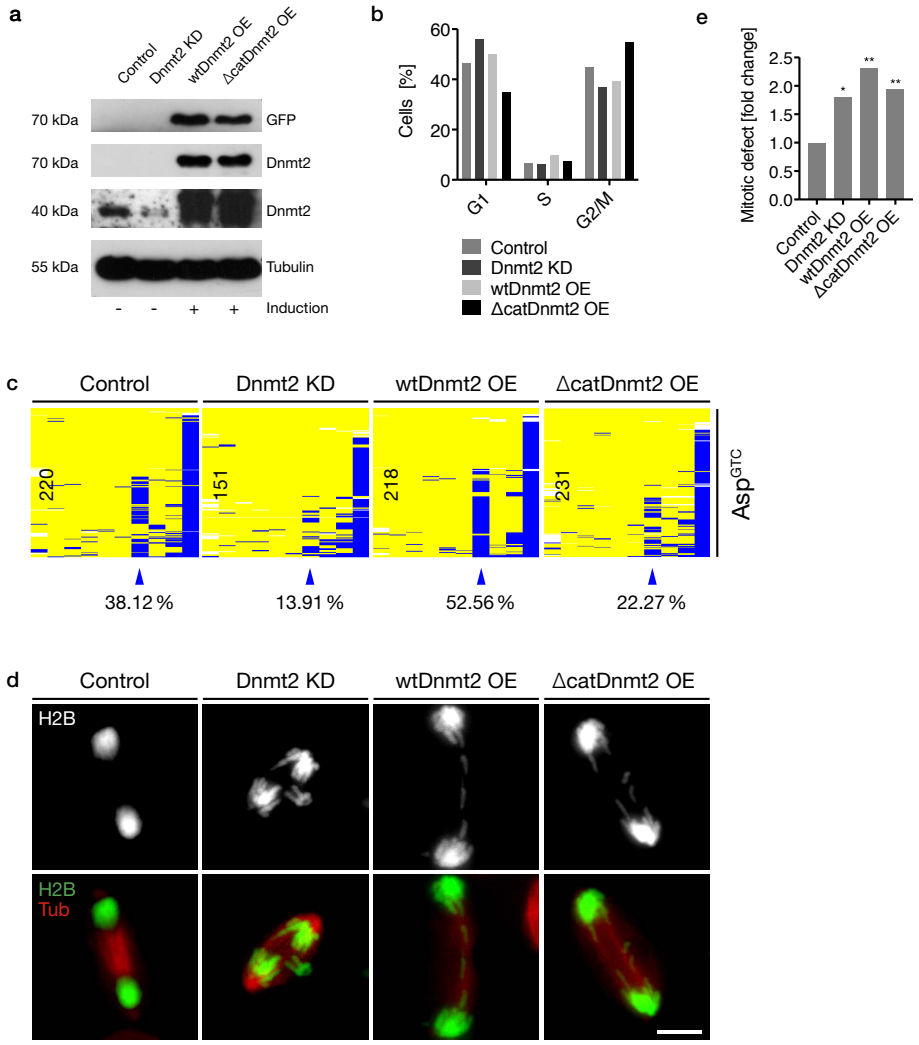


Figure 3.8: Dysregulation of Dnmt2 leads to mitotic chromosome segregation defects in S2 cells. a-e, S2 cells transfected with inducible recombinant wild type (wt) and catalytic mutant (Δcat) pMT-Dnmt2-GFP. Uninduced control, knock down (KD) and overexpression (OE) of wild type (wt) and catalytic mutant (Δcat) Dnmt2 conditions were examined for expression, cell cycle progression, methylation activity, and mitotic chromosome segregation. **a**, Western blot showing endogenous (40 kDa) and recombinant GFP-tagged (70 kDa) Dnmt2 expression levels in indicated conditions. **b**, Quantification of representative FACS profiles per indicated condition. **c**, 5mC heatmaps from 454 bisulfite sequencing of $tRNA^{Asp(GTC)}$ in indicated conditions. Columns indicate cytosine residues (Cs), rows single sequencing reads, and numbers in the left of each map represent the coverage. Converted Cs are shown in yellow and unconverted Cs in blue. Arrowheads mark known Dnmt2-dependent methylation sites (C38) and their unconverted C levels [%] reflecting the degree of methylation. **d**, Representative micrographs from live imaging showing anaphase H2B-GFP (green) and mCherry-tubulin (red) expressing S2 cells with inducible recombinant wt or Δcat Dnmt2-V5/His in indicated conditions. Scale bar, 5 µm. **e**, Quantification of the number of anaphase defects per cell from two independent experiments. (control n=52, Dnmt2 KD n=115 (p=0.0199 Dnmt2), wtDnmt2 n=56 (p=0.0012), ΔcatDnmt2 n=66 (p=0.0020), chi-square test). Defects are represented as fold change over control.

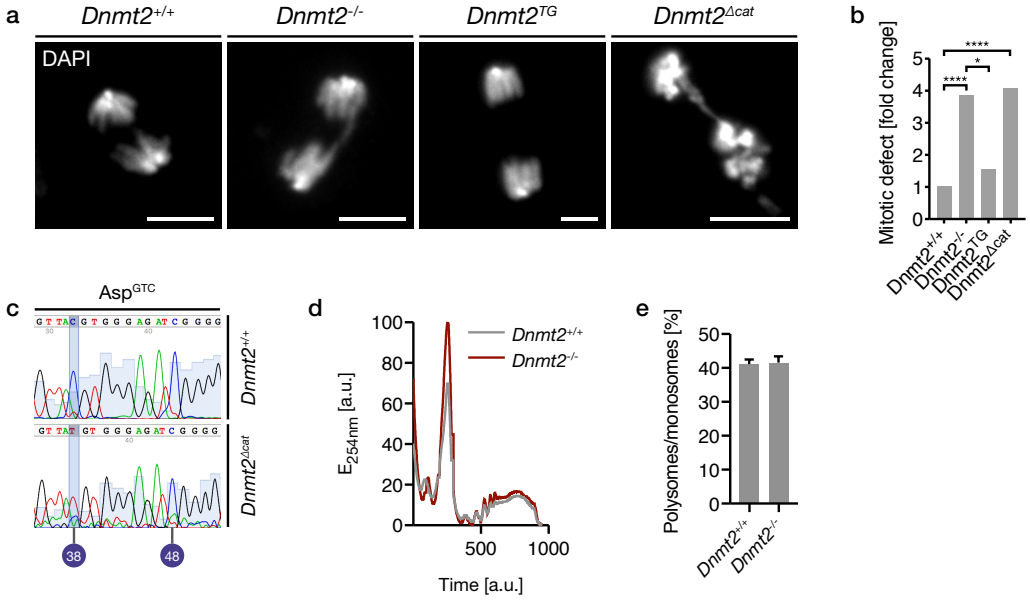


Figure 3.9: tRNA methylation by Dnmt2 is required for mitotic chromosome segregation in *Drosophila*. **a**, DAPI staining of mitotic neuroblasts in wild type ($Dnmt2^{+/+}$), mutant null ($Dnmt2^{-/-}$), transgenic Dnmt2-GFP rescue ($Dnmt2^{TG}$), and catalytic mutant ($Dnmt2^{\Delta cat}$) *Drosophila* third instar larval brain. Scale bar, 5 µm. **b**, Quantification of observed mitotic defects in at least four individual brains per genotype ($Dnmt2^{+/+}$ n=295, $Dnmt2^{-/-}$ n=68 (p<0.0001), $Dnmt2^{TG}$ n=106 (p=0.2482 to $Dnmt2^{+/+}$, p= 0.0109 to $Dnmt2^{-/-}$), $Dnmt2^{\Delta cat}$ n=197, (p<0.0001)), chi-square test). Defects are represented as fold change over control. **c**, Sanger bisulfite sequencing of larval neuroblasts demonstrating loss of C38 (blue highlight) but not C48 methylation of $tRNA^{Asp(GTC)}$ in $Dnmt2^{\Delta cat}$ compared to $Dnmt2^{+/+}$. **d**, Representative polysome profiles from $Dnmt2^{+/+}$ and $Dnmt2^{-/-}$ *Drosophila* embryos. **e**, Quantification of polysome over monosome ratio of $Dnmt2^{+/+}$ and $Dnmt2^{-/-}$ embryos from two independent experiments (n=2, mean±SD, p=0.8114, Student's t-test).

$Dnmt2^{\Delta cat}$ mutant fly resembles the *Dnmt2*-null mutant fly with respect to the mitotic function (Figure 3.7.c). Brains from both $Dnmt2^{\Delta cat}$ and *Dnmt2*-null mutant flies showed significantly increased mitotic defects confirming the role of tRNA methylation in mitosis (Figure 3.9.b). A transgenic *Dnmt2* ($Dnmt2^{TG}$) in the null mutant background [Schaefer et al., 2008] rescued the segregation defects, demonstrating a direct role of Dnmt2 in mitosis (Figure 3.9.a-b). As tRNA methylation influences tRNA homeostasis and thus protein synthesis [Tuorto et al., 2015], polysome profiles were analysed that displayed intact profiles and unchanged polysome to monosome ratios in the *Dnmt2* null mutants. This indicated intact translational machineries and hence a translation-

independent role of Dnmt2 in chromosome segregation (Figure 3.9.d-e). In summary, the results presented here suggest a direct role of Dnmt2-mediated tRNA methylation in mitotic chromosome segregation in *Drosophila* S2 cells and larval tissue.

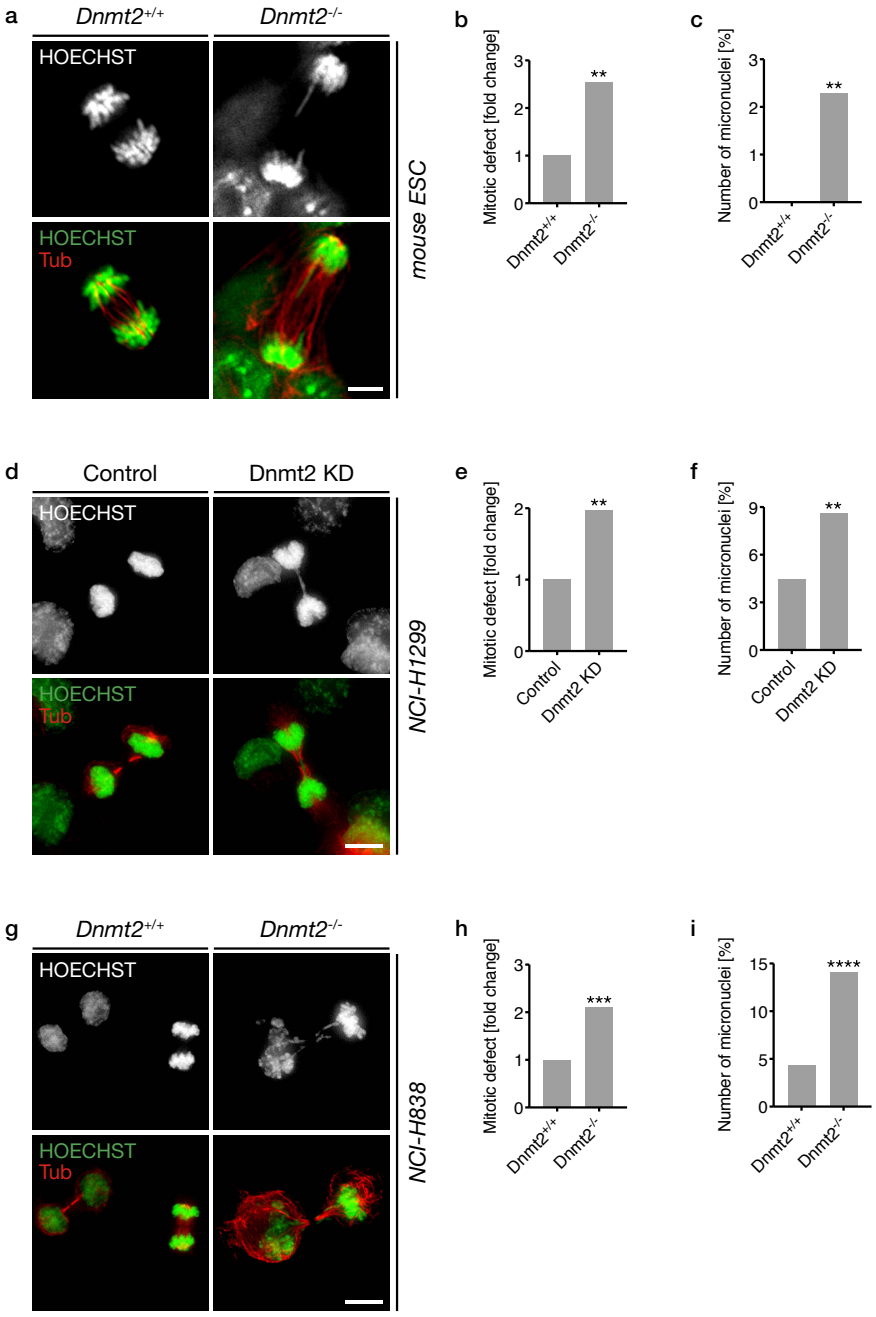


Figure 3.10: The role of Dnmt2 in mitosis is conserved in mammalian cells.

Figure 3.10: The role of Dnmt2 in mitosis is conserved in mammalian cells. a, Immunofluorescence on mouse embryonic stem cells (mESC) in wild type (*Dnmt2*$^{+/+}$) and mutant (*Dnmt2*$^{-/-}$) cells, stained with HOECHST (green), and anti-tubulin (red). Scale bar, 5 µm. **b**, Quantification of mitotic defects in mESC (*Dnmt2*$^{+/+}$ n=105, *Dnmt2*$^{-/-}$ n=120 (p=0.0021), chi-square test). Quantified defects are represented as fold change over control. **c**, Quantification of micronuclei in interphases of mESC (*Dnmt2*$^{+/+}$ n=296, *Dnmt2*$^{-/-}$ n=349 (p=0.0088), chi-square test). Numbers describe the amount (%) of all micronuclei from all imaged interphases. **d**, Immunofluorescence on human NCI-H1299 non-small cell lung cancer cells depleted for Dnmt2 using shRNA (unspecific shRNA as control), stained with HOECHST, and anti-tubulin. Scale bar, 10 µm. **e**, Quantification of mitotic defects in NCI-H1299 cells (control n=106, Dnmt2 KD n=125 (p=0.0011), chi-square test). Quantified defects are represented as fold change over control. **f**, Quantification of micronuclei in interphases of NCI-H1299 cells (*Dnmt2*$^{+/+}$ n=496, *Dnmt2*$^{-/-}$ n=699 (p=0.0052), chi-square test). Numbers describe the amount (%) of all micronuclei from all imaged interphases. **g**, Immunofluorescence on human CRISPR NCI-H838 non-small cell lung cancer cells depleted for Dnmt2 (*Dnmt2*$^{-/-}$) using specific (or unspecific as control) sgRNA, stained with HOECHST, and anti-tubulin. Scale bar, 10 µm. **h**, Quantification of mitotic defects in NCI-H838 cells (*Dnmt2*$^{+/+}$ n=86, *Dnmt2*$^{-/-}$ n=94 (p=0.0004), chi-square test). Quantified defects are represented as fold change over control. **i**, Quantification of micronuclei in interphases of NCI-H838 (*Dnmt2*$^{+/+}$ n=436, *Dnmt2*$^{-/-}$ n=205 (p<0.0001), chi-square test). Numbers describe the amount (%) of all micronuclei from all imaged interphases. (Lung cancer cells were obtained from Manuel Rodriguez.)

Since Dnmt2 is the most conserved member of the Dnmt family of proteins, the question arised, if the functional role of Dnmt2 in mitosis is conserved. Therefore, different human and mouse cell lines depleted for Dnmt2 were examined. Dnmt2 null mouse embryonic stem cells (mESC) were provided by Francesca Tuorto, and shRNA-depleted NCI-H1299 and Dnmt2 null NCI-H838 non-small cell lung cancer cells were provided by Manuel Rodriguez (Lyko laboratory). Both were analysed for chromosome segregation defects and compared to corresponding controls (Figure 3.10.a, d, g). Consistently, all three systems confirmed increased chromosome segregation defects upon Dnmt2 depletion (Figure 3.10.b, e, h). Moreover, as a result of aberrant mitosis, increased amounts of micronuclei were found for all cell lines (Figure 3.10.c, f, i). Both lung cancer cells already showed distinct amounts of micronuclei in control samples, reflecting their pathological origin (Figure 3.10.f, i). On the contrary, wild type mESCs showed no micronuclei, confirming the technical suitability of the applied method (Figure 3.10.c). In conclusion, the functional role of Dnmt2 in mitosis is conserved from insects to mammals.

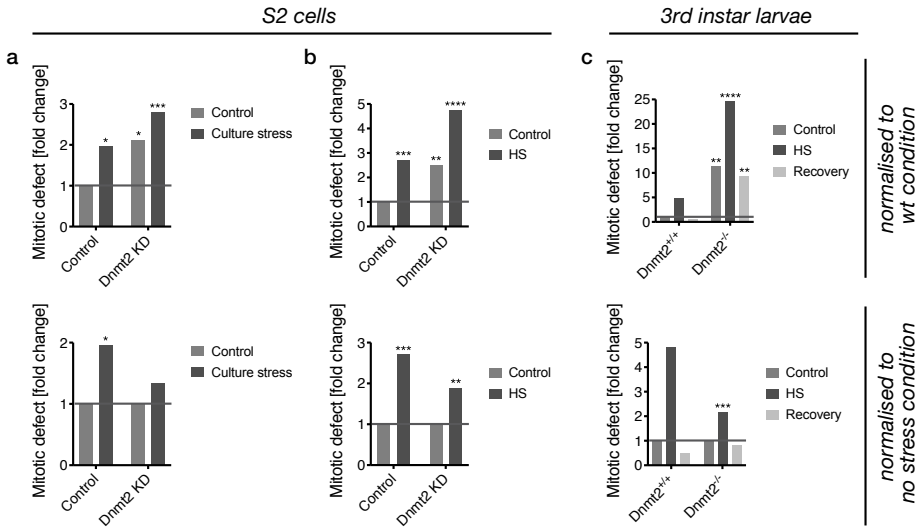


Figure 3.11: Dnmt2 depleted cells accumulate stress that affects chromosome segregation. a-c, Quantification of chromosome segregation defects in different Dnmt2 depleted cells under the influence of different kinds of cellular stress. The two different graphs of each panel represent two different normalisations of the same data: In each upper graph all data is normalised to control KD without stress (normalised to wt condition), in each lower graph every stressed condition is normalised to its corresponding no stress condition (normalised to no stress condition). Quantified defects are represented as fold change over control, dashed lines mark normalisations. **a**, Quantification of anaphase defects in fixed S2 cells in control or Dnmt2 knock down (KD) cells using dsRNA. Normal and overgrowing culture conditions are compared (control n=40, control CS n=43 (p=0.0368), Dnmt2 KD n=42 (p<0.0174), Dnmt2 KD CS n=38 (p<0.0003 to control, p=0.1630 to Dnmt2 KD), chi-square test). **b**, Quantification of anaphase defects in live cell imaging of S2 cells in control or Dnmt2 knock down (KD) cells using dsRNA. Compared are control and 30 minutes heat shock conditions at 37°C (control n=151, control HS n=128 (p=0.0006), Dnmt2 KD n=83 (p<0.0048), Dnmt2 KD HS n=76 (p<0.0001 to control, p=0.0092 to Dnmt2 KD), chi-square test). **c**, Quantification of mitotic defects in neuroblasts of wild type (*Dnmt2*$^{+/+}$) or *Dnmt2* mutant (*Dnmt2*$^{-/-}$) third instar larvae. Compared are control and 45 minutes heat shock conditions at 37°C, recovery after heat shock was performed for 4 hours at 25°C (*Dnmt2*$^{+/+}$ n=48, *Dnmt2*$^{+/+}$ HS n=60, *Dnmt2*$^{+/+}$ rec n=98, *Dnmt2*$^{-/-}$ n=68, *Dnmt2*$^{-/-}$ HS n=135, *Dnmt2*$^{-/-}$ rec n=134, (*Dnmt2*$^{+/+}$ to *Dnmt2*$^{+/+}$ HS p=0.0968), (*Dnmt2*$^{+/+}$ to *Dnmt2*$^{+/+}$ rec p=0.6037), (*Dnmt2*$^{+/+}$ to *Dnmt2*$^{-/-}$ p=0.0013), (*Dnmt2*$^{+/+}$ to *Dnmt2*$^{-/-}$ HS p<0001), (*Dnmt2*$^{+/+}$ to *Dnmt2*$^{-/-}$ rec p=0.0038), (*Dnmt2*$^{-/-}$ to *Dnmt2*$^{-/-}$ HS p=0.0002), (*Dnmt2*$^{-/-}$ to *Dnmt2*$^{-/-}$ rec p=0.04947) chi-square test).

Dnmt2-mediated tRNA methylation has been described to protect tRNA from stress-induced cleavage [Schaefer et al., 2010, Tuorto et al., 2012]. To determine whether Dnmt2 at centromeres is functionally connected to stress, chromosome segregation defects in different mitotic cells (S2 cells or third instar larval neuroblasts) were quantified with and without different stress-inducing measures (culture stress or heat shock). All quantifications confirmed increased defects upon Dnmt2 depletion and showed even stronger defects when stress was applied to depleted cells (Figure 3.11.a, b, c, upper row). Normalisation of stressed conditions to each corresponding no stress control, however, revealed that the relative stress-dependent increase of defects was smaller in Dnmt2 depleted than wild type cells (Figure 3.11.a, b, c, bottom row). This may result from 'stress saturation' in Dnmt2 depleted cells, however shows a role of Dnmt2 in stress mediation at centromeres.

In summary, Dnmt2-mediated tRNA methylation is required for chromosome segregation in mitosis, a function that may be connected to cellular stress response. Moreover, the suggested role of Dnmt2 in this process is conserved from *Drosophila* to human.

3.6 Dnmt2 regulates centromeric chromatin states during mitosis

The regulation of constitutive pericentromeric chromatin is essential for centromeric function [Rošić and Erhardt, 2016]. Classical assays to examine chromatin states and regulatory factors in *Drosophila* make use of reporter genes displaying 'Position-Effect Variegation' (PEV). Translocation of the *white* gene, which is required for red eye pigmentation, to heterochromatic domains causes partial silencing in some cells due to stochastic spreading of heterochromatin and thereby to mottled (variegated) eye-pigmentation. The introduction of 'suppressor-of-variegation' (Su(var)) mutations increases the eye-pigmentation that reflects an opening of the chromatin at the transcriptional start site and thus a role in heterochromatin-mediated silencing of this factor [Cryderman et al., 1998].

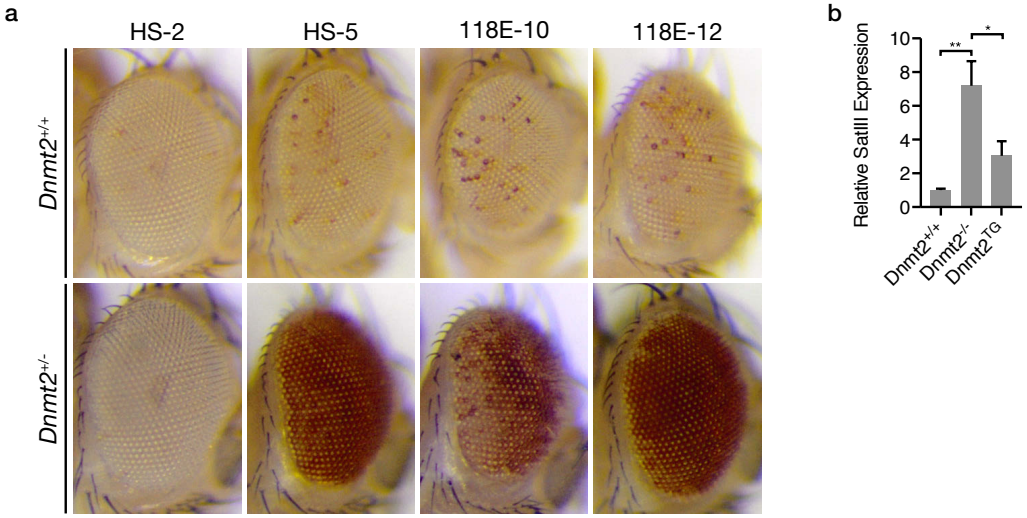


Figure 3.12: Dnmt2 influences chromatin signatures of pericentromeric chromatin. a, Dominant modifier effects of Dnmt2 on variegation of white reporter gene expression (red eye pigmentation) inserted into various partially repressed pericentric heterochromatin domains (HS2, HS5, 118E10, 118E12). Wild type ($Dnmt2^{+/+}$), and heterozygous mutant ($Dnmt2^{+/-}$) male fly eyes are shown. **b**, qPCR analysis of pericentric SatIII expression in wild type ($Dnmt2^{+/+}$), null mutant ($Dnmt2^{-/-}$), and transgenic Dnmt2-GFP rescue ($Dnmt2^{TG}$) flies. SatIII expression was normalised to actin. (n=3, mean±SD, $Dnmt2^{-/-}$ p=0.0018, $Dnmt2^{TG}$ p=0.0132, Student's t-test).

Dnmt2 has previously been shown to regulate heterochromatin at retrotransposons, however, PEV of pericentric domains of the X chromosome appeared to be unaffected by Dnmt2 depletion [Phalke et al., 2009]. To further examine whether Dnmt2 regulates pericentromeric chromatin, various flies with *white* as a reporter gene within different loci of constitutive heterochromatin were crossed with *Dnmt2* null mutants. In three out of four crossings, heterozygous $Dnmt2^{+/-}$ flies showed an increase of red eye pigmentation, demonstrating an upregulation of *white* expression at these sites (Figure 3.12.a). Therefore, Dnmt2 is a locus-specific dominant modifier (Su(var)) of pericentromeric chromatin. To examine whether changes in heterochromatin lead to altered pericentromeric gene expression, transcript levels of the pericentromeric lncRNA SATIII were assessed. $Dnmt2^{-/-}$ flies expressed about seven-fold more SATIII than wild type control flies. This effect could partially be rescued in the transgenic $Dnmt2^{TG}$ fly, confirming a regulatory role of Dnmt2 on chromatin states (Figure 3.12.b).

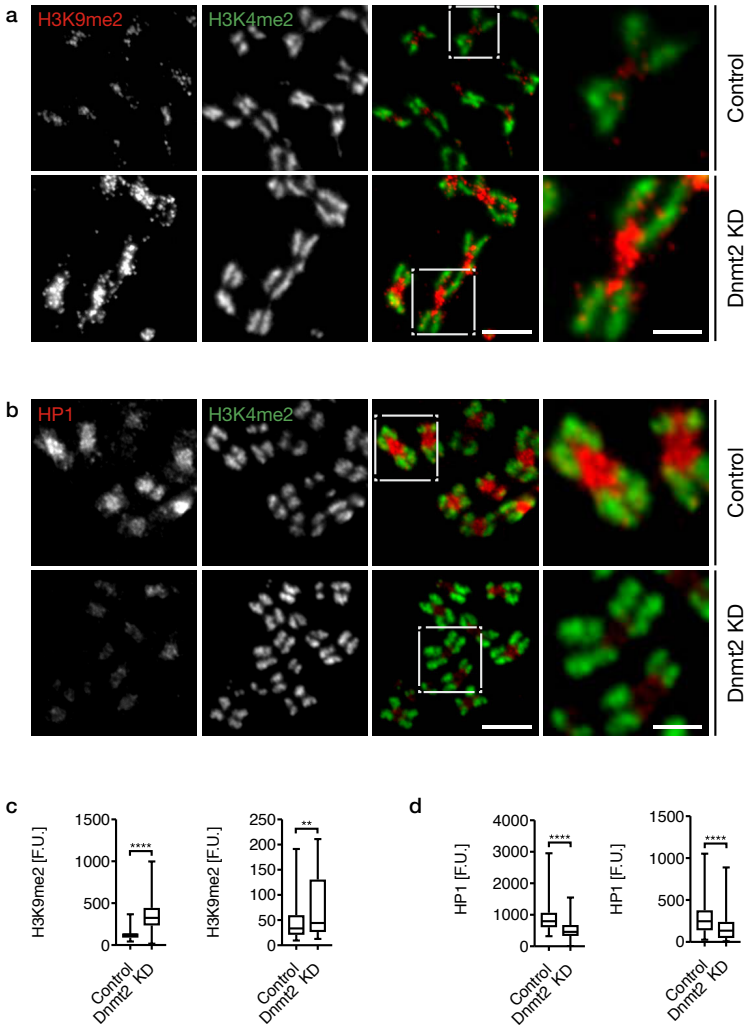


Figure 3.13: Mitotic chromatin states at centromeres are altered in Dnmt2 depleted cells. **a-b**, Immunofluorescence on mitotic chromosome spreads of control and Dnmt2-depleted S2 cells, stained with (**a**) anti-H3K9me2 or (**b**) anti-HP1, and anti-H3K4me2 staining. Scale bars, 5 µm and 2 µm (zoom). **c**, Quantification of two independent experiments of mean chromosomal H3K9me2 fluorescence intensities in control and Dnmt2 knock down (KD) cells. F.U., fluorescence units. (Replicate 1: control: n=41, Dnmt2 KD: n=135 ($p<0.0001$); Replicate 2: control: n=59, Dnmt2 KD: n=53 ($p=0.0021$), Student's t-test). **d**, Quantification of two independent experiments of mean chromosomal HP1 fluorescence intensities in control and Dnmt2 knock down (KD) cells. F.U., fluorescence units. (Replicate 1: control: n=289, Dnmt2 KD: n=308 ($p<0.0001$); Replicate 2: control: n=125, Dnmt2 KD: n=168 ($p<0.0001$), Student's t-test).

Defined chromatin states are especially important during mitosis and essential for proper chromosome segregation. To examine chromatin states of mitotic chromosomes in Dnmt2 depleted cells, chromosome spreads were analysed using immunofluorescence. Histone 3 di-methylated at lysine 9 (H3K9me2) and heterochromatin protein 1 (HP1) were examined in control and Dnmt2 KD cells (Figure 3.13.a, b). Quantification of fluorescence signals of H3K9me2 showed an increase of pericentromeric signal upon Dnmt2 depletion (Figure 3.13.c). HP1 levels, in contrast, were decreased in Dnmt2 knock down cells (Figure 3.13.d). These presumably counter-intuitive observations have also been made in yeast and are discussed below [Keller et al., 2013, Stunnenberg et al., 2015]. In summary, Dnmt2 is a strong regulator of pericentromeric heterochromatin affecting global chromatin states that alter transcriptional regulation. Moreover, Dnmt2 dysregulation causes changes in mitotic chromatin states, which may in turn lead to the observed chromosome segregation defects.

3.7 Components of the RNAi pathway associate with mitotic centromeres

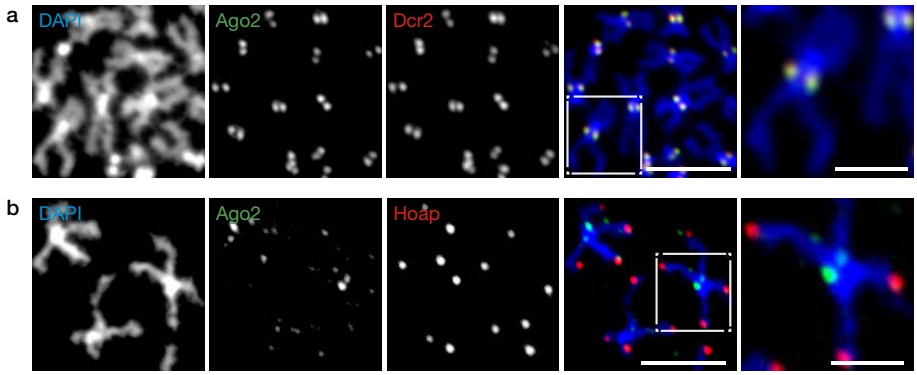


Figure 3.14: RNAi factors Ago2 and Dcr2 localise to mitotic centromeres. a-b, Immunofluorescence on mitotic chromosome spreads of S2 cells, stained with DAPI (blue), α-Ago2, and (**a**) α-Dcr2 (red) or (**b**) α-Hoap (red). Scale bars, 5 µm and 2 µm (zoom).

Components of the siRNA-pathway have been connected to heterochromatin formation and mitotic function in *Drosophila* [Fagegaltier et al., 2009, Deshpande et al., 2005]. Interestingly, Dnmt2 is required for the Dicer-2-dependent

siRNA pathway [Durdevic et al., 2013b]. Here, Ago2 and Dcr2 were detected co-localising specifically with centromeres during mitosis as observed for Dnmt2 (Figure 3.14.a). Co-staining of Ago2 with the telomeric capping protein Hoap confirmed the specificity of immunofluorescence experiments investigating centromeric interactions [Rashkova, 2002] (Figure 3.14.b). The co-localisation of RNAi components with the methyltransferase Dnmt2 could indicate a functional connection of tRNA methylation and siRNA-mediated heterochromatin formation, which requires further investigations.

3.8 Indications for a functional connection between RNAPIII-mediated transcription and centromeric Dnmt2

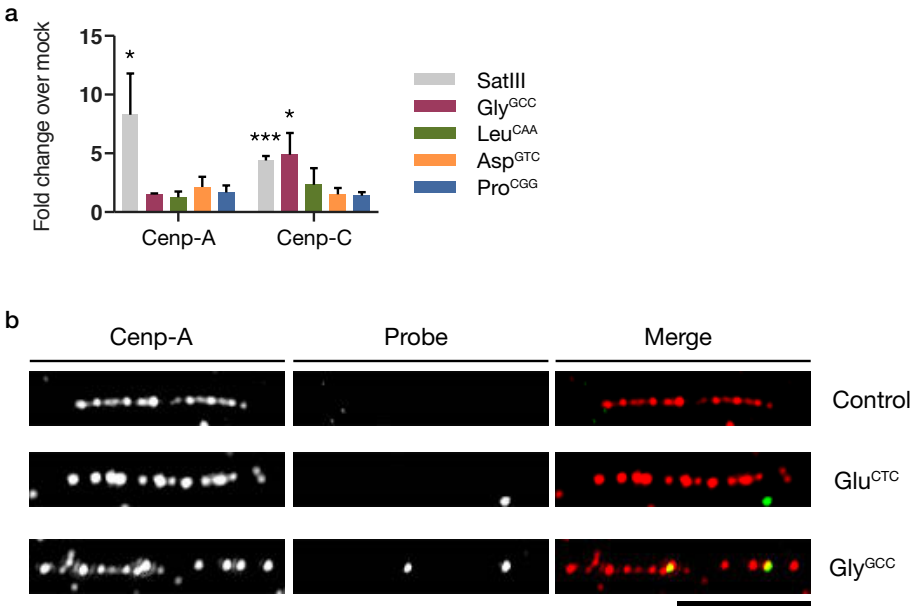


Figure 3.15: tRNA genes could potentially be encoded at Drosophila centromeres. **a**, ChIP-DNA qPCR: Enrichment analysis of selected tRNA genes in ChIP over control (n=3, mean±SD, $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$ (****), Student's t-test). **b**, Combined DNA-FISH-IF on chromatin fibres of S2 cells using Alexa488-labeled unspecific control, $tRNA^{Glu(CTC)}$- or $tRNA^{Gly(GCC)}$ -specific DNA oligo probes (green), anti-Alexa488 (green), and anti-Cenp-A (red). Scale bar, 5 µm.

The detection of tRNAs and tRNA-modifying enzymes raised the question whether these transcripts are associated with centromeres in trans or if they are encoded at or close to centromeres as shown for yeast [Kuhn et al., 1991, Takahashi et al., 1991, Partridge et al., 2000]. As described in the introduction, large pericentric domains of DNA sequences are not assembled in *Drosophila* (or other multicellular eukaryotes) due to their repetitive nature, including centromeric sequences. However, a number of single and clustered tRNA genes (tDNA) are present in pericentric heterochromatin and could potentially be encoded at or associated with centromeres during mitosis (Figure 3.7, supplements Figure B.1). To investigate these possibilities, several tRNA genes were tested using qPCR on centromere-ChIP samples. $tDNA^{Leu(CAA)}$, $tDNA^{Asp(GTC)}$ and $tDNA^{Pro(CGG)}$ were not increased in either Cenp-A or Cenp-C IPs compared to control. However, $tDNA^{Gly(GCC)}$ was significantly amplified in Cenp-C IPs. However, this result could not be confirmed in Cenp-A IPs (Figure 3.15.a). To further verify centromeric $tDNA^{Gly(GCC)}$, DNA-FISH on chromatin fibres with anti-Cenp-A immunostaining was performed. Indeed, FISH signals were detected localising to or between Cenp-A nucleosomes, when applying specific $tDNA^{Gly(GCC)}$ probes (Figure 3.15.b). Unspecific control or a $tDNA^{Glu(CTC)}$-specific probe did not show such localisation patterns. Nevertheless, these results need to be taken cautiously. A number of fluorophores need to accumulate at the site of interest to get a reliable signal-to-noise ratio. Here, single-labelled probes were amplified by applying indirect immunofluorescence. Whether this amplification is sufficient to detect a single tRNA gene with one hybridised probe per locus is not clear. However, chromatin fibres provide the highest possible resolution and signal-to-noise ratio for fluorescence microscopy on chromatin. This could be further increased through tRNA gene clusters as they frequently appear in the *Drosophila* genome (Figure 3.7, supplements Figure B.1). This allows the assumption that tRNA genes are encoded in centromeric domains.

Additional confirmation about the presence of tRNA genes can indirectly be assessed by the presence of the appropriate RNA polymerase III (RNAPIII). Immunofluorescence microscopy was used to examine the distribution of RNAP-III-related factors during mitosis. An antibody targeting TBP-related factor 1 (TRF1) was used, which is a transcription factor specific for RNAPIII [Isogai

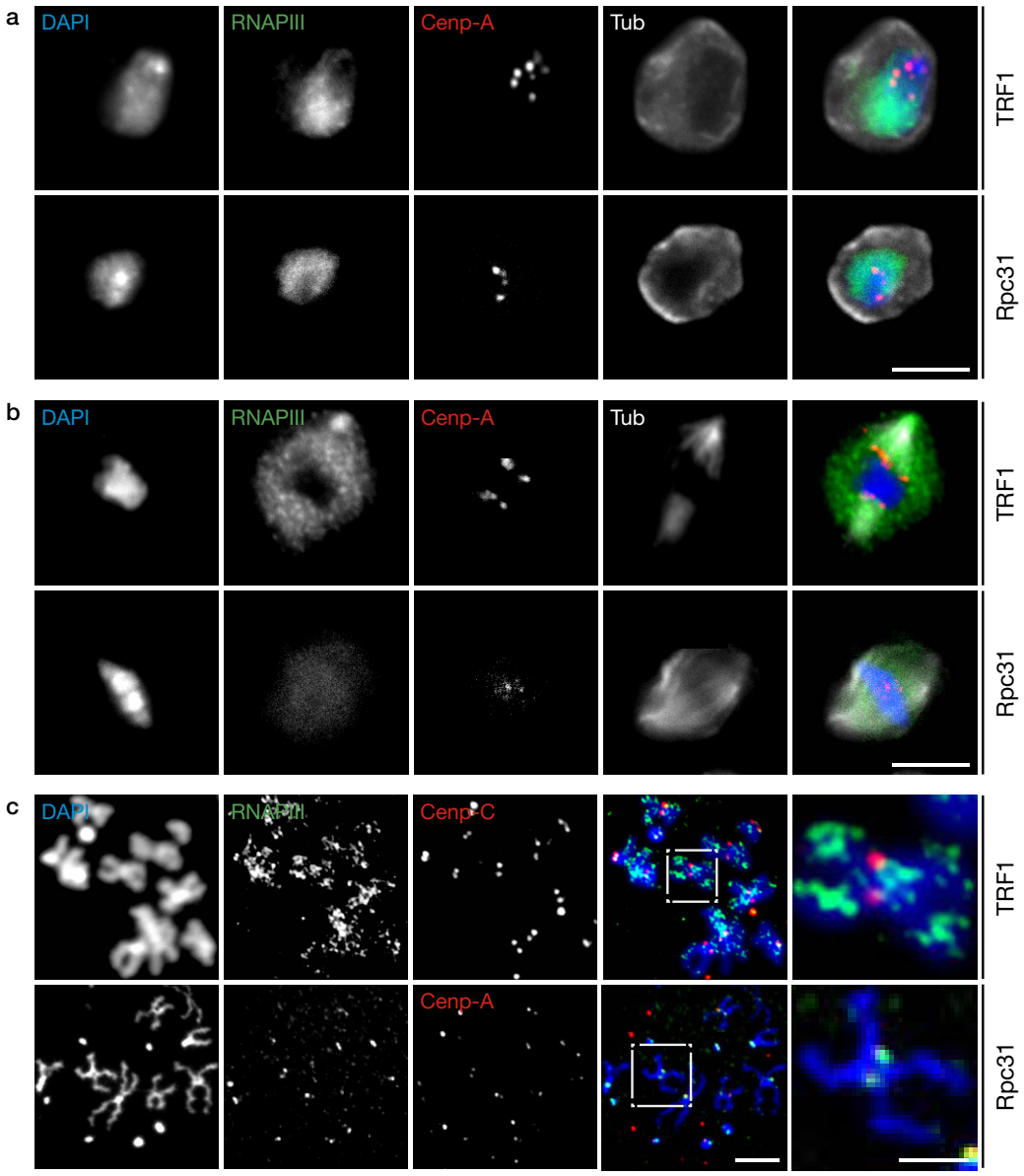


Figure 3.16: RNAPIII-specific transcription factor TRF1 and Rpc31 localise to mitotic centromeres. a-b, Immunofluorescence on wild type or Rpc31-GFP transfected S2 cells in (**a**) interphase and (**b**) metaphase, stained with DAPI (blue), anti-TRF1 or (Rpc31-GFP) anti-GFP (green), anti-Cenp-A (red), and anti-tubulin (grey). Scale bar, 5 µm. **c**, Immunofluorescence on mitotic chromosome spreads of wild type or Rpc31-GFP transfected S2 cells, stained with DAPI (blue), anti-TRF1 or (Rpc31-GFP) anti-GFP (green), and anti-Cenp-C (red). Scale bars, 5 µm and 2 µm (zoom).

et al., 2007]. Additionally, Rpc31 is an RNAPIII-specific polymerase subunit [Teichmann et al., 2010], which was tagged with GFP and stably transfected into S2 cells. Both factors showed nuclear signals with weakest levels at the highly dense pericentric chromatin domains with embedded centromeres (Figure 3.16.a). Upon nuclear breakdown during mitosis, TRF1 and Rpc31 were released from chromatin and distributed within the entire cell (Figure 3.16.b). Mitotic chromosome spreads revealed that a remarkable subset of transcription factors remained dispersed over the entire chromosome, including centromeres. Furthermore, Rpc31-GFP remained chromatin-bound and exhibited the highest concentration at centromeres (Figure 3.16.c). The presence of RNAPIII itself together with the corresponding transcription factor strongly suggests RNAPIII-mediated transcription during mitosis.

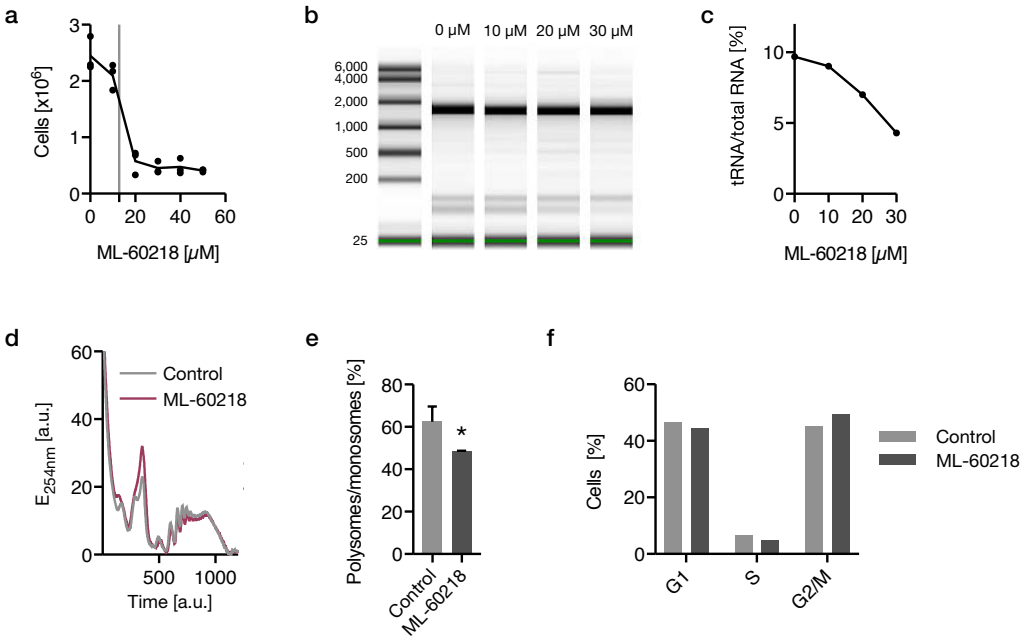


Figure 3.17: ML-60218 inhibits RNAPIII-mediated transcription in Drosophila S2 cells. **a-c**, S2 cells treated with ML-60218 at indicated concentrations for 48 hours. **a**, Growth curve, dashed line marks the IC_{20} (12.8 µM). **b**, Agilent TapeStation electropherogram of total RNA samples. **c**, Relative amounts of tRNA over total RNA. **d-f**, S2 cells treated with ML-60218 at IC_{20} or DMSO (control) for 24 hours. **d**, Representative polysome profiles (a.u., arbitrary units). **e**, Quantification of polysome over monosome ratio from three independent experiments (n=3, mean±SD, p=0.0287, Student's t-test). **f**, Quantification of representative FACS profiles.

Since RNAPIII and corresponding transcription products (tRNAs) were shown to be present at mitotic centromeres, the question was raised whether RNAPIII-mediated transcription is needed for chromosome segregation. Active RNA polymerase II (RNAPII) can specifically be distinguished from the inactive form by immunofluorescence using antibodies targeting phosphorylated serine 2 [Phatnani and Greenleaf, 2006], which is not possible for RNAPIII. For yeast and mammals a cell-permeable small molecule inhibitor for RNAPIII-transcription (ML-60218) has been described [Wu et al., 2003]. To test its potency in *Drosophila*, ML-60218 was applied in different concentrations on S2 cells for 48 hours. The growth curve revealed IC_{20}, IC_{50}, and IC_{90} values of 12.8, 19.0, and 26.2 µM, respectively (Figure 3.17.a). Gel electrophoresis displayed a specific decrease of tRNAs whereas 18S and 28S rRNA remained mostly unaffected (Figure 3.17.b). With respect to total RNA, tRNA levels decreased distinctly (Figure 3.17.c). After applying ML-60218 at IC_{20} to S2 cells for 24 hours, polysome profiles and polysome to monosome ratios changed only slightly (Figure 3.17.d-e) and cell cycle progression was not affected (Figure 3.17.f). In contrast, chromosome segregation was strongly disturbed when applying ML-60218 at IC_{20} and immediately recording cell divisions using live cell imaging (Figure 3.18.a). Comparable to the effects observed for Dnmt2 knock down, quantifying anaphase bridges, lagging chromosomes and chromosome fragments revealed a more than 2.5-fold increase of mitotic defects (Figure 3.18.b).

The comparability of mitotic defects of RNAPIII-inhibited and dysregulated cells for Dnmt2 raised the question of a functional interaction of RNAPIII and Dnmt2. Indeed, both factors co-localised on mitotic chromosome spreads (Figure 3.19.a). Moreover, inhibition of RNAPIII using ML-60218 at IC_{20} for 20 minutes (which is shorter than the duration of mitosis in S2 cells) depleted Dnmt2-GFP from centromeres and increased centromere-associated Rpc31-GFP (Figure 3.19.b). Likewise, knock down of Dnmt2 increased the signal of Rpc31 at centromeres (Figure 3.19.c). Moreover, centromeric Dnmt2 was found to be sensitive to RNase A treatment, which is in line with the depletion of Dnmt2 caused by RNAPIII transcriptional inhibition (Figure 3.19.d-e).

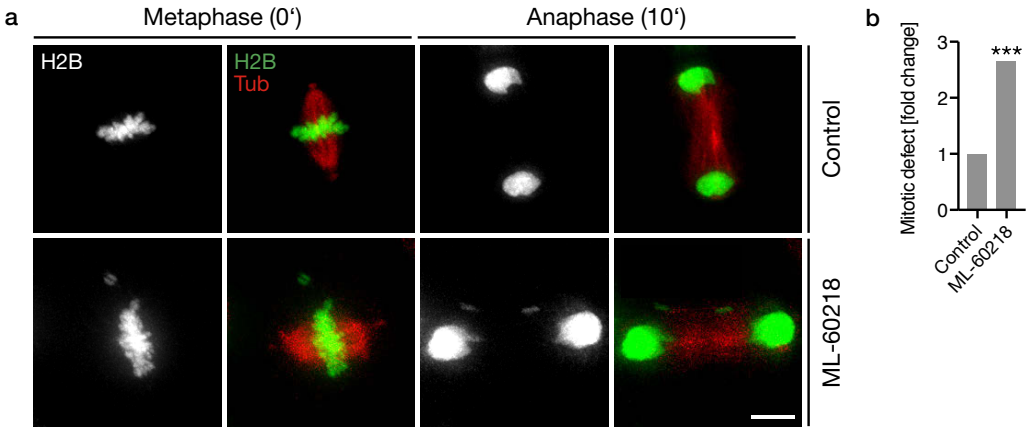


Figure 3.18: RNAPIII-mediated transcription is required for mitotic chromosome segregation. **a**, Representative micrographs from live cell imaging showing metaphase (left) and anaphase (right) of H2B-GFP (green), mCherry-tubulin (red) expressing S2 cells treated with DMSO (control) or RNAPIII inhibitor ML-60218 at IC_{20}. Numbers indicate time laps. Scale bar, 5 µm. **b**, Quantification of anaphase defects from two independent experiments (control: n=86, ML-60218: n=92 (p=0.0005), chi square test). Defects are represented as fold change over control.

In conclusion, RNAPIII was found to co-localise with Dnmt2 at centromeres and moderate but global inhibition of RNAPIII transcription disrupted chromosome segregation as seen for Dnmt2. Centromeric localisations of Dnmt2 and RNAPIII were disturbed after only 20 minutes of drug treatment, which is slightly shorter than a typical mitosis and drastically shorter than the expected half life of a eukaryotic tRNA (approximately two to three days; [Nwagwu and Nana, 1980, Kanerva and Mäenpää, 1981]), suggesting a regulatory role of active RNAPIII transcription at centromeres. Vice versa, Dnmt2 depletion also increased centromeric RNAPIII demonstrating reciprocal effects. These observations suggest a mutual role of Dnmt2-mediated tRNA methylation and RNAPIII-dependent transcription in the regulation of centromere function.

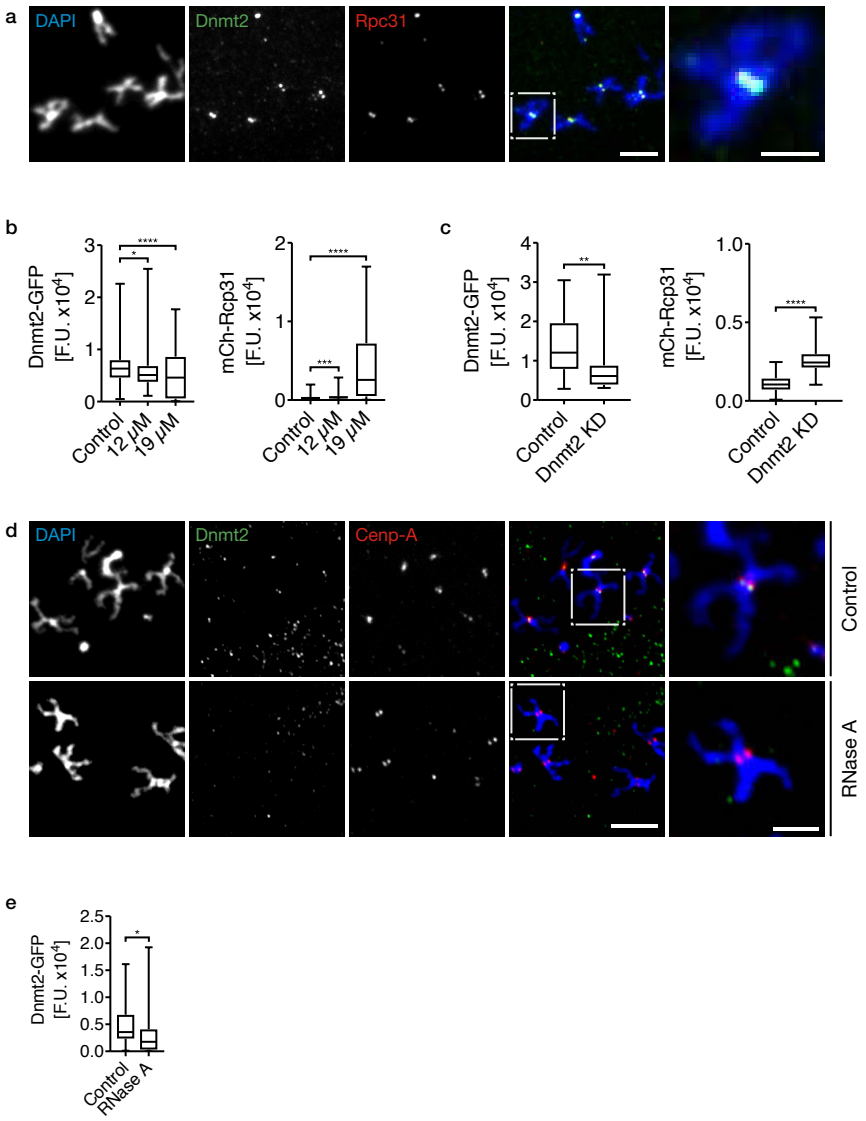


Figure 3.19: Centromeric localisation of Dnmt2 and RNAPIII depend on each other. a, Immunofluorescence on mitotic chromosome spreads of S2 cells expressing Dnmt2-GFP (green) and mCherry-Rpc31 (red), stained with DAPI (blue), anti-GFP (green), and anti-Cenp-C (not shown). Scale bars, 5 µm and 2 µm (zoom). **b-c**, Quantification of centromeric Dnmt2-GFP and mCherry-Rpc31 signals. **b**, Cells were treated with RNAPIII inhibitor ML-60218 at IC_{20} or DMSO control for 20 minutes prior to chromosome spreading. (Dnmt2: control: n=330, 12 µM: n=281, p=0.0105, 19 µM: n=378, p<0.0001; Rpc31: control: n=325, 12 µM: n=281, p=0.0002, 19 µM: n=386, p<0.0001 Student's t-test). **c**, Dnmt2 knock down (KD) cells were compared with Brown knock down (control) using dsRNA (Dnmt2: control: n=58, Dnmt2 KD: n=18, p=0.0022, Rpc31: control: n=58, Dnmt2 KD: n=17, p<0.0001, Student's t-test). **d**, Immunofluorescence on mitotic chromosome spreads of S2 cells expressing Dnmt2-GFP and mCherry-Cenp-A in control and RNase A-treatment, stained with DAPI (blue), and anti-GFP (green). Scale bars, 5 µm and 2 µm (zoom). **e**, Quantification of centromeric Dnmt2-GFP signals with and without RNase A-treatment prior to IF (control: n=105, RNAse: n=40, p=0.0146, Student's t-test).

4
Discussion

Regulation of chromatin states is a highly complex cellular process and directly connected to chromatin function. Especially during mitosis, the interplay between the open chromatin state of centromeres and the highly condensed surrounding pericentric heterochromatin are essential for chromosome segregation. Emerging evidence is arising that not only chromatin-associated transcripts but also the act of transcription itself is crucial for chromatin regulation [Hall et al., 2012, Rošić and Erhardt, 2016]. The impact of RNA modifications on chromatin regulation, however, remains elusive.

In this thesis, it was demonstrated that RNAPIII associates to mitotic centromeres and that RNAPIII-mediated transcription may be required for chromosome segregation. RNAPIII transcription products remained centromere-associated during mitosis. Moreover, centromeric $tRNA^{Gly(GCC)}$ appeared to be methylated at levels comparable to cytosolic tRNAs. The centromeric localisation of the tRNA methyltransferases Dnmt2 and NSun2 during mitosis points toward a role of RNA methylation in chromosome segregation. Indeed, depletion of these RNA MTases led to severe chromosome segregation defects. Detailed analysis of dysregulated Dnmt2 revealed disturbed chromatin states and mitotic defects that correlated with altered tRNA methylation levels. Finally, the examination of catalytic mutant larvae suggested a direct role of Dnmt2-mediated tRNA methylation in mitotic chromosome segregation. This finding reflects an epitranscriptomic regulation of centromeric function.

Interestingly, the centromeric localisation of Dnmt2 and RNAPIII appeared to be dependent on each other. This observation points towards a model of co-transcriptional regulation of centromeric transcription by RNA modifica-

tion. Both RNAPIII-mediated transcription as well as Dnmt2-mediated tRNA methylation is closely connected to the cellular stress response. Here the mitotic function of Dnmt2 appeared to be stress-related, which suggests a model of RNA biogenesis as a centromeric stress sensor. This might reflect a general centromeric regulatory mechanism since the mitotic function of Dnm2 was conserved in mammalian cells.

4.1 Dnmt2 regulates heterochromatin states during mitosis

In this thesis, Dnmt2 was found as a dominant modifier of heterochromatin states in mitosis. In agreement with this, the Reuter laboratory has previously demonstrated that Dnmt2 affects chromatin states in *Drosophila* [Phalke et al., 2009]. Null mutant flies revealed altered chromatin states at retrotransposons, telomeres, and repeat arrays, which was visible in reporter gene assays for PEV. Importantly, reporters in pericentric domains of the X chromosome remained unaffected. Here, Dnmt2 showed dominant modifier effects at different but not all tested pericentric loci. An explanation for this variance in PEV can be locus-dependent effects and hence the choice of reporter [Howe et al., 1995]. Of note, a different Dnmt2 mutant fly strain was used in this study, which is why an impact of the genetic background on PEV cannot be fully excluded. This underlines the need of additional complementing methods (e.g. qPCR and immunofluorescence) and different experimental systems (e.g. animal models and cell culture) to back these observations. According to this, Phalke et al confirmed the impact of Dnmt2 on heterochromatin states with immunofluorescence stainings of polytene chromosomes displaying distinctly decreased H4K20me3 levels in Dnmt2 mutant larvae. The methylation of histone H4 at lysine 20 is an evolutionarily conserved pericentric heterochromatin mark that depends on H3K9 methylation and subsequent HP1 association in human cells [Schotta et al., 2004]. Therefore, pericentric HP1 levels were examined in this thesis and found decreased on mitotic chromosomes upon Dnmt2 depletion, demonstrating a regulatory role of Dnmt2 for mitotic chromatin states. The observed increase of SATIII transcription, which is encoded in pericentric heterochromatin, further supports this conclusion.

Since constitutive heterochromatin is generally required for proper chromosome segregation [Kellum and Alberts, 1995, Allshire et al., 1995, Dernburg and Sedat, 1996, Ekwall et al., 1997, Melcher et al., 2000], the disruption of mitotic chromatin in Dnmt2-depleted cells is probably the reason for the detected mitotic defects.

In contrast to the decreased HP1 levels on mitotic chromosomes, H3K9me2 was slightly increased upon Dnmt2 depletion. This appears inconsistent at first sight, as H3K9 di- and tri-methylation is the conserved HP1 binding site from yeast to human [Bannister et al., 2001, Lachner et al., 2001]. However, exactly the same negative correlation has been observed in yeast [Keller et al., 2013, Stunnenberg et al., 2015]. The Bühler laboratory found that depletion of HP1 leads to a spreading of H3K9me2 marks across hetero- to euchromatin boundaries. Interestingly, HP1 was not necessary for spreading of H3K9 methylation but for restriction and demarcation of heterochromatin from neighbouring euchromatin. HP1 strengthens heterochromatin domains and hence functions as a regulatory element at chromatin boundaries.

Remarkably, the binding of HP1 to methylated H3K9 was antagonised by heterochromatic lncRNA [Keller et al., 2012, Stunnenberg et al., 2015]. In this thesis, decreased chromatin-associated HP1 levels were not only accompanied by spreading H3K9me2, but also increased pericentric transcription (SATIII). Therefore, elevated SATIII levels upon Dnmt2 depletion may be either cause or consequence of disturbed chromatin states.

Of note, Dnmt2 associated to mitotic chromatin in an RNase-sensitive manner. The agreement of the published yeast model with the data presented here suggests an RNA-dependent role of Dnmt2 at chromatin boundaries.

4.1.1 Indications for a function of Dnmt2 as a chromatin boundary factor

In yeast, chromatin boundary elements demarcate centromeric from pericentromeric chromatin [Donze, 2012]. A prerequisite for a boundary factor is the physical interaction with boundary elements, and Dnmt2 specifically localised to mitotic centromeres as observed here. Chromatin boundaries prevent spreading of heterochromatin into adjacent chromatin domains. Boundaries

can be DNA sequence elements that act as higher-order insulators or *cis*-acting barriers [Sun and Elgin, 1999]. Both types of boundaries are possible models for the nuclear function of Dnmt2.

Dnmt2 at centromeric tDNA barrier elements

The mitotic localisation of the RNAPIII transcription machinery and tRNAs themselves suggests centromere-encoded tRNA genes. As described in the introduction, tRNA genes at yeast centromeres build up chromatin barrier elements separating centromeric from pericentric heterochromatin [Partridge et al., 2000, ichi Noma et al., 2006, Scott et al., 2006]. Interestingly, the capability of chromatin boundaries is probably regulated by active transcription [Keller et al., 2013, Stunnenberg et al., 2015, Bernard et al., 2001, Scott et al., 2006]. Continuous transcription can perfectly be implemented by RNAPIII, which is characterised by highly progressive transcription cycles of re-initiation [Orioli et al., 2016].

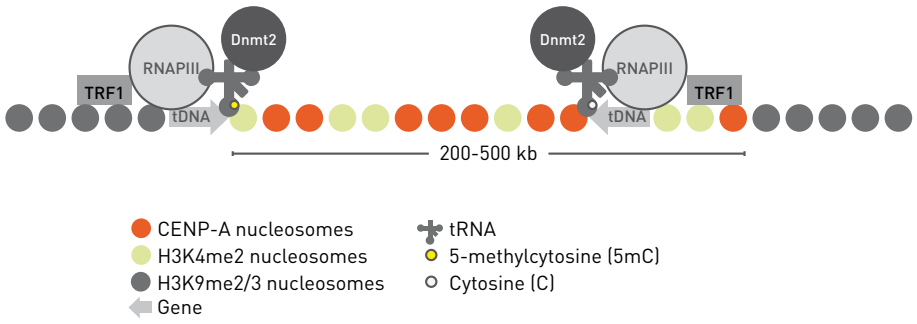


Figure 4.1: Model: RNAPIII transcription and tRNA methylation by Dnmt2 at centromeres are required for mitotic chromosome segregation in *Drosophila*. A tRNA transcription and modification complex functions as a chromatin barrier, preserving centromeric and pericentromeric chromatin states. Alternatively these complexes can act as regulatory elements keeping centromeric chromatin open.

Strikingly, the specific RNAPIII subunit Rpc31 and the tRNA methyltransferase Dnmt2 co-localised interdependently at centromeres, suggesting an interaction in mitosis. This was supported by the phenotypic similarity of chromosome segregation defects upon RNAPIII inhibition and Dnmt2 depletion. Therefore, the alterations of chromatin states discussed above could be explained by disrupted *cis*-regulatory elements such as chromatin barriers (Fig-

ure 4.1). It will be interesting to see, whether inhibition of RNAPIII leads to the same effects on chromatin as described for Dnmt2.

Dnmt2 at higher-order tDNA insulators

The concept of chromatin barriers assumes centromere-encoded tRNA genes. tDNA-FISH and ChIP-DNA-qPCR indicated but have not proved the existence of centromeric tDNA. This allows the assumption that tRNA genes encoded in non-centromeric loci might associate to centromeres by higher-order chromatin organisation in *trans* (Figure 4.2).

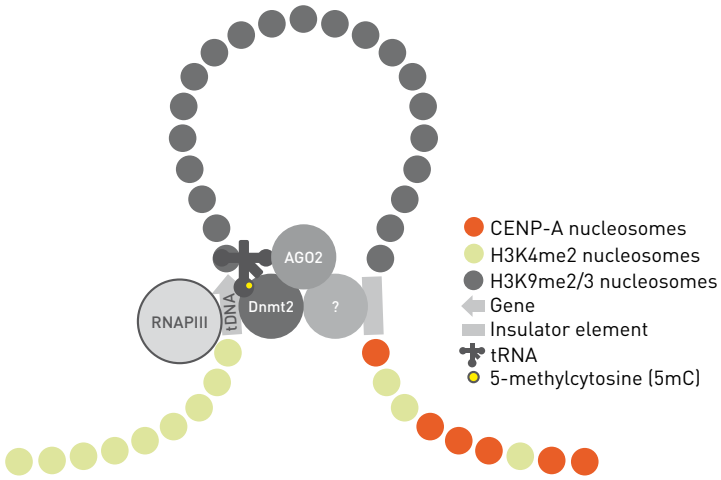


Figure 4.2: Model: *Trans*-acting tDNA regulates higher-order chromatin structures at mitotic centromeres. Actively transcribed tDNA associates in *trans* to mitotic centromeres. Dnmt2 functions as a component of insulator complexes (e.g. with AGO2) regulating higher order chromatin structures.

This model is supported by a study in yeast that functionally connected mitotic chromosome condensation with the centromeric localisation of RNAPIII genes in *trans* [Iwasaki et al., 2010]. The association of dispersed RNAPIII genes with centromeres became particularly prominent in mitosis, and it was shown that RNAPIII transcription regulated this interaction. The association of RNAPIII genes to centromeres was proposed to be required for mitotic chromosome condensation, which is essential for chromosome segregation. These *trans*-interacting elements presumably function as chromatin boundaries just like *cis*-acting tDNA chromatin barriers [Iwasaki and Noma, 2012].

Alike the molecular mechanisms of chromosome segregation, tDNA insulators are highly conserved from yeast to human [Donze et al., 1999, Raab et al., 2011, Ebersole et al., 2011, Moqtaderi et al., 2010, Yanagida, 2005]. In addition, the RNAPIII transcription factor TFIIIC has insulator activity in fly, mouse, and human (Van Bortle et al. 2014). Dnmt2 may be recruited to centromeres by *trans*-associated tDNA, which regulates higher-order chromatin organisation that in turn provides structural stability in chromosome segregation (Figure 4.2).

In summary, disrupted boundaries in Dnmt2 depleted cells can cause dysregulated chromatin states, which lead to global chromosomal instability and thereby chromosome segregation defects. The open question of centromere-encoded tRNA genes might soon be clarified by emerging long-read sequencing techniques, which can overcome the technical issues of sequencing of repetitive DNA such as centromeres.

4.1.2 Indications for an interaction between RNA methylation and RNAi-mediated heterochromatin formation

In this thesis, the RNAi-components Ago2 and Dcr2 were detected at mitotic centromeres, akin Dnmt2. Both factors have previously been connected with *Drosophila* insulators [Moshkovich et al., 2011, Cernilogar et al., 2011], which is concordant with the tDNA insulator model discussed above. However, insulator functions of Ago2 and Dcr2 were connected to euchromatic rather than repetitive chromatin domains. The specific localisation of these factors to repetitive centromeric chromatin therefore suggests a role of the siRNA pathway in heterochromatin formation and chromosome segregation [Fagegaltier et al., 2009, Deshpande et al., 2005], independent of the non-enzymatic insulator function. This is supported by the different localisations of the insulator factors CTCF and CP190, which are Ago2 interaction partners at insulators but did not co-localise to all centromeres as Ago2 and Dcr2 (supplements B.4).

Several studies identified tRNA fragments associating with Argonaute and Dicer proteins, among them fragments of Dnmt2 substrates [Haussecker et al.,

2010, Burroughs et al., 2011, Durdevic, 2013, Kumar et al., 2014, Karaiskos et al., 2015, Durdevic et al., 2013b, Cole et al., 2009]. It has been proposed that tRNA-derived fragments themselves could function as small RNAs in gene silencing [Haussecker et al., 2010, Burroughs et al., 2011, Cole et al., 2009]. Certainly, they are part of the RNAi-related competition of small RNAs and could block siRNA-dependent RNAi function [] [Haussecker et al., 2010, Durdevic and Schaefer, 2013b]. Strikingly, tRNAs and Dnmt2-dependent tRNA-derived fragments served as Dcr2 substrates and were able to inhibit Dcr2 function on dsRNA [Durdevic et al., 2013b].

The loss of Dnmt2 methylation could lead to a centromeric accumulation of tRNA fragments, which interfere with RNAi-mediated regulation of heterochromatin states (Figure 4.3). Accordingly, tRNA fragment-mediated disruption of the RNAi pathway could prevent recruitment of Ago2 and Dcr2 and hence cause altered protein levels at mitotic centromeres, which can be examined on chromosome spreads of Dnmt2 depleted cells.

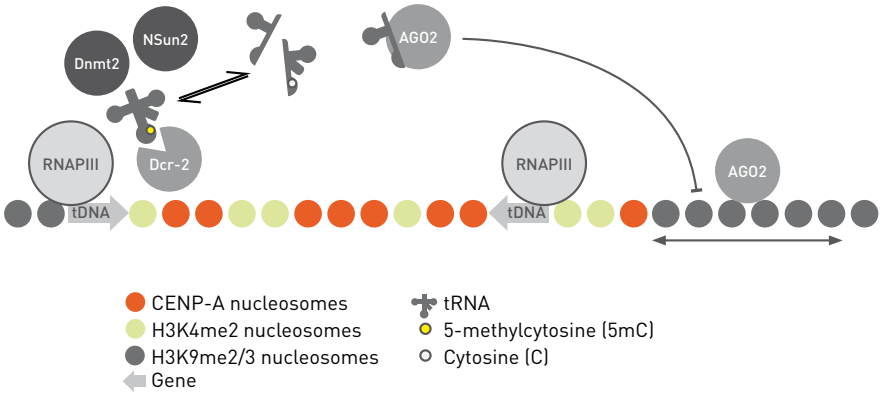


Figure 4.3: Model: Heterochromatin formation by RNAi components is inhibited by tRNA fragments. Loss of tRNA methylation by Dnmt2 and NSun2 leads to increased fragmentation of tRNAs. tRNA fragments competitively bind RNAi components thereby blocking canonical interactions and inhibiting heterochromatin formation.

To answer which chromatin alterations observed in this study are causes and which are consequences requires further investigations. Dnmt2-dependent heterochromatin marks and pericentric transcription levels can depend on boundary effects or the RNAi pathway, or on both. Heterochromatin regulation appears as a complex and interdependent network of partially redundant as

well as complementing mechanisms, and is often accompanied by shared components in multiple pathways, as described in the introduction. For example, the mutual regulation of HP1 deposition and lncRNA transcription at heterochromatin boundaries in yeast was connected to Dicer-generated siRNA-like small RNAs that originate from lncRNAs transcribed from a pericentromeric boundary element [Keller et al., 2013]. The specific composition of chromatin modifiers at centromeres, which was identified here, strongly suggests a specific regulation of centromeric chromatin states that includes transcription and RNA processing.

4.2 Active RNAPIII transcription at mitotic centromeres

Several observations in this thesis indicate active RNAPIII transcription at mitotic centromeres in *Drosophila*. This is in agreement with the conserved transcriptional activity of centromeric chromatin in general, and with the RNAPIII-mediated transcription of boundaries in yeast. During mitosis, overall transcription is minimal but not completely absent [Rošić and Erhardt, 2016], which is also true for RNAPIII transcription [White et al., 1995a] [Gottesfeld et al., 1994, White et al., 1995b, Fairley et al., 2003, Fairley et al., 2012].

In this thesis, the RNAPIII-specific subunit Rpc31 and the TFIIIB transcription factor TRF1 (TBP-related factor 1) were present at mitotic centromeres. TFIIIB is essential for transcriptional initiation [Moir and Willis, 2013]. Of note, transcription factors can remain at transcriptionally inactive loci of mitotic chromosomes to 'bookmark' these sites for facilitated re-initiation of transcription after mitosis [Chen et al., 2005, Teves et al., 2016]. In addition, transcription factor-associated sites have been connected to a multitude of extra-transcriptional functions including chromatin boundaries and higher-order organisation [Donze, 2012]. Accordingly, the presence of TRF1 confirms RNAPIII promoters at centromeres, but it does not prove RNAPIII-mediated transcription.

However, not only TRF1 but also the RNAPIII-specific subunit Rpc31 has been detected at mitotic centromeres [Werner et al., 1993, Werner et al., 1992]. It has been shown that RNAPIII occupancy correlates strongly with ongo-

ing transcription and that RNAPIII arrest at DNA can be largely excluded under standard conditions [Orioli et al., 2016]. Thus, the centromeric localisation of the transcription machinery in combination with RNAPIII-specific products (tRNAs) supports the hypothesis of active RNAPIII transcription at centromeres in *Drosophila*.

4.2.1 Indications for co-transcriptional RNAPIII regulation by tRNA methylation

The Dnmt2-dependent chromatin alterations provide a plausible explanation for the observed mitotic defects. However, the underlying molecular mechanism is elusive. The interdependent co-localisation of Dnmt2 and the RNAPIII machinery, observed in this thesis, indicates a connected function at centromeres. This is supported by the common mitotic defects upon transcriptional inhibition and dysregulation of tRNA methylation. The model of co-transcriptional tRNA methylation integrates the Dnmt2- and RNAPIII-dependent mitotic defects and the interdependent localisation (Figure 4.4).

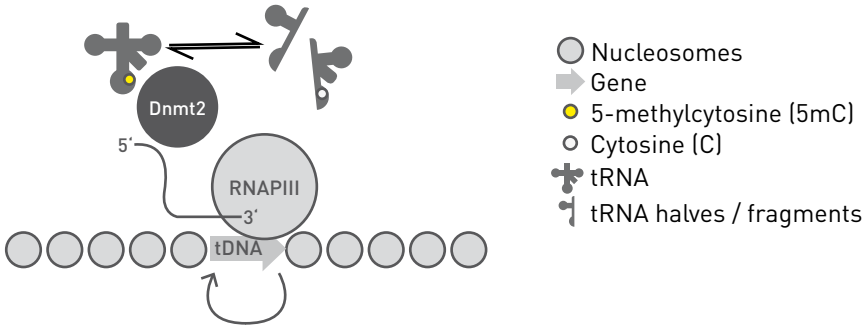


Figure 4.4: Model: Co-/post-transcriptional modification regulates RNAPIII transcription at centromeres. Dnmt2 binding or methylation of nascent tRNA regulates transcriptional termination, facilitating re-initiation of RNAPIII. Increased amounts of tRNA fragments may feed back to the RNAPIII transcription process by promoting or blocking the recruitment of further factors.

tRNA processing regulates RNAPIII transcription

Continuous RNAPIII-mediated transcription requires efficient termination to facilitate re-initiation. Nascent RNA needs to be released for transcriptional

termination of RNAPIII [Campbell and Setzer, 1992], and pre-tRNA processing probably facilitates this crucial step [Arimbasseri et al., 2013]. A result of inefficient termination is the accumulation of the polymerase at the transcription site [Turowski et al., 2016]. The increased RNAPIII levels upon transcriptional inhibition or Dnmt2 depletion, which was detected in this thesis, reflect this phenomenon. Vice versa, inhibited RNAPIII leads to decreased tRNA levels and thus decreased tRNA-mediated recruitment of Dnmt2.

Many different RNA processing mechanisms can occur co-transcriptionally [Perales and Bentley, 2009] [Ameur et al., 2011, Rodriguez et al., 2012, Fu et al., 2014] Among them is the cleavage of pre-tRNA by RNase P [Esakova and Krasilnikov, 2010]. RNAPIII-mediated transcription was significantly decreased upon RNase P depletion, which indeed connects tRNA processing with transcriptional regulation [Reiner, 2006, Jarrous and Reiner, 2007]. This might also be true for tRNA methylation, but requires further investigations like pre-tRNA or nascent tRNA analysis, or the inhibition of RNAPIII transcription in ChIP-RNAseq experiments.

Co-transcriptional tRNA processing regulates heterochromatin states

Co-transcriptional RNA surveillance and polymerase processivity have been connected with heterochromatin formation [Reyes-Turcu et al., 2011]. A recent publication demonstrated that disrupted tRNA processing by RNase P decreased heterochromatin levels at tRNA transcription sites and simultaneously increased the expression of transposable elements in *Drosophila* [Molla-Herman et al., 2015]. Mechanistically, it was suggested that the depletion of RNase P results in stalled RNAPIII caused by unprocessed tRNAs that are misfolded and therefore not efficiently released for termination [Nielsen et al., 2013].

The model proposed by Molla-Herman et al. reflects some important observations of this thesis. Dysregulation of Dnmt2 leads to increased RNAPIII levels at centromeres, altered chromatin states, and up-regulated transcription of pericentric RNAPII-dependent transcripts. Data from the Lyko laboratory revealed up-regulated transposon expression upon Dnmt2 depletion [Durdevic, 2013]. A direct regulation of transposable elements via Dnmt2-mediated methylation appears unlikely, since cytosine-5 methylation of transposon-originated

transcripts could not be detected by the transcriptome-wide RNA methylation study performed in this thesis (supplements B.5). Accordingly, chromatin-mediated effects rather than direct methylation of these transcripts likely regulate transposon and SATIII transcription.

Co-transcriptional regulation of centromeric chromatin

The methylation of tRNAs is thought to stabilise the tRNA structure, which may regulate RNAPIII-mediated transcription, as described for RNase P [Molla-Herman et al., 2015]. Indeed, tRNA modification enzymes are functionally redundant with other nascent pre-tRNA stabilisation mechanisms [Anderson et al., 1998, Copela et al., 2006]. The centromeric RNA processing factors, detected in this thesis, may function as specific regulators of transcription during mitosis. Co-transcriptional methylation as a step of tRNA maturation could facilitate transcriptional termination specifically at centromeres (Figure 4.4). In this manner, Dnmt2 could contribute to both of the proposed models – the maintenance of transcription at chromatin boundaries and the regulation of RNAi-mediated heterochromatin formation (Figure 4.1, 4.2, 4.3).

Interestingly, human Ago2 has previously been found to bind nascent tRNAs and to regulate gene expression in *cis* [Woolnough et al., 2015]. In conclusion, co-transcriptional tRNA processing is a general and possibly also centromeric regulatory mechanism. In this model, disruption of the centromeric RNA processing machinery deregulates RNAPIII transcription, which induces altered chromatin states and RNAPII transcription in *cis*. This deregulation leads to a global propagation of altered chromatin states as detected upon Dnmt2 depletion.

4.3 tRNA biogenesis as a stress sensor at centromeres

As described in the introduction, the biogenesis of tRNAs is directly connected to the cellular stress responses. Several cross-talking stress-signalling pathways regulate RNAPIII-mediated transcription [Moir and Willis, 2013]. In addition, not only tRNA transcription but also tRNA modification is dynamically regulated during various kinds of stress [Chan et al., 2010]. More-

over, Dnmt2-mediated tRNA methylation regulates the biogenesis of stress-dependent tRNA-derived fragments [Schaefer et al., 2010].

Accordingly, the mitotic role of Dnmt2 described in this thesis is connected to cellular stress. Both heat shock and cell culture stress increased the amount of mitotic defects in S2 cells and larval tissue. Surprisingly, the relative increase of defects upon stress was smaller in cells lacking Dnmt2 than in wild type cells in three independent experimental setups. This observation indicates a saturated stress situation upon Dnmt2 depletion.

Stress-dependent regulation of centromere function

Mitosis is the most vulnerable cell cycle phase. As observed in this study, mitotic chromosome segregation is highly sensitive to cellular stress, which was reflected by severely increased mitotic defects. Entry and transition through mitosis are highly regulated by a multitude of sensor and effector proteins, and a range of post-translational modifications. Mitotic kinases such as Aurora-B, macromolecular complexes such as the anaphase promoting complex/cyclosome (APC/C), and protein components of the G2/M and spindle assembly checkpoints (SAC) such as PLK1 and Mad2 control mitosis upon harmful incidents such as DNA damage, in order to ensure genome stability [Ferrari and Gentili, 2016]. These factors also transiently associate to the kinetochore during mitosis [Kang et al., 2006] [McKinley and Cheeseman, 2014, Zhang, 2004, Sharp-Baker and Chen, 2001, Adams et al., 2001, Murata-Hori et al., 2002, Acquaviva et al., 2004]. However, specific centromeric stress-sensors are barely described.

Interestingly, a recent study demonstrated that the localisation of Aurora-B, which controls proper spindle attachment to the centromeres [Lampson and Cheeseman, 2011], is dependent on centromeric transcription [Blower, 2016]. In addition, transcription at centromeres is stress-dependent regulated [Bouzinba-Segard et al., 2006]. The conservation of (peri-) centromeric transcription in general, and the response to stress in particular [Hall et al., 2012], might indicate that the transcriptional process itself rather than individual RNAs or protein factors functions as a stress sensor at centromeres. The complex regulation of transcription provides a system to respond to stress, which can in principle be adopted for centromere regulation during mitosis. The observed

accumulation of the pericentromeric lncRNA SATIII upon Dnmt2 depletion can therefore be a consequence of mitotic stress.

Stress-dependent regulation of RNAPIII transcription

As discussed above, components of the RNAPIII transcription machinery and tRNAs were detected at mitotic centromeres, indicating spatially restricted RNAPIII transcription during mitosis. Generally, RNAPIII transcription is highly active (15% of total cellular transcription; [Moir and Willis, 2013]) to maintain the constantly high demand for tRNAs (4-10% of all cellular RNA; [Durdevic and Schaefer, 2013b]). A variety of repressive regulation mechanisms provide the opportunity to balance the protein synthesis capacity with respect to environmental, nutritional, and stress-related influences [Moir and Willis, 2013, Orioli et al., 2016, Ernens et al., 2006].

The biogenesis of tRNAs could provide a suitable system to regulate centromere function upon stress. The high activity and stress-dependent regulation of RNAPIII-mediated transcription accomplish the general requirements for centromeric transcription. The regulatory mechanisms of the essential processes of tRNA transcription and chromosome segregation need to be connected for cell cycle regulation. As discussed above, tRNA methylation might contribute to transcriptional regulation (Figure 4.4), providing a stress sensor for centromeres during mitosis.

tRNA fragmentation as a stress sensor at centromeres

tRNA biogenesis and processing is not limited to the canonical maturation of full-length tRNAs. The generation of tRNA-derived fragments is closely related to stress [Thompson and Parker, 2009a]. tRNA fragments play important roles in stress responses [Durdevic and Schaefer, 2013b] and are regulated by Dnmt2-mediated methylation [Schaefer et al., 2010]. Accordingly, stress has been shown to be a central aspect for the biological role of Dnmt2 [Durdevic and Schaefer, 2013a]. The stress-related mitotic function of Dnmt2, as described in this thesis, suggests a central role of tRNA methylation and fragmentation as a stress response at centromeres.

Generally, RNA processing could provide the required flexibility and immediacy to respond to cellular stress. Of note, all RNA processing enzymes, which

have been identified to be centromeric in this thesis (Dnmt2, NSun2, Ago2, Dcr2), have been connected to stress and tRNA fragmentation [Schaefer et al., 2010, Tuorto et al., 2012, Blanco et al., 2014, Blanco et al., 2016, Cernilogar et al., 2011, Kumar et al., 2014, Durdevic et al., 2013b, Burroughs et al., 2011, Cole et al., 2009, Haussecker et al., 2010]. Importantly, not only Dnmt2 but also its enzymatic activity and hence tRNA methylation are required for faithful chromosome segregation, as demonstrated by catalytically inactive *Dnmt2* mutants. tRNA methylation could regulate the stress-induced generation of centromeric tRNA fragments by Dcr2 [Durdevic et al., 2013b], which subsequently regulate Ago2 or RNAPIII at centromeric chromatin.

Vice versa, the depletion of these factors, as shown here upon Dnmt2 depletion, provokes cellular stress situations that can result in the observed mitotic defects. This might also be true for the inhibition of RNAPIII using a small molecule drug. The applied concentrations of this inhibitor were low and no distinct effects on cell cycle progression, translation, or rRNA transcription could be observed here. However, unspecific effects upon drug treatment that trigger the cellular stress response cannot entirely be excluded [Brose et al., 2012].

Small RNAs have been shown to function in heterochromatin formation, gene regulation, and genome stability [Castel and Martienssen, 2013]. Additionally, tRNA fragments are functionally active cellular components rather than simple degradation products, as described in the introduction. Mechanistically, an increased amount of tRNA fragments can affect the regulation of transcription or heterochromatin, both of which can be regulated via the RNAi pathway (as discussed above) or directly in a feedback mechanism to the transcriptional machinery [Janowski et al., 2006, Cho et al., 2014, Haussecker et al., 2010, Layat et al., 2013]. The regulation of tRNA stability by cytosine-5 methylation can therefore regulate the centromeric stress response (Figure 4.3 & 4.4).

4.4 Indications for additional tRNA fragmentation-independent functions of Dnmt2

In this thesis, the overexpression of wild type Dnmt2 did not rescue Dnmt2-dependent mitotic defects but revealed comparable defects as Dnmt2 depletion or catalytic inactivation. Of note, both the depletion of endogenous Dnmt2 and the dominant-negative delta-cat Dnmt2 decreased C38 methylation levels. On the contrary, overexpression of the wild type Dnmt2 increased the methylation at the same site. A previous study demonstrated that overexpression of Dnmt2 globally rescues tRNA fragmentation of Dnmt2 substrates [Schaefer et al., 2010]. Assuming the same at centromeres, the mitotic defects upon overexpression cannot be caused by increased levels of tRNA fragments. Although this does not exclude a role of tRNA fragmentation upon decreased methylation levels per se, at least one alternative mechanism probably co-exists that explains the mitotic defects upon Dnmt2 overexpression. In the following, potential mechanisms that might explain this observation are discussed.

First of all, one needs to take into account that overexpression can generally provoke non-natural situations within the cell. This may also cause mitotic stress that leads to the observed defects, probably independent of tRNA fragmentation. The kinetochore consists of over 100 proteins in prophase [Ferrari and Gentili, 2016]. This macromolecular complex needs to be tightly regulated and overexpression of Dnmt2 might disrupt the canonical protein composition. Of note, Dnmt2 can tightly bind to DNA *in vitro* [Dong et al., 2001], although its DNA methylation activity is very weak [Fisher et al., 2004, Hermann et al., 2003, Raddatz et al., 2013]. Therefore, DNA binding upon overexpression could disrupt kinetochore formation or centromeric DNA structures through perturbation of the relative complex compositions. Alternatively, the increased number of active Dnmt2 could methylate off-targets, as shown for the Dnmt2 homolog Pmt1 in *S.pombe*, which methylates $tRNA^{Glu(TTC)}$ when over-expressed [Becker et al., 2012]. Non-canonical and potentially centromere-related substrates can be identified using whole-transcriptome bisulfite sequencing (WTBS) of Dnmt2-overexpressing cells. Another possibility is related

to the discussed co-transcriptional methylation model. Centromeric chromatin is rather euchromatic in contrast to the surrounding pericentric heterochromatin [Sullivan and Karpen, 2004] and non-canonical secondary structures of centromeric DNA require active transcription [Gallego et al., 1997, Garavís et al., 2015, Sun and Hurley, 2009, Kouzine et al., 2008]. Up-regulation of Dnmt2 could lead to deregulated RNAPIII as observed upon Dnmt2 depletion. Another possible explanation is a non-enzymatic function of Dnmt2, which is discussed below.

4.4.1 Enzymatic-independent function of Dnmt2

In this thesis, a role of tRNA methylation in chromosome segregation was demonstrated using catalytically inactive Dnmt2 mutant flies. Interestingly, mitotic defects upon Dnmt2 overexpression indicated a possible additional non-enzymatic but dosage-dependent function of centromeric Dnmt2.

It has previously been speculated that the evolutionarily conserved Dnmt2 might also have functions other than RNA methylation activity [Durdevic and Schaefer, 2013a]. The examined control of retrotransposon and viral RNA, as well as the propagation of RNA-induced paramutations by Dnmt2 could not be clearly connected to Dnmt2's methylation activity [Phalke et al., 2009, Durdevic et al., 2013a, Kiani et al., 2013, Liebers, 2015], and could therefore be regulated by enzymatic-independent functions.

In principle, the chromatin-boundary models discussed above (Figure 4.1 & 4.2) are compatible with enzymatic-independent functions of RNA processing factors, which has previously been demonstrated for the role of Ago2 at insulator elements and promoters [Moshkovich et al., 2011, Taliaferro et al., 2013]. Alternatively, the hypothesised role of Dnmt2 in RNAPIII transcriptional regulation (Figure 4.4) could be accomplished by a chaperone-like function, which has been described for the RNA chaperone protein La [Bayfield et al., 2010, Fan et al., 1998, French et al., 2008]. Indeed, the function of La is based on structural stabilisation through binding of nascent tRNAs and has been described to be redundant with tRNA modification enzymes [Anderson et al., 1998, Copela et al., 2006]. The concept of co-existing non-enzymatic functions is supported by such observations for other RNA processing proteins such as the 6mA RNA methyltransferase METTL3 and epigenetic factors such as the DNA methyl-

transferase Dnmt1 [Lin et al., 2016, Thompson and Parker, 2009b, Acquati et al., 2005, Smirnoff et al., 2006, Espada et al., 2011].

At first sight, an enzymatic-independent function of Dnmt2 is supported by the ubiquitous localisation of Dnmt2 to all centromeres of all chromosomes, which does not entirely match the centromeric localisation of $tRNA^{Gly(GCC)}$ at approximately a third of all chromosomes. The RNase-sensitivity of centromeric Dnmt2 affected all centromeres equally. Therefore the other two substrates, $tRNA^{Asp(GTC)}$ and $tRNA^{Val(AAC)}$, non-substrate tRNAs, or non-tRNA transcripts might also interact with Dnmt2 at centromeres. Generally, Dnmt2 is able to bind other RNAs than tRNA substrates [Durdevic et al., 2013a, Durdevic, 2013], which allows all of these possibilities. However, undetectable FISH signals do not necessarily contradict the presence of a given transcript, since the concentration might be below the detection limit of this method. This is supported by the ChIP-RNAseq experiment: Although $tRNA^{Asp(GTC)}$ lacked FISH signals on spreads (data not shown), RNAseq revealed reads for the same tRNA in the centromere pull-downs that were quantitatively comparable to overall nuclear tRNA, which eventuates in a log2 fold change close to zero. This was confirmed by qPCR analysis that revealed low levels of $tRNA^{Asp(GTC)}$ in the Cenp-C IP.

The investigation of chromosome segregation in catalytically Dnmt2-inhibited S2 cells and especially in catalytically mutant *Dnmt2* flies clearly demonstrated a crucial role for Dnmt2-mediated methylation in mitosis. However, co-existing catalytically independent functions of centromeric Dnmt2 cannot be excluded and may become relevant especially in non-canonical environments such as cellular stress. To finally elucidate the centromeric role of tRNA fragmentation, FISH experiments targeting different tRNA fragments or ChIP-RNAseq analysis in catalytically inactive and wild type systems should be analysed.

4.5 tRNA methylation by the evolutionarily conserved Dnmt2 is required for mitosis

This study addresses two highly conserved and essential cellular processes – mitosis and transcription. The presented data suggests Dnmt2-mediated cytosine-5 methylation of tRNAs as the functional connection between tRNA biogenesis

and chromosome segregation. Importantly, Dnmt2 is a highly conserved RNA methyltransferase, and Dnmt2-dependent segregation defects were conserved in mouse and human cell lines, suggesting a general role of tRNA modification in mitosis.

Research on tRNA modifying enzymes has often encountered the phenomenon that strong phenotypes, such as lethality or sterility, cannot be observed in single mutant conditions [Phizicky and Alfonzo, 2010, Phizicky and Hopper, 2010, Grosjean et al., 2010, El Yacoubi et al., 2012]. The same is true for the tRNA methyltransferase Dnmt2, as described in the introduction [Wilkinson et al., 1995, Goll et al., 2006, Kunert et al., 2003, Schaefer et al., 2010, Durdevic and Schaefer, 2013a]. This seems to contradict the high conservation of tRNA modifications and the respective enzymes at first. An explanation for the comparably weak phenotypes in single modification mutants is the concept that a multitude of tRNA modifications function 'in concert', not only cooperatively, but possibly also redundantly [Alexandrov et al., 2006, Chernyakov et al., 2008]. This is confirmed by the synthetic lethality of Dnmt2/NSun2 double mutant mice [Tuorto et al., 2012], and by the increase of mitotic defects upon Dnmt2 and NSun2 double knock down, observed here. In addition, the strong connection of tRNA biogenesis to environmental cues indicates a central role of tRNA modifications under non-laboratory conditions [Durdevic and Schaefer, 2013b].

tRNAs themselves are probably the most ancient ncRNAs and many modification sites are highly conserved in all domains of life [Motorin and Helm, 2011]. RNA methylation evolved independently multiple times, which underlines its significance [Motorin and Helm, 2011]. The conservation of cytosine-5 methylation was also demonstrated in this thesis, revealing conserved Dnmt2- and NSun2-dependent methylation sites in yeast and flies, as previously described for mouse and human.

The highly conserved but non-essential role of Dnmt2 in *Drosophila* does not contradict the novel role in chromosome segregation described in this thesis. Mitosis is a fundamental cellular process that is essential for life of multicellular organisms. The same is true for the role of centromeres as the sites of spindle attachment, which is indispensable for chromosome segregation [Allshire and Karpen, 2008]. Remarkably, a couple of mutations of key mitotic regulators are accompanied with severe mitotic defects but do not lead to lethality [Yohn

et al., 2003]. Examples are the spindle assembly checkpoint protein Mad2 and the RNAi component Ago2 [Buffin et al., 2007, Deshpande et al., 2005]. Internal data from the Erhardt laboratory reveals the same for flies that are depleted for SATIII DNA (Sreemukta Acharya, unpublished), which is required for proper chromosome segregation [Rošić et al., 2014].

Even though these defects do not cause lethality in flies under standard laboratory conditions, they may explain the reduced stress tolerance of these model organisms [Schaefer et al., 2010]. It can be speculated that this disadvantage is sufficient to make Dnmt2 essential outside of the laboratory.

4.5.1 RNA methyltransferases in mitosis

As described in the introduction, little is known about the mitotic role of RNA methyltransferases. Mitotic Dnmt2 is enriched and gets access to chromatin in *Drosophila* embryos [Schaefer et al., 2008]. However, the mitotic function remained elusive.

Dnmt2-mediated methylation in the nucleus

The vast majority of nuclear $tRNA^{Asp(GTC)}$ and $tRNA^{GlyGCC)}$ was found to be methylated, similar to tRNAs in total RNA samples. This indicates that co- or post-transcriptional Dnmt2-mediated methylation can occur in the nucleus for all nuclear tRNAs throughout the cell cycle. Dnmt2 is a predominantly cytosolic protein with a small fraction localising to the nucleus [Schaefer et al., 2008], which was confirmed here. In theory, the nuclear fraction of Dnmt2 can principally mediate nuclear tRNA methylation and the cytosolic pool a more specific, context-dependent methylation or alternative enzymatic-independent functions [Durdevic and Schaefer, 2013b, Durdevic and Schaefer, 2013a]. However, the high abundance of tRNAs and Dnmt2 in the cytosol argues for cytosolic Dnmt2-mediated methylation in most of the cell cycle phases. Further investigations like cell cycle-dependent bisulfite analysis of subcellular fractions are needed to answer this question.

Specific spatiotemporal regulation of centromeric chromatin during mitosis

In this thesis, the specific centromeric localisation of the tRNA methyltransferase Dnmt2 and of its substrate $tRNA^{GlyGCC)}$ supported the hypothesis of a mitotic function of Dnmt2, which was confirmed by the examination of chromosome segregation in cells dysregulated for Dnmt2. The presence of Dnmt2-mediated tRNA methylation at centromeres suggested an enzymatic role of Dnmt2, which was confirmed by the analysis of catalytically inactive Dnmt2. In conclusion, Dnmt2-mediated tRNA methylation is required for the regulation of centromere function in mitosis.

The unique centromeric chromatin is differently regulated compared to non-centromeric domains (see introduction). This can explain the specific appearance of Dnmt2 at mitotic centromeres and the ensuing impact on the centromeric RNAPIII localisation. Importantly, global effects on transcription and tRNA levels upon Dnmt2 depletion could not be detected in previous studies [Liebers, 2015, Durdevic, 2013, Schaefer et al., 2010]. The impact of Dnmt2 on RNAPIII is probably restricted in place and time, since immunofluorescence studies of Dnmt2 and RNAPIII revealed a clear co-localisation exclusively at centromeres during mitosis. Especially RNAPIII transcription, which occurs at genome-wide distributed tRNA genes during interphase (supplements B.1), requires a centromere- and mitosis-specific regulation to maintain mitotic transcription. This is supported by the hypothesis that transcriptional activity designates the functionally active state of the respective chromatin domain [Rošić and Erhardt, 2016]. The recruitment of Dnmt2 to centromeres is presumably a component of such a spatiotemporal regulation, as indicated by the interdependent co-localisation of Dnmt2 and RNAPIII.

Indications for an epigenetic role of Dnmt2 in meiosis

The only function of Dnmt2 in cell division published so far is the role of Dnmt2 in asymmetric sister chromatid segregation in male *Drosophila* germline stem cells [Yadlapalli and Yamashita, 2013a]. Besides Dnmt2, components of the nuclear envelope and the centrosome were found to be essential for non-random chromatid segregation. In the proposed model, the mother centrosome is stably anchored between the hub cell and the nuclear envelope, which is con-

nected to one of each pair of chromatids depending on different epigenetic marks at the sister chromatids [Yadlapalli and Yamashita, 2013b]. The centromere presumably serves as the perfect chromosomal component for such a selective capture [Thorpe et al., 2009]. Yadlapalli & Yamashita speculate that Dnmt2 could set the required epigenetic mark, although the molecular mechanism is completely elusive. In this thesis, Dnmt2 and its substrates were shown to localise to mitotic centromeres, which may represent the missing link in the non-random segregation model. To prove this hypothesis, the centromeric localisation of Dnmt2 needs to be examined in meiotic stem cells of the germline, where different kinds of asymmetry are a common feature [Fichelson and Huynh, 2007] [Spradling et al., 2011].

The data of this thesis demonstrates the ability of Dnmt2 to bind and epigenetically mark centromeres with methylated tRNAs. The ability of Dnmt2 to associate with the nuclear matrix [Schaefer et al., 2008] could accomplish the asymmetry needed for the non-random chromatid segregation. However, a role of Dnmt2 in meiosis was not part of this thesis and is hence only speculative. It will be interesting to examine whether Dnmt2 is also present at centromeres in the germline.

The mitotic function of Dnmt2 is conserved from flies to human

In contrast to Dnmt2, distinct mitotic defects have previously been found in NSun2 depleted cells [Hussain et al., 2009]. Remarkably, the mitotic role of NSun2 appeared to be independent of its methylation activity [Hussain et al., 2009]. In conclusion, this is the first detailed report of an enzymatic function of a tRNA methyltransferase in mitotic chromosome segregation. Remarkably, not only the molecular function and substrate-specificity, but also the mitotic function of Dnmt2 is conserved from fly to human, as demonstrated here, which suggests a general role of tRNA methylation in mitosis.

5
Conclusions

Mitosis is a key process for the inheritance of epigenetic information from one cell generation to the next. A role of Dnmt2 in mitosis was proposed almost a decade ago (Schaefer et al. 2008), however a mitotic function and molecular mechanisms remained elusive. Here, not only a direct role of Dnmt2 and tRNA methylation in chromosome segregation but also several indications for a general and interdependent role of tRNA transcription and processing in chromosome segregation were demonstrated.

Transcriptional regulation and chromatin function are closely related to each other. Although RNAPII and RNAPIII repression do not require condensed chromatin structures or repressive chromatin factors (Spencer et al. 2000; Hartl et al. 1995), several publications report a crucial role of transcription in chromatin regulation (e.g. RNAi-mediated heterochromatin formation, chromatin insulators and boundaries) (Slotkin & Martienssen 2007; Ebersole et al. 2011; Scott et al. 2006). This is especially true at centromeres (Rosic & Erhardt 2016), as recent publications demonstrated different functionally essential mechanisms of centromeric transcription at mitotic centromeres (Liang et al., 2015, Liu et al., 2015, Blower, 2016).

It is intriguing to consider RNA to be functioning as a carrier of epigenetic memory, because nucleic acids are absolutely precise transmitters of epigenetic information (Kouzarides 2007). Both the phenomena of RNA-dependent paramutations (though molecular mechanisms remain elusive) and RNA-mediated heterochromatin formation support this hypothesis and have previously been connected to Dnmt2 (Rassoulzadegan et al. 2006, Kiani et al. 2013; Liebers

et al. 2014, Phalke et al. 2010, Volpe & Martienssen 2011). Following this idea, transcription would be both a prerequisite and an executing mechanism of RNA-mediated inheritance. In this regard, the diversity of RNA modifications provides a complex regulatory system for the regulation of epigenetic inheritance.

Cross-talk of transcriptomic and epitranscriptomic mechanisms regulating centromeric chromatin

The presence of at least four different RNA processing factors at mitotic centromeres, namely Dnmt2, NSun2, Ago2, and Dcr2, is the first indication for a large number of RNA processing events during mitosis. Dnmt2 is a tRNA-specific methyltransferase, NSun2 has been found to additionally methylate a limited number of ncRNAs, and the RNAi components are associated with a variety of small RNAs and related pathways. Such types of RNAs have been detected at centromeres in different species, with crucial functional roles. It is appealing to speculate that specific RNA processing events regulate these RNAs.

The number of RNA modifications (especially on tRNAs) demonstrates the complexity of epitranscriptomic regulation. This may especially be essential at highly specified chromatin sites, such as centromeres, for example for fine-tuning of chromatin compositions for kinetochore formation, or to respond to cell cycle dependent or environmental signals like nutrition or stress.

The discrimination of centromeric from other chromatin is reflected by the specific histone H3-variant Cenp-A, the specific composition of post-translational modifications, the transcriptional activity during mitosis, and the unique nature of underlying DNA sequences and emerging transcripts. Epitranscriptomic regulation of the transcribed centromeric RNAs may very well be involved in regulation of centromeres, especially as the unique nature of centromeres requires a unique form of regulation. Epitranscriptomic mechanisms can provide the needed diversity and flexibility for this purpose.

In this thesis, the only distinct chromatin-association of Dnmt2 was with centromeres and during mitosis, which may indicate a spatiotemporally restricted function. Transcriptional regulation of tRNA genes encoded within the chromosome arms is probably independent of Dnmt2, especially outside of

mitosis. Remarkably, depletion of Dnmt2 led to globally affected chromatin states, also in somatic tissue as seen in PEV experiments, which may indicate the regulation of an early step in chromatin formation and heritable effects of mitotically active Dnmt2.

In conclusion, Dnmt2-mediated regulation of centromeric chromatin may create an environment, which is necessary for centromeric identity, kinetochore formation, and subsequent chromosome segregation during mitosis.

6

Materials & Methods

6.1 Materials

All materials used in this study are generally used in the Erhardt and Lyko laboratories unless otherwise specified.

Equipment and laboratory materials

Equipment and the laboratory materials frequently used in this study are listed in table 1.

Table 6.1: Equipment and laboratory materials

Equipment or material	Provider
-80 °C freezer	Heraeus
0.2 ml PCR reaction tubes	Sarstedt, Thermo Scientific
1.5 and 2 ml reaction tubes	Sarstedt, Eppendorf
15 and 50 ml tubes	Sarstedt
150 cm2 flask (cell culture)	Orange Scientific
25 cm2 flask (cell culture)	Orange Scientific
384-well Plates	Steinbrenner Laborsysteme
454 Genome Sequencer FLX Titanium	Roche
75 cm2 flask (cell culture)	Orange Scientific
8 well chambered slides	Ibidi
96-well Plates	Thermo Scientific
Agarose Gel Electrophoresis	BioRad, Workshop ZMBH
AR1 microscope	Nikon
Balance	Sartorius, Kern EG
Bioruptor	Next Gen
Blotting materials	BioRad
ChIP-IT Magnetic Beads	Active Motif
Cover slips	Thermo Scientific
Deltavision microscope	GE healthcare lifescience
Eppendorf Pipettes	Gilson
FACSAria™ Illu Cell Sorter	BD Biosciences
FlowJo software	FlowJo LLC
FLUOstar OPTIMA	BMG Labtech
Heraeus multifuge 1L	Thermo Scientific
Illumina HiSeq 2000 System	Illumina
Leica M420 macroscope system	Leica
LightCycler 480 instrument.	Roche

Micropipettes	Gilson
NanoDrop ND-1000 Spectrophotometer	Thermo Scientific
Nitrocellulose membrane	Amersham Biosciences
Nunc CryoTubes	Sigma
PAGE gel cast	BioRad
PCR-cycler	BioRad
pH-meter	Sartorius
Pipette tips	BioRad, EMBL PS143, Nerbe
Poly-lysine coated microscopy slides	Thermo scientific
Power supplies	Sarstedt, TipOne, Avant Guard, Consort
Protein gel equipment	BioRad
Shandon 4 Cytospin	Thermo Scientific
Shandon EZ Double Cytofunnel	Thermo Scientific
Shandon EZ Megafunnel	Thermo Scientific
Stereo microscope	Zeiss
Superfrost Plus Slides	Thermo Scientific
SW60 rotor	Beckman
Tabletop centrifuges	Eppendorf
TapeStation	Agilent
Thermo Mixer	Eppendorf
Thermocycler, DNA Engine	BioRad
UA-6 gradient fractionator	ISCO
UV stratalinker 2400	Stratagene
Vortex	Scientific industries
Waterbath	Memmert
Western blot wet system	BioRad
Western Turbo blot system	BioRad
Whatman Paper	Roth
XCell SureLock Mini-Cell	Thermo Scientific

Chemicals

Chemicals used in this study were purchased from Agilent, Ambion, AppliChem, Baker, Bioline, BioRad, Fermentas, Fluka, Invitrogen/Life technologies, J.T. Barker, Labconsult, Merck, New England Biolabs, Poly Sciences, Roche, Roth, Sigma, SouthernBiotech, Thermo Scientific, and VWR. For a detailed overview see table 2.

Table 6.2: Chemicals

Chemical	**Provider**
2-Propanol	AppliChem
30% Acrylamide solution	AppliChem
Acetic Acid	Merck
Acrylamide (37,5:1) Rotiphorese 30	Roth
Agarose	Roth
Agarose Ultra Pure	Invitrogen
Albumin Fraction V (pH 7.0) (BSA)	Invitrogen
Ammonium Acetate	Fluka
Ammoniumpersulfat	Sigma
Bio Spin 6 Chromatography Columns	BioRad
Bromophenol Blue	AppliChem
Calcium chloride	Fluka
Chloroform	VWR
CoT-1 DNA	Invitrogen
CuSO4	Applichem
DAPI	AppliChem
dATP	New England Biolabs
Deoxynucleotides Mix (dNTPs)	Agilent
Diethylpyrocarbonate (DEPC)	AppliChem
Dimethyl Sulfoxide (DMSO)	Baker
Dithiothreitol (DTT)	Fluka

DNA Ladder, O'GeneRuler 1 kb	Fermentas
DNA Ladder, O'GeneRuler 1oo bp Plus	Fermentas
DNA Ladder, O'RangeRuler 100bp+500bp	Fermentas
DNA Ladder, O'RangeRuler 10bp	Fermentas
EDTA	Roth
Ethanol absolute	AppliChem
Ethidium bromide	Roth
Ethidium bromide (EtBr)	AppliChem
Fluoromount-G	SouthernBiotech
Formaldehyde	J.T. Barker
Formaldehyde 37%	AppliChem
Formamide	Sigma
Glycerol	AppliChem
GlycoBlue	Ambion
HEPES	AppliChem
Hoechst 33258	Invitrogen
Methanol	ZMBH
Milk Powder	AppliChem
MOPS	AppliChem
Mounting medium –Aqua/polymount	Poly Sciences
N-Ethylmaleimide	Sigma
Na2 EDTA	Roth
Na2 HPO4	AppliChem
Nonidet P-40	AppliChem
Novex TBE Gels, 6%, 12 well	Invitrogen
phenol/chloroform	Sigma
Phenol/Chloroform/Isoamylalcohol	Ambion
Phenylmethylsulfonylflouride (PMSF)	Roth
Potassium chloride	Fluka
Protease inhibitor cocktail complete Sodium acetate-3H2O	Roche
Protein Ladder, PageRuler Plus Prestained	Fermentas
RNA ladder, RiboRuler HR #SM1821	Thermo Scientific
RNA ladder, RiboRuler LR #SM1831	Thermo Scientific
RNA Loading Buffer, 2x	Thermo Scientific
RNase Inhibitor, RiboLock	Thermo Scientific
Sodium azide	AppliChem
Sodium chloride	AppliChem
Sodium citrate	AppliChem
Sodium dodecyl sulfate ß-Mercaptoethanol	AppliChem
ß-Mercaptoethanol	AppliChem
Sybr Gold	Thermo scientific
TEMED	AppliChem
Triethanolamine	AppliChem
Tris	AppliChem
Trisure	Bioline
Triton X-100	Merck
Trizol	Invitrogen
Tween 20	AppliChem
Vectashield mounting medium	Labconsult
Yeast tRNA	Ambion

Tissue culture reagents

Tissue culture reagents frequently used in this study are listed in table 3.

Table 6.3: Tissue culture reagents

Reagent	**Provider**
Cellfectin II	Invitrogen
Colchemid	Capricorn Scientific
Fetal Bovine Serum (FBS)	Biochrom AG
Heparin	Sigma
Hygromycin B solution	Sigma
Penicillin, Streptomycin	Invitrogen

Schneider's *Drosophila* medium	Invitrogen

Buffers and solutions

Frequently used buffers and solutions used in this study are listed in table 4. All buffers were prepared with double destilled water of DNase/RNase-free water (Gibco).

Table 6.4: Buffers and solutions

Buffer	Ingredients
Apple juice agar plate for embryo collection	3g Agar in 50ml Water + 50ml Apple juice
ChIP buffer A	5 mM PIPES (pH 8.0) 85 mM KCl 0.5% NP40 1x Roche Protease Inhibitor Cocktail 10 mM Ribonucleoside Vanadyl Complex
ChIP buffer B	1% SDS 10 mM EDTA 50 mM Tris-HCl (pH 8.1) 1x Roche Protease Inhibitor Cocktail 10 mM Ribonucleoside Vanadyl Complex
ChIP elution buffer	1% SDS 100 mM NaHCO3 10 mM Ribonucleoside Vanadyl Complex
ChIP high salt wash	0.1 % SDS 1% Triton X-100 20 mM Tris-HCl (pH 8.1) 2 mM EDTA 500 mM NaCl
ChIP IP buffer	0.01 % SDS 1.1% Triton X-100 1.2 mM EDTA 16.7 mM Tris-HCl (pH 8.1) 167 mM NaCl 1x Roche Protease Inhibitor Cocktail 10 mM Ribonucleoside Vanadyl Complex
ChIP LiCl wash	250 mM LiCl 1 % NP40 1% deoxycholate 1 mM EDTA 10 mM Tris-HCl (pH 8.1)
ChIP low salt wash	0.1 % SDS 1% Triton X-100 20 mM Tris-HCl (pH 8.1) 2 mM EDTA 150 mM NaCl
Chromatin fibres salt detergent lysis buffer	25mM Tris, pH 7.5 500mM NaCl 1% Triton X-100
FISH hybridization buffer	2x SSC 50% formamide 10% dextran sulfate in 2x SSC
Fractionation lysis buffer	50 mM Tris-HCl (pH 7.5) 1 % NP-40 150 mM NaCl 2 mM PMSF 1 µg/ml aprotinin 1 µg/ml leupeptin 1 µg/ml pepstatin Roche Protease Inhibitor Cocktail (1:50)

IF PBS blocking solution	1x PBS
	0.1% Triton X 100
	1% BSA Fraction V
IF PBS permeabilization solution	1x PBS
	0.1% Triton X 100
Laemmli sample loading buffer 4x	50 mM Tris-HCl pH 6.8
	10% glycerol
	2% SDS
	0.5% ß-Mercaptoethanol
	0.02% Bromphenol Blue
Paraformaldehyde (PFA) 4% in 50 ml PBS	2g PFA
	75.7µl 1N KOH
	5ml 10X PBS
	45ml H2O
PBS	137 mM NaCl
	2.7 mM KCl
	10 mM Na2HPO4
	1.7 mM KH2PO4
	adjusted to pH 7.5 (HCl)
Polysome lysis buffer	20 mM TRis-HCl (pH 7.5)
	150 mM NaCl
	5 mM MgCl2
	1 mM DTT
	1% Triton X-100
Ponceau	0.2% Ponceau
	3% Trichloroacetic acid
RIPA buffer	50 mM TrisHCl (pH7.5)
	150 mM NaCl
	1% NP-40
	0.5% Sodium dodecylsulfate
	0.1% SDS
	2 mM PMSF
SDS gel running buffer 1x	25 mM Tris
	190 mM glycine
	0.1% SDS
SDS-PAGE separation gel (12%)	0.375 M Tris-HCl pH 8.8
	10.5% acrylamide/bisacrylamide 30:0.8%
	0.1% SDS
	0.05% APS
	0.05% TEMED
SDS-PAGE stacking gel	0.123 M Tris-HCl pH 6.8
	4.4% acrylamide/bisacrylamide 30:0.8%
	0.1% SDS
	0.03% APS
	0.1% TEMED
Spreads hypotonic solution	0.5% (w/v) Sodium citrate
	in ddH2O
Spreads KB buffer	10 mM Tris-HCl (pH 7.7)
	0.15 M NaCl
	0.1% BSA
Spreads KCM buffer	120 mM KCl
	20 mM NaCl
	10 mM Tris-HCl (pH 7.7)
	0.1% Triton X-100
Spreads TEEN buffer	1mM Triethanolamine-HCl (pH 8.5)
	0.2 mM EDTA
	25 mM NaCl
	0.1% Triton X-100
	0.1% BSA
SSC 20x	3M NaCl
	0,3M sodium citrate
SSCT 4%	2x SSC
	0.1% Tween-20
Standard *Drosophila* Medium	18 g Agar
	150 g Dextrose
	170 g Maize Meal
	30 g Dry Yeast
	50 ml 10% Nipagin M 1700 ml H2O
Sucrose gradient (17.5-50%)	15 mM Tris-HCl (pH 8.0)
	15 mM MgCl2
	300 mM NaCl

TBE-Agarose Gel	1% (w/v) Agarose 1x TBE
TBS 10x	30 g/l Tris 88 g/l NaCl 2 g/l KCl pH 7.5
Tris-acetate-EDTA (TAE) 50x	242 g/l Tris-HCl 18.6 g/l EDTA pH 7.7 adjusted with acetic acid 890 mM Tris Base 890 mM Boric Acid 20 mM EDTA
Tris-EDTA buffer (TE)	10 mM Tris (pH 8.0) 1 mM EDTA
UREA-PAGE 15% (Rotiphorese)	30 ml Gel Solution 15 ml Diluent 5 ml Buffer
UREA-PAGE 20% (Rotiphorese)	40 ml Gel Solution 5 ml Diluent 5 ml Gel Solution
Western blocking buffer	1x PBS 1% Tween-20 5% Milk powder
Western borate transfer buffer 20x	20 mM Boric acid 1 mM EDTA 0.1 mM DTT pH 8.8
Western mild stripping buffer	15 g/l glycine 0.1% SDS 1% Tween-20 pH 2.2
Western tris-glycine-methanol transfer buffer	25 mM TrisHCl 0.192 M glycine 20% methanol absolute
Western washing buffer	1x TBS/PBS 0-0.1% Tween-20

Primary antibodies

Primary antibodies frequently used in this study are listed in table 5.

Table 6.5: Primary antibodies

Primery antibody	Species	Dilution (Application)	Source
α-Ago2 (9D6)	mouse	1:50 (IF)	H. Siomi (Kawamura et al. 2008)
α-Alexa Fluor 488	rabbit	1:500 (IF)	Molecular Probes
α-alpha tubulin	mouse	1:1000 (IF); 1:5000 (WB)	Sigma
α-CENP-C	guinea pig	1:2000 (IF)	G. Karpen
α-CID	rabbit	2.5 µl (ChIP)	Active Motif
α-CID	chicken	1:200 (IF)	P. Heun
α-CP190	guinea pig	1:250 (IF)	E. Lei (Lim et al. 2013)
α-CTCF	guinea pig	1:50 (IF)	E. Lei (Lim et al. 2013)
α-Dcr2	rabbit	1:200 (IF)	Abcam
α-Digoxigenin	mouse	1:200 (FISH)	Abcam
α-Dnmt2 peptide 2	rabbit	1:100 (WB)	F. Lyko (Schaefer et al. 2008)
α-GFP	rabbit	1:10000 (IF)	A. Straight
α-H3	rabbit	1:1000 (WB)	Abcam
α-H3K4me2	goat	1:200 (IF)	Abcam
α-H3K9me2	rabbit	1:500 (IF)	Abcam
α-His	rabbit	2.5 µg (ChIP)	Abcam
α-Hoap	rabbit	1:100 (IF)	Yikang Rong
α-HP1	mouse	1:1000 (IF)	Hybridoma bank (C1A9)
α-TRF1	rabbit	1:200 (IF)	W. Stumph (Verma et al. 2013)
α-Tubulin	rabbit	1:1000 (IF); 1:5000 (WB)	Abcam
α-Tubulin	goat	1:500 (IF)	Santa Cruz
α-YFP	rabbit	1:5000 (WB)	S. Erhardt

Secondary antibodies

Secondary antibodies frequently used in this study are listed in table 6.

Table 6.6: Secondary antibodies

Secondary antibody	Species	Dilution (Application)	Source
α-chicken Alexa Fluor 488 IgG	goat	1:500 (IF)	Invitrogen
α-chicken Alexa Fluor 546 IgG	goat	1:500 (IF)	Invitrogen
α-chicken Alexa Fluor 647 IgG	goat	1:500 (IF)	Invitrogen
α-goat Alexa Fluor 488 IgG	donkey	1:500 (IF)	Invitrogen
α-goat Alexa Fluor 546 IgG	donkey	1:500 (IF)	Invitrogen
α-goat Alexa Fluor 647 IgG	donkey	1:500 (IF)	Invitrogen
α-guinea pig Alexa Fluor 488 IgG	goat	1:500 (IF)	Invitrogen
α-guinea pig Alexa Fluor 546 IgG	goat	1:500 (IF)	Invitrogen
α-guinea pig Alexa Fluor 647 IgG	goat	1:500 (IF)	Invitrogen
α-mouse Alexa Fluor 488 IgG	goat	1:500 (IF)	Invitrogen
α-mouse Alexa Fluor 546 IgG	goat	1:500 (IF)	Invitrogen
α-mouse Alexa Fluor 647 IgG	goat	1:500 (IF)	Invitrogen
α-mouse polyclonal IgG-HRP	goat	1:10000 (WB)	Abcam
α-rabbit Alexa Fluor 488 IgG	goat	1:500 (IF)	Invitrogen
α-rabbit Alexa Fluor 546 IgG	goat	1:500 (IF)	Invitrogen
α-rabbit Alexa Fluor 647 IgG	goat	1:500 (IF)	Invitrogen
α-rabbit polyclonal IgG-HRP	goat	1:10000 (WB)	Abcam

Enzymes

Enzymes frequently used in this study are listed in table 7.

Table 6.7: Enzymes

Enzyme	Provider
BaseMuncher endonuclease	Expedeon
Benzonase	Sigma
DreamTaq PCR Master Mix	Thermo Scientific
Fire Taq blue	Steinbrenner
Pfu X polymerase	Jena Bisciences
Proteinase K	Sigma
PyroMark PCR Kit	QIAGEN
Restriction enzymes	New England Biolabs
RNase A	AppliChem
SuperScript III Reverse Transcriptase	Invitrogen
T4 Polynucleotide kinase	TaKaRa
Taq Master Mix 2x	Fermentas
TURBO DNase	Ambion

Kits

Kits frequently used in this study are listed in table 8.

Table 6.8: Kits

Kit	Provider
DES TOPO TA expression kit	Invitrogen
EZ RNA Methylation Kit	Zymo Research
LightCycler 480 SYBR Green I Master	Roche
MEGAscript RNAi Kit	Ambion
mirVana	Ambion

NEBNext Magnesium RNA Fragmentation Module	New England Biolabs
NEBNext Small RNA Library Prep Set	New England Biolabs
NG dART RT Kit	roboklon
QIAquick PCR Purification Kit	Qiagen
Quant-iT PicoGreen	Invitrogen
QuantiTect Reverse Transcription Kit	Qiagen
RiboMinus Eukaryote System v2	Ambion

Inhibitors

Protease and phosphatase inhibitors were supplemented to protein biochemistry buffers. ML-60218 is an RNAPIII inhibitor used in functional studies on S2 cells. RNase inhibitors were supplemented to buffers and solutions of RNA-related methods. Inhibitors used in this study are listed in table 9.

Table 6.9: Inhibitors

Inhibitor	Provider
Aprotinin	AppliChem
Complete Protease Inhibitor	Roche
Leupeptin	AppliChem
ML-60218	Calbiochem, Merck
Pepstatin	AppliChem
PMSF	Sigma
Ribonucleoside Vanadyl Complex	New England Biolabs
RNaseOut	Invitrogen

DNA and LNA oligonucleotides

All primers used in this study (Table 10) and other commonly used primers can be found in the Erhardt laboratory primer collection. FISH probes are labelled as LNA or DNA oligonucleotides, respectively. DNA oligonucleotides were synthesised by MWG or Sigma and LNA probes by EXIQON. Tags are separated from the oligonucleotide by "/": Dig is digoxigenin, A488 is Alexa Fluor 488.

Table 6.10: DNA and LNA oligonucleotides

Application	Name	Sequence
454	Asp Cenp-A fw	CGTATCGCCTCCCTCGCGCCATCAGgttcTGATAGTATAGTGGTtAGTAT
	Asp Cenp-A re	CTATGCGCCTTGCCAGCCCGCTCAGgaacCTCCCCAACAAAAAATTAAACC
	Asp Cenp-C fw	CGTATCGCCTCCCTCGCGCCATCAGccagAGTATAGTGGTTAGTATt
	Asp Cenp-C re	CTATGCGCCTTGCCAGCCCGCTCAGctggCTCCCCAACAAAAAATTA
	Asp control fw	CGTATCGCCTCCCTCGCGCCATCAGagagAGTATAGTGGTTAGTATt
	Asp control re	CTATGCGCCTTGCCAGCCCGCTCAGctctNNNNNCTCCCCAACAAAAAATTA
	Asp Input fw	CGTATCGCCTCCCTCGCGCCATCAGtcagAGTATAGTGGTTAGTATt
	Asp Input re	CTATGCGCCTTGCCAGCCCGCTCAGctgaCTCCCCAACAAAAAATTA
	Asp KD fw	CGTATCGCCTCCCTCGCGCCATCAGtgagAGTATAGTGGTTAGTATt
	Asp KD re	CTATGCGCCTTGCCAGCCCGCTCAGctcaNNNNNCTCCCCAACAAAAAATTA
	Asp mito Cenp-C fw	CGTATCGCCTCCCTCGCGCCATCAGgggtAGTATAGTGGTTAGTATt
	Asp mito Cenp-C re	CTATGCGCCTTGCCAGCCCGCTCAGacccCTCCCCAACAAAAAATTA
	Asp total fw	CGTATCGCCTCCCTCGCGCCATCAGgtcaTGATAGTATAGTGGTtAGTAT
	Asp total re	CTATGCGCCTTGCCAGCCCGCTCAGCTCCCCAACAAAAAATTAAACC

	Asp wt OE fw	CGTATCGCCTCCCTCGCGCCATCAGcaaaAGTATAGTGGTTAGTATt
	Asp wt OE re	CTATGCGCCTTGCCAGCCCGCTCAGtttgNNNNNCTCCCCAACAAAAAATTA
	Asp Δcat OE fw	CGTATCGCCTCCCTCGCGCCATCAGccaaAGTATAGTGGTTAGTATt
	Asp Δcat OE re	CTATGCGCCTTGCCAGCCCGCTCAGttggNNNNNCTCCCCaACaaaaAATTa
	cal1 fw	CGTATCGCCTCCCTCGCGCCATCAGCACAAGtGTAGtGTGtAAtTttAtAGtTtTG
	cal1 re	CTATGCGCCTTGCCAGCCCGCTCAGTGTGNNNNNNCCaTAaTaCTAACAaCaaCTaCCC
	CG13377 fw	CGTATCGCCTCCCTCGCGCCATCAGCACATTttGAGGttAGGTTGAATtGtAGA
	CG13377 re	CTATGCGCCTTGCCAGCCCGCTCAGTGTGNNNNNCCTCCACTTACTCCTCCCAAa
	CG15546 fw	CGTATCGCCTCCCTCGCGCCATCAGCACAtGtTTTGATGGTGGGtGGTA
	CG15546 re	CTATGCGCCTTGCCAGCCCGCTCAGTGTGNNNNNCTCAaTCCaaCAaACCCACa
	CG8668 fw	CGTATCGCCTCCCTCGCGCCATCAGCACAAGtGAAtGAGGAAGGGAGAtG
	CG8668 re	CTATGCGCCTTGCCAGCCCGCTCAGTGTGNNNNNNTaCaCTCaTCaaTTaCTTAAaCAaAC
	cow fw	CGTATCGCCTCCCTCGCGCCATCAGCACAtTGtAAGGAGATTGTtGATGGAAA
	cow re	CTATGCGCCTTGCCAGCCCGCTCAGTGTGNNNNNaTTaCCaCCCATTATaTTaCCaC
	Dfd fw	CGTATCGCCTCCCTCGCGCCATCAGCACAAAAAGtAAAAGGTTtGGAGTATGTG
	Dfd re	CTATGCGCCTTGCCAGCCCGCTCAGTGTGNNNNNCCAAaCCATTaCCCATaaACA
	EndoA 1 fw	CGTATCGCCTCCCTCGCGCCATCAGCACAAtGATGAGATTtGTGGTGttGAG
	EndoA 1 re	CTATGCGCCTTGCCAGCCCGCTCAGTGTGNNNNNTCCaCCTCaaAaCaCTTCTCC
	EndoA 2 fw	CGTATCGCCTCCCTCGCGCCATCAGGAGAAtGATGAGATTtGTGGTGttGAG
	EndoA 2 re	CTATGCGCCTTGCCAGCCCGCTCAGTCTCNNNNNTCCaCCTCaaAaCaCTTCTCC
	Gly Cenp-A fw	CGTATCGCCTCCCTCGCGCCATCAGgttcGGTGGTTTAGTGGTAGAATG
	Gly Cenp-A re	CTATGCGCCTTGCCAGCCCGCTCAGgaacTACATCAACCAAAAATC
	Gly Cenp-C fw	CGTATCGCCTCCCTCGCGCCATCAGccagGGTGGTTTAGTGGTAGAATG
	Gly Cenp-C re	CTATGCGCCTTGCCAGCCCGCTCAGctggTACATCAACCAAAAATC
	Gly Input fw	CGTATCGCCTCCCTCGCGCCATCAGtcagGGTGGTTTAGTGGTAGAATG
	Gly Input re	CTATGCGCCTTGCCAGCCCGCTCAGctgaTACATCAACCAAAAATC
	Gly mito Cenp-C fw	CGTATCGCCTCCCTCGCGCCATCAGgggtGGTGGTTTAGTGGTAGAATG
	Gly mito Cenp-C re	CTATGCGCCTTGCCAGCCCGCTCAGacccTACATCAACCAAAAATC
	Gly total fw	CGTATCGCCTCCCTCGCGCCATCAGtctaGGTGGTTTAGTGGTAGAATG
	Gly total re	CTATGCGCCTTGCCAGCCCGCTCAGtagaTACATCAACCAAAAATC
	Mur29B fw	CGTATCGCCTCCCTCGCGCCATCAGCACAGTtAGATTtTTtAAttTttGATT
	Mur29B re	CTATGCGCCTTGCCAGCCCGCTCAGTGTGNNNNNAaAACTaaCaaTTaAACCTaC
	neur fw	CGTATCGCCTCCCTCGCGCCATCAGCACATTTAAtGttTATGATTttGGAAGTGA
	neur re	CTATGCGCCTTGCCAGCCCGCTCAGTGTGNNNNNTCACaTAaACCAaCCCTCaCA
	NOFb fw	CGTATCGCCTCCCTCGCGCCATCAGCACATTAtAAGATTGGtAATtGGAAttAAT
	NOFb re	CTATGCGCCTTGCCAGCCCGCTCAGTGTGNNNNNCTATTaCCCACCATATAAACACaT
	opus fw	CTATGCGCCTTGCCAGCCCGCTCAGTGTGNNNNNACACAaCTCACaCAaCACTTCTTT
	opus re	CGTATCGCCTCCCTCGCGCCATCAGCACAGAGTGATAAGGTGGTGTGTGG
	pr-set7 fw	CGTATCGCCTCCCTCGCGCCATCAGCACAAGtGGGGtAAttttGAtGtAGAAAA
	pr-set7 re	CTATGCGCCTTGCCAGCCCGCTCAGTGTGNNNNNCaACaCaACTTaCAaaCTaCCACC
	pros fw	CGTATCGCCTCCCTCGCGCCATCAGCACATttGAtGAGGAGtAGTGGAGGA
	pros re	CTATGCGCCTTGCCAGCCCGCTCAGTGTGNNNNNCaCCaTTaCCTaaCCAaCaaCTaC
	ry fw	CGTATCGCCTCCCTCGCGCCATCAGCACAtTGttGGAATtGGAGAttAGATTG
	ry re	CTATGCGCCTTGCCAGCCCGCTCAGTGTGNNNNNCCACAAAACTaaCCACCTCCA
	shot fw	CGTATCGCCTCCCTCGCGCCATCAGCACAGAtAGAGATtGttTGttGtAtTATG
	shot re	CTATGCGCCTTGCCAGCCCGCTCAGTGTGNNNNNCAaACATCaCaCCACTTaaTCC
	sick fw	CGTATCGCCTCCCTCGCGCCATCAGCACATtGTGGAGGAAtTtGTtAtGAtG
	sick re	CTATGCGCCTTGCCAGCCCGCTCAGTGTGNNNNNCaaACAaCCaACTaCCAaCCa
	spen fw	CGTATCGCCTCCCTCGCGCCATCAGCACAtGGTAtATTTGGAtTtGGTTtGG
	spen re	CTATGCGCCTTGCCAGCCCGCTCAGTGTGNNNNNaCTATaCTCTTTCaATAATTTCCAA
	ssp3 fw	CGTATCGCCTCCCTCGCGCCATCAGCACATGGAAttGTTTtGATGtTtTATGG
	ssp3 re	CTATGCGCCTTGCCAGCCCGCTCAGTGTGNNNNNaACCCTCCACAaTTAaaaTCTCC
	Toll-6 fw	CGTATCGCCTCCCTCGCGCCATCAGCACATGAGTATtAGGttGGtAATGGTG
	Toll-6 re	CTATGCGCCTTGCCAGCCCGCTCAGTGTGNNNNNACaACCTCCTCCAaCTaCAaC
	wmd fw	CGTATCGCCTCCCTCGCGCCATCAGCACAGAAtAAGAAtAAtTtGTAGTGtGG
	wmd re	CTATGCGCCTTGCCAGCCCGCTCAGTGTGNNNNNAaTTATTTATTTCTATTaCATTTCACAAT
CRISPR	gRNA sequence	TGTCAGCCCCACACTCGCC
	pDCC6 cloning fw	CTTCGTGTCAGCCCCACACTCGCC
	pDCC6 cloning re	AAACGGCGAGTGTGGGGCTGACAC
FISH	Control DNA	TTTACGGAGTCAGCAGGTCCAGCTTCATG/A488
	GluCTC DNA	GGATATCCTAACCACTAGACAATATGGGA/A488
	GlyGCC DNA	GAGCATTCTACCACTGAACCACCGATGC/A488
	GlyGCC LNA	/5DigN/AGCATTCTACCACTGAACCACCGAT/3Dig_N/
	Scrambled LNA	/5DigN/GTGTAACACGTCTATACGCCCA/3Dig_N/
PCR	Xneg fw	CAGGTGGGTTCCCTCAACTA
	Xneg re	AAGCATCAGCTCGCGTTAG
qPCR	Actin fw	TGGCACCGTCGACCATGAAGATC
	Actin re	TTAGAAGCACTTGCGGTGCAC
	AspGTC fw	TTAGTATCCCCGCCTGTCAC
	AspGTC re	CGACGGGGAATTGAACAC
	Dnmt2 fw	TACGGCAGTAATTTGGTGAA

	Dnmt2 re	ACAGATGAGTAAGTGCATCC
	EndoA 1 fw	CCAAGGACGATGAGATTCGT
	EndoA 1 re	GCTTCTCCTGCAGTGTCTCC
	EndoA 2 fw	CGAGGACAAGTTCGGTGAAT
	EndoA 2 re	AGGCCTCGAAGGACATCC
	GlyGCC fw	TCGGTGGTTCAGTGGTAGAA
	GlyGCC re	TGCATCGGCCGGGAATCG
	LeuCAA fw	GCCAGACTCAAGAGCGAAAG
	LeuCAA re	CCTCAGAGAGGACCAGAACG
	LeuTAG fw	GGTCTAAGGCGCTGGTTTTA
	LeuTAG re	GCCCTTTCGGACTGGTG
	NSun2 fw	GCCGTATGAGGAGATCAAAA
	NSun2 re	TCAATAATGGATAGCAGGGC
	ProCGG fw	GGCTCGTTGGTCTAGGGGTA
	ProCGG re	AATTGAACCCGGGACCTCT
	Rp49 fw	CGGATCGATATGCTAAGCTGT
	Rp49 re	GCGCTTGTTCGATCCGTA
	SatIII fw	AATGGAAATTAAATTTTTTGGCC
	SatIII re	GTTTTGAGCAGCTAATTACC
RNAi	DRSC03374 fw	CTAATACGACTCACTATAGGGAGGGATTCGTCCTCCGAAAG
	DRSC03374 re	CTAATACGACTCACTATAGGGAGAAAGGGACACGGAAGACAA
	DRSC28657 fw	CTAATACGACTCACTATAGGGAGGGTCACGAGATTGGGAAAGA
	DRSC28657 re	CTAATACGACTCACTATAGGGAGTTTCCGTGTGACAGGATTCA
	dsBrown fw	TAATACGACTCACTATAGGGAGCTCTCCTTCGTGCCCGT
	dsBrown re	TAATACGACTCACTATAGGGATCAATAGTAACCACTGCGGTGAAT

E.coli strain

DH5α was used in this study as listed in table 11.

Table 6.11: E.coli strain

Name	Genotype
DH5α	F- Phi80dlacZ DeltaM15 Delta(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK-mK+)phoA supE44 lambda- thi-1

DNA vector constructs

Different DNA vector constructs were used in this study to generate stably transfected *Drosophila* S2 cells. All plasmids are described in detail in the collection of the Erhardt laboratory. Constructs frequently used in this study are listed in table 12.

Table 6.12: DNA vector constructs

Name	Source
pAc-GFP-H2B	Goshima et al, 2007
pAc-mCherry-tubulin	Goshima et al, 2007
pCopia-Hygro	Erhardt et al. 2008
pCopia-LAP-Dnmt2	S. Rosic (Erhardt laboratory)
pCopia-LAP-NSun2	M. Romeike (Erhardt laboratory)
pCopia-LAP-Rpc31	A. Bergner (Erhardt laboratory)
pCopia-mCherry-CID	S. Erhardt
pCopia-mCherry-Rpc31	A. Bergner (Erhardt laboratory)
pMT-Cenp-C-V5-His	S. Erhardt
pMT-Dnmt2-wt-GFP-V5-His-hygro	A. Bergner (Erhardt laboratory)
pMT-Dnmt2-wt-V5-His-hygro	A. Bergner (Erhardt laboratory)
pMT-Dnmt2-Δcat-GFP-V5-His-Hygro	A. Bergner (Erhardt laboratory)

pMT-Dnmt2-Δcat-V5-His-hygro	A. Bergner (Erhardt laboratory)

pCopia refers to a pCopia-localisation and purification (LAP) vector with a basal expression Copia promoter and an N-terminal EGFP tag [Erhardt et al., 2008]. pAc refers to pAc5.1 C plasmid (Invitrogen) with a strong, constitutive *Drosophila* actin 5C gene promoter and various cloned tags. pMT refers to a CuSO4-inducible pMT-V5-His vector (Life technologies). pCopia-Hygro [Erhardt et al., 2008] has been used to introduce Hygromycin b (Sigma-Aldrich) resistance for S2 cells.

Cell lines

The majority of experiments was performed on *Drosophila* S2 cells. Therefore, different DNA vector constructs (table 12) were stably transfected in various combinations. In addition, mouse embryonic stem cells and human lung cancer cells depleted for Dnmt2 were examined. These cell lines are listed in table 13.

Table 6.13: Cell lines

Cells	Source	Genotype
Schneider 2 (S2) cells	Schneider, 1972	wild type
Mouse embryonic stem (ES) cells	En Li laboratory (Okano et al. 1998)	*Dnmt2*$^{-/-}$
Human non-small cell lung cancer cells (NCI-H1299)	Lyko laboratory (M. Rodriguez, unpublished)	stably transduced Dnmt2shRNA
Human non-small cell lung cancer cells (NCI-H838)	Lyko laboratory (M. Rodriguez, unpublished)	*Dnmt2*$^{-/-}$

Drosophila fly stocks

Drosophila fly lines frequently used in this study are listed in table 14.

Table 6.14: *Drosophila* fly stocks

Name (Chromosome)	Source	Genotype
118E-10 (4th)	Wallrath laboratory (Wallrath & Elgin 1995)	*y, w67c23; +/+; +/+* hsp70-white
118E-12 (3R)	Wallrath laboratory (Wallrath & Elgin 1995)	*y, w67c23; +/+; hsp70-white*
Balancer (2nd and 3rd)	Teleman laboratory	*If/Cyo; Sb/TM6B*
Dnmt2^{99}	Lyko laboratory (Schaefer et al., 2010)	*w*^{1118}*; Dnmt2-/-*
Dnmt2^{TG}	Lyko laboratory (Schaefer et al., 2008)	*w*^{1118}*; Dnmt2*$^{-/-}$*, Dnmt2*$^{genTG\text{-}EGFP}$
Dnmt2$^{\Delta cat}$	Schaefer laboratory (B. Genenncher, unpublished)	*w*^{1118}*; Dnmt2*$^{\Delta cat}$
HS-2 (3L)	Wallrath laboratory (Cryderman et al. 1998)	*y, w*^{67c23}*; +/+; hsp70-white*
HS-5 (2L)	Wallrath laboratory (Cryderman et al. 1998)	*y, w*^{67c23}*; hsp70-white*
NSun2^{ex1}	Sigrist laboratory (Abbasi-Moheb et al. 2012)	*NSun2*$^{-/-}$
w^{1118}	Lyko laboratory	*w*^{1118}

6.2 Methods

The methods listed here are standard protocols used in the Erhardt and Lyko laboratories unless otherwise specified. The used buffers and solutions, as well as materials and corresponding providers are listed in section 5.1.

6.2.1 Molecular biology techniques

All standard techniques were essentially performed as described in Molecular cloning: A Laboratory Manual by Sambrook and Russell (2001) [Sambrook and Russell, 2001].

Molecular cloning techniques

Gene fragments of interest were amplified with specific primers containing recognition sites for restriction endonucleases (*AscI* and *PacI* for pCopia; *KpnI* and *NotI* for pAc; *SpeI* and *NotI* for pMT, New England Biolabs) into a vector carrying the same restriction sites. pMT-V5-His constructs were cloned as described in DES TOPO TA expression kit. *Drosophila* S2 cells were co-transfected with the pCopia-Hygro plasmid for selection of Hygromycin b resistant stably transfected cells.

Mutagenesis

The mutagenesis of the ΔcatDnmt2 construct was performed by use of synthetic double-stranded DNA (dsDNA) (provided by IDT using the 'gBlocks Gene Fragments' Service) containing restriction sites for cloning into pMT-Hygro-V5-His vectors. The following dsDNA sequence was used as a ΔcatDnmt2 construct:

ATGGTATTTCGGGTCTTAGAACTATTTAGTGGCATTGGCGGCATGCATTATGCCTTTAA

TTATGCCCAATTGGATGGACAAATAGTTGCCGCCTTGGATGTCAACACCGTGGCCAATG

CGGTTTATGCGCACAATTACGGCAGTAATTTGGTGAAAACTAGGAATATTCAAAGCCTG

AGTGTAAAGGAAGTCACAAAGCTTCAGGCCAACATGCTGCTGATGTCCCCGCCAgcTCA

GCCCCACACTCGCCAGGGATTGCAAAGGGACACGGAAGACAAGCGATCGGATGCACTTA

CTCATCTGTGTGGACTGATCCCAGAGTGCCAGGAACTGGAGTACATACTCATGGAAAAC

GTCAAGGGTTTCGAGAGCTCACAGGCGCGAAATCAGTTTATTGAATCGCTGGAGCGGTC

GGGATTCCATTGGCGGGAGTTTATTCTAACGCCGACGCAATTCAATGTGCCAAATACTC

GATATCGCTACTATTGCATCGCCCGCAAGGGTTCAGACTTTCCATTCGCCGGTGGAAAG

ATCTGGGAAGAAATGCCGGGAGCTATAGCCCAGAATCAGGCTCTTTCACAAATTGCCGA

GATTGTGGAGGAAAATGTATCACCCGATTTCCTGGTGCCCGACGATGTCTTGACCAAAA

GAGTGCTGGTCATGGACATAATACATCCTGCTCAAAGTAGATCCATGTGCTTTACAAAG

GGCTACACCCATTACACCGAGGGCACGGGCTCTGCATACACACCGCTTTCGGAGGACGA

ATCCCACCGCATCTTCGAGTTGGTCAAGGAAATTGACACAAGTAATCAGGATGCATCGA

AGTCGGAGAAGATTTTGCAGCAACGCTTGGACCTGTTGCACCAGGTGAGACTGCGCTAT

TTCACGCCACGGGAAGTTGCTCGTCTAATGAGTTTTCCGGAGAATTTTGAATTTCCGCC

AGAAACAACGAATCGACAAAAGTATCGACTGCTGGGAAATAGTATTAATGTAAAGGTTG

TCGGTGAACTTATTAAATTGCTGACGATAAAATAA

Preparation of dsRNA for RNA interference

The MEGAscript kit (Ambion) was used to generate double-stranded RNA (dsRNA) according to the manufacturers manual. Prepared dsRNA was aliquoted and stored at -20 °C. dsRNA was generated against Dnmt2 (dsDnmt2, DRSC03374), NSun2 (dsNSun2, DRSC28657), and Brown (dsBrown) as a control (Table 10).

RNA isolation for reverse-transcription PCR

TRIzol (Ambion) or TRIsure (Bioline) were used to isolate total RNA from cells and flies applying standard protocols. cDNA synthesis was performed with the cDNA synthesis kit NG dART RT Kit (roboklon) according to the manufacturer's manual.

PCR analysis

Analysis of Cenp-A-negative genomic locus Xneg was performed with specific primers from Olszak et al. (2011) (Table 4.10). The DreamTaq DNA polymerase (Thermo scientific) was used for PCR reactions.

Quantitative Real-Time PCR (qPCR)

The QuantiTect Reverse Transcription Kit (QIAGEN) was used to synthesise cDNA according to the manufacturer's manual. The gDNA wipeout reaction

was performed and random hexamer primers or specific reverse primers were used for reverse transcription. The LightCycler 480 SYBR Green I Master (Roche) was used to conduct qPCRs, which were analysed in triplicates on a LightCycler 480 instrument. Actin or RP49 were used as references to normalise expression levels. Merrit Romeike performed qPCRs for tRNAs and SATIII.

6.2.2 Cell biology techniques

Drosophila S2 cell culture

Schneider 2 (S2) cells are a *Drosophila* embryonic cell line. Cells were grown under sterile conditions in tissue culture flasks as semi-adherent monolayers at standard conditions (25 °C, in dark, 10% fetal bovine serum-containing medium (SM), supplemented with 200 µg/ml of each penicillin and streptomycin). Cells were split twice a week to a density of approximately 10^6 cells/ml.

Freezing and thawing of S2 cells

S2 cell stocks were regularly replaced with freshly thawed cells. Stably transfected cell lines were frozen approximately 6 weeks after transfection for long-term storage at -80 °C or -196 °C (liquid nitrogen) in 45% fresh SM, 10% DMSO, and 45% conditional medium (CM), which is used medium containing growth factors of S2 cells. Cells were grown to maximal confluence in a 150 cm2 flask, washed off, pelleted at 1,000 x g for 5 minutes and resuspended in overall 5 ml of the DMSO-SM-CM mixture. Aliquots of 1 ml in 2 ml Nunc CryoTubes (Sigma-Aldrich) were frozen to -80 °C in isopropanol filled freezing containers at a freezing rate of 1 °C per minute and transferred to liquid nitrogen for long-term storage. S2 cells frozen in liquid nitrogen were quickly thawed in a 30 °C water bath, pelleted at 500 x g for 5 minutes, resuspended in 3 ml of fresh SM and transferred to a 25 cm2 flask. Thawed cells were allowed to recover for one to two weeks at standard growing conditions before experiments were conducted.

Transfection of S2 cells

Plasmid transfections of S2 cells were performed using the cationic lipid formulation Cellfectin II (Invitrogen). 1.5x10^6 actively dividing cells were plated in 2 ml fresh SM in 6-well plates and grown at standard conditions over night (O/N). Two solutions were used for transfection. Solution I contained 300 µl of serum-free medium (SFM), 5 µg of the desired plasmid, and 5 µg of pHygro. Solution II contained 300 µl of SFM and 30 µl Cellfectin II Reagent. These solutions were mixed in a polystyrene tube by drop-wise addition of solution I to solution II and incubated for 30 minutes at room temperature (RT). Cells were washed with SFM without disturbing the cell layer. 2.4 ml of fresh SFM and the transfection mixture were added to the cells and incubated for 3.5 hours. The medium was removed and 3 ml of fresh SM were supplemented. After 2 days of recovery, stable cell lines were generated by adding 250 µg/ml Hygromycin B (Sigma) every time the cells were split. This was continued for at least 6 weeks and tested with direct immunofluorescence or Western blot for transfection efficiency.

RNA interference (RNAi) in S2 cells

For RNAi, 1.5-2.0x10^6 actively dividing cells were plated one day in advance in 6-well plates and grown O/N. 15-20 µg of dsRNA in 1 ml SFM were incubated in polystyrene tubes for 15 minutes at RT. Meanwhile, CM of plated cells was removed and used to prepare 15% serum-containing SM with fresh medium and additional serum. Cells were washed once with SFM. Following the incubation, the SFM/dsRNA mix was supplemented to the washed cells. After 1 hour of incubation, 2 mL of 15% CM was added to obtain 3 ml of 10% CM and cells were grown for 2-4 days at standard conditions. dsRNA against Dnmt2 and NSun2 were controlled with Brown. Knock down efficiency was controlled using qPCR and Western blot.

Drug treatment of S2 cells and cellular stress

The microtubule depolymerisation reagent Colcemid (Capricorn Scientific) was used to enrich mitotic cells for mitotic chromosome spreads at 3 µg/µl for 30 min, and at 1 µg/ml for 10 hours to block cells in mitosis. As a control, no

Colcemid was applied. Merrit Romeike performed the Colcemid-block. Overexpression of pMT constructs was performed with 1 mM Cu_2SO_4 supplemented directly to settled cells in standard medium O/N. As a control, no Cu_2SO_4 was applied. Expression levels were controlled using Western blot. ML-60218 (Calbiochem, Merck) is a cell-permeable small molecule drug that is a specific RNAPIII inhibitor in yeast and human [Wu et al., 2003]. The IC20 of RNAPIII-specific inhibitor ML-60218 for S2 cells was determined by testing indicated concentrations of the drug for 48 hours at standard conditions. After counting viable cells, total RNA was extracted from the same samples and analysed on a TapeStation (Agilent). The integrated area under the curve [%] reflects the relative concentration of tRNA peak (72 nt) to total RNA. For FACS and polysome analysis, cells were treated at IC20 O/N. For live cell analysis, cells were treated at IC20 and subsequently recorded. For mitotic chromosome spreads cells were incubated for 20 minutes prior to lysis. In all cases, DMSO served as a control. To examine the impact of cellular stress, heat shock and culture stress were applied to S2 cells. Heat shock prior to live cell analysis was performed in a water bath at 37 °C for 30 min. Following heat shock, cells were immediately recorded at 25 °C as described below. Culture stress was defined by the state of overgrown cultures where cells passed 100% confluence, also visible by increased number of cells in suspension, whereas control cells remained in an exponential growth state. For this purpose, $1x10^6$ or $2.5x10^6$ cells were seeded at day 1 and grown in parallel until the overgrown state was reached for the stress condition.

Flow cytometry analysis (FACS) for cell cycle analysis of S2 cells

10^6 cells were pelleted at 1,000 x g for 5 min, washed once in PBS at RT, and resuspended in 100 µl of ice cold PBS. Fixation was performed by drop-wise addition of a total of 1 ml ice cold 70% ethanol and continuous mixing by vortexing. Following 30 min incubation on ice, fixed cells were washed twice in ice cold PBS and resuspended in 100 µl PBS containing 0.2 mg/ml RNase A (AppliChem), 0.02 mg/ml propidium-iodide, and 0.1% Triton X-100 and incubated at 37 °C for 15 min. 10,000 to 30,000 cells were measured on a FACSAria Illu Cell Sorter and analysed using FlowJo software by the ZMBH FACS Core Facility.

Preparation of mitotic chromosome spreads

For mitotic chromosome spreads, two 2x10^6 cells were seeded, grown O/N, and 100 µl cell suspension was arrested in mitosis with 2.5 µg/µl Colcemid (Capricorn Scientific) for 30 min. Harvested cells were resuspended in 500 µl hypotonic sodium citrate solution (0.5%) and incubated for 7 min. Swollen cells were spun on microscopy slides in a cytocentrifuge (Shandon 4 Cytospin; Thermo Fisher Scientific) at 900 rpm for 10 min. Spreads were fixed with 4% PFA for 10 min (FISH, α-HP1, α-H3K9me2, α-H3K4me2) or used without fixation (Rpc31-GFP/mCherry-Cenp-A cells, Dnmt2-GFP/mCherry-Rpc31, Dnmt2-GFP/mCherry-Cenp-A cells, NSun2-GFP cells; with α-GFP, α-TRF1, α-Ago2, anti, Dcr2, α-CTCF, α-CP190, α-Hoap, α-CID, α-Cenp-C). Fixed spreads were subsequently used for IF or FISH. Unfixed spreads were immediately covered with KCM buffer for 10 min. RNase A-treatment was done at RT using 100 ng/µl RNase A in PBS for 15 min, followed by a short wash in PBS. As a control, PBS only was used. This was followed by IF.

Indirect immunofluorescence (IF) on *Drosophila* S2 cells

Mitotic chromosome spreads were subjected to IF using a fixed or an unfixed protocol. Fixed spreads were washed three times after fixation with PBS and subsequently permeabilised with 0.1% Triton X-100 in PBS for 5 min. Unspecific binding was blocked using 1% BSA and 0.1% Triton X-100 in PBS for 30 min. Primary antibodies in blocking solution were incubated for 1 hour at RT and subsequently washed three times in 0.1% Triton X-100 in PBS. Secondary antibody was incubated for 45 min in the dark and washed three times again. Unfixed spreads were directly subjected to primary antibody incubation in TEEN buffer at 37 °C for 30 min. Following three washes in KB buffer, secondary antibody in KB buffer was incubated at 37 °C for 30 min, and washed three times in KB again. IF on fixed S2 cells was done with 100-200 µl of exponentially growing cells. Cells were centrifuged (3 min, 800 x g), washed once with PBS, centrifuged again and resuspended in 50 µl PBS. Cells were settled on positively charged glass slides for 10 min and fixed with 4% PFA for 10 min. Following two washes in PBS, cells were permeabilised with 0.1% Triton X-100 for 5 min. Blocking was done with 3% BSA in PBS at 37 °C for 30 min. Primary antibodies were incubated in blocking solution for 2 hours at 37 °C

and subsequently washed three times in PBS. Secondary antibodies were incubated in blocking solution for 45 min at RT and subsequently washed again. DAPI staining was always done in PBS (1 µg/ml), followed by two washes in PBS. Slides were mounted in Aqua/Polymount (Polysciences), covered with a glass coverslip (1.5 mm thickness), and stored at 4 °C until imaging.

RNA FISH coupled with IF on mitotic chromosomes spreads

LNA FISH probes were synthesised with a digoxigenin double tag (EXIQON) and used for RNA FISH coupled with IF on mitotic chromosomes. Specific $tRNA^{Gly(GCC)}$ probes complement the 5' half of tRNA, scrambled LNA probes and no probe were used as controls. Following fixation, slides were washed with PBS, permeabilised with 0.1% Triton X-100 in PBS, and subsequently washed in 2x saline-sodium citrate (SSC). 100 nM of LNA probes in 2x SSC were diluted in FISH hybridization buffer supplemented with 1 µg human CoT-1 DNA (Invitrogen) and 10 µg yeast tRNA (Ambion), and denatured at 80 °C for 10 min. After short dehydration of the slide, the probe solution was applied to the slide and incubated for 5 min, all at 80 °C. Hybridisation was performed at 51 °C for 3 hours. Slides were washed three times in 50% formamide/2x SSC and three times in 2x SSC at 51 °C, and again fixed with 4% PFA for 5 min. For subsequent IF, slides were blocked with 4% BSA in PBS and incubated with the appropriate antibodies in the same solution at RT for 1 hour. LNA probes were detected with α-digoxigenin (DIG) and centromeres with α-Cenp-A antibody. Subsequently, slides were washed three times in PBS. Secondary antibodies were incubated and washed in the same manner. Slides were counterstained with DAPI for 5 min, briefly washed in PBS, mounted, and stored at 4 °C until imaging. RNA-FISH experiments on spreads were performed in collaboration with Sarah Doppler.

Preparation of chromatin fibres

10^5 actively dividing cells were harvested at 1,000 x g for 1 min and resuspended in 1 ml 0.5% sodium citrate solution by vortexing. Following 10 min of incubation, cells were spun with use of EZ MegafunnelTM (Thermo Scientific) on poly-lysine coated microscopy slides (Thermo Scientific) with high acceleration and at 800 RPM for 4 min using a cytocentrifuge (Shandon 4 Cytospin;

Thermo Fisher Scientific). Slides were immediately removed and placed into lysis buffer for 15 min. The slides were slowly pulled out by hand to spread the chromatin. Fixation in 4% PFA in PBS was done for 2 min and subsequently washed twice in PBST (0.1% Triton X-100) for 5 min each.

DNA FISH coupled with IF on chromosome fibres

DNA FISH probes were synthesised with a single Alexa488 tag (Sigma-Aldrich) and used for RNA FISH coupled with IF on chromosome fibres. Specific oligonucleotides complemented the 5' half of $tRNA^{Gly(GCC)}$ and an unspecific DNA oligonucleotide were used as a control. DNA FISH coupled with IF was performed as described for RNA FISH with the following changes. 1 µg of DNA probes were used and denaturation of the probes and the chromosome fibres was performed at 95 °C for 5 min. Hybridisation was done at 42 °C O/N.

Preparation of mammalian cells for immunofluorescence

Dnmt2$^{-/-}$ mouse embryonic stem (ES) cell lines were provided by En Li [Okano et al., 1998]. For IF, single cells were plated after trypsination on gelatine-coated coverslips at a density of 10,000 cells/cm2 and grown O/N before performing IF. Mouse ES cells were cultured by Francesca Tuorto and provided for IF experiments. Mutant and depleted human non-small cell lung cancer cells (NCI-H1299 and NCI-H838) were generated and cultured by Manuel Rodriguez from the Lyko laboratory and provided for IF experiments. In brief, shRNA constructs were generated using pLVX-shRNA2 vector, in which 2 templated oligos carrying the 19 nt shRNA sequence against human Dnmt2 were inserted (sense-loop-antisense). Control shRNAs missed the connecting loop. Transduced cells were selected for positive GFP signals and used as a pool. CRISPR constructs were generated with LentiV1 vectors and sgRNA against Dnmt2 from the GECKO library. Clones were isolated and controlled by Western blot for null mutation.

Indirect immunofluorescence (IF) on mammalian cells

Cover slips with mouse ES or human lung cancer cells were washed once with PBS and fixed with 3.75% PFA in PBS for 10 min. Cells were permeabilised for 15 min in 0.3% Triton X-100 in PBS and subsequently washed three times in

PBS. Unspecific binding was blocked for 1 hour with 10% FBS in 0.3% Triton X-100 in PBS. Primary antibody was incubated in blocking solution at RT for 90 min and subsequently washed three times in PBS. Secondary antibody incubation was done in blocking solution at RT for 45 min and subsequently washed again. DNA was stained with Hoechst 33258 (Invitrogen), and washed three times with PBS. Slides were mounted with Fluoromount-G (SouthernBiotech) and stored at 4 °C until imaging.

6.2.3 *Drosophila* animal methods

Drosophila cultures and fly husbandry Flies were kept on standard *Drosophila* medium at 60% humidity and under a 12 hour light-dark cycle. Fly stocks were kept at 18 °C and food was changed every 3-4 weeks. For bisulfite sequencing and brain squashes, flies were kept at 18 °C and food was changed every 2 weeks. For PEV experiments, flies were kept at 25 °C. Female virgins were isolated based on the light body colour and the dark spot of the translucent abdomen and held isolated from males to control their virginity. Confirmed flies were used for crosses, which were set up at 25 °C.

'Position Effect Variegation' (PEV)

PEV was examined using following reporter flies for pericentric heterochromatin (white P element insertions at different genetic loci): 118E-10, 118E-12, HS-2, and HS-5 (Wallrath & Elgin 1995; Cryderman et al. 1998). Males of PEV reporter flies were crossed with virgins of $Dnmt^{99}$ and vice versa. Modifier effects of $Dnmt^{99}$ null alleles were examined in 2-3 day old w^{1118}/w^{1118} female and w^{1118}/Y male offspring and compared. Representative male offspring are displayed in the results. Images of fly eyes were made with an Olympus SC30 digital camera on a SZX7 stereo microscope system (Olympus).

Larval brain squashes

Drosophila larvae were grown at 18 °C on standard fly food with yeast paste. Crawling 3rd instar larvae were collected and larval brains were dissected in PBS as described before [Henderson, 2004]. Following Colcemid (Capricorn Scientific) treatment for 1 hour, brains were incubated for 5 min in 1% Na-

citrate solution. Subsequently, brains were fixed in 3.7% PFA in PBS for 30 min, then transferred to 45% acetic acid for 30 sec, and finally to 60% acetic acid for 3 min on a siliconised coverslip. All incubations were done at RT. Coverslips were covered with poly-lysine coated microscopy slides (Thermo Fisher scientific) and tissue was squashed with high pressure and pointed forces using a pencil tip. Slides were frozen in liquid nitrogen, the coverslip was removed, and slides were washed three times in 0.1% Triton X-100 in PBS for a total of 1 hour. Third instar larval brain squashes were immediately used for staining. DAPI staining was done in PBS (1µg/ml), followed by two washes in PBS. Slides were mounted in Aqua/Polymount (Polysciences), covered with a glass coverslip (1.5 mm thickness), and stored at 4 °C until imaging. Andrea Bergner performed tissue preparation and DAPI staining for $Dnmt2^{\Delta cat}$ larvae. Heat shock was applied to 3rd instar crawling larvae in a water bath at 37 °C for 45 min. Larvae were dissected directly after heat shock in PBS at RT. Recovery after heat shock was 4 hours at 25 °C. As control, larvae were kept at 25 °C and directly dissected.

EndoA and Satellite boutons stainings on larval filets

Ine Maes from the Verstreken laboratory in Leuven, Belgium performed the experiments for EndoA on *Drosophila* larvae. In brief, larvae were grown on grape juice plates and females picked for immunohistochemistry. Larvae were dissected, fixed in 3.7% formaldehyde, washed 3-5 times with HL3, 4 times with 0.4% Triton X-100 in PBS, blocked with 10% normal goat serum in 0.4% Triton X-100 in PBS for 1 hour, and incubated with α-EndoA antibody O/N. Following 6 times washing and 15 min block, α-HRP and α-DLG antibodies were incubated for 2 hours and washed again. Following a third block, secondary antibodies were incubated for 2 hours and washed again. Vectashield mounting medium (Labconsult) was used on a glass slide and covered with a cover slip. Imaging was performed on a confocal AR1 microscope from Nikon with a Plan APO 60x A/1.20 Water immersion DIC N2 lens. For EndoA stainings, 4 pictures per filet from 6 larvae were taken, 2 on each side (segments A2 and A3, muscle 6 and 7). ImageJ/FIJI (Schindelin et al., 2012) was used for mean intensity quantifications. For Satellite boutons, HRP staining was used to visualise boutons in 4 larvae per genotype. 4 NMJs (muscle 2, segments A2

and A3) were analysed 'live' per filet, which resulted in 24 values per genotype in total. Prism (GraphPad) was used to determine statistics. The following antibodies were used: α-EndoA (guinea-pig, 1:2,500, Verstreken et al. 2002), α-Horse Radish Peroxidase (rabbit, 1:500, Lucron), α-Dic Large (mouse, 1:500, DSHB), α-guinea-pig (Alexa488, 1:500, Invitrogen), α-rabbit (Alexa555, 1:500, Invitrogen), α-mouse (Pacific Blue, 1:500, Invitrogen). The following fly stocks were used: w^{1118}, d$NSun2^{ex1}$, $Dnmt^{99}$.

6.2.4 Microscopy techniques

A DeltaVision Core system (GE healthcare lifescience) with softWoRx v5.5 suite (AppliedPrecision) and a charge-coupled device camera (CoolSNAP HQ2; Photometrics) were used for microscopy. Acquisition was done with 100x UPlan-SApochromat (NA 1.4; Olympus) or 60x Plan-Apochromat N (NA 1.42; Olympus) lenses, and binning of 1x1 or 2x2.

Live cell imaging of S2 cells

Live cell imaging was performed with 250 µl of exponentially growing cells in sterile 8-well chamber slides (Ibidi). The cells were settled at 25 °C for about 30 min. Imaging was performed at 25 °C O/N for 12 to 16 hours with a time lapse of 10 to 20 min and the following settings: 12.8 to 17.5 µm in Z, 0.4 to 0.5 µm stack interval spacing, and approximately 0.02 sec exposure for H2B-EGFP and 0.3 sec for mCherry-tubulin constructs.

Image processing

Images of fibres, spreads, brain squashes, and mammalian cells were taken as z-stacks with 0.1, 0.2, or 0.4 µm spacing. Shown are single slices per z-stack, focused on centromeric signals. FISH spreads were deconvoled (ratio, aggressive) and 3 Z-slices were projected (additive) using softWoRx v5.5 suite (AppliedPrecision) prior to quantification and presentation. Live cell imaging movies were projected and in some cases time points were intensity equalized. All images were adjusted in brightness and contrast using ImageJ/FIJI [Schindelin et al., 2012].

Quantification of fluorescence intensities at centromeres and whole chromosomes

To quantify centromeric signals of FISH and IF on chromosome spreads, the ImageJ/FIJI macro spotCharacterize was applied on deconvolved and projected images. Centromeric signals were identified by α-CID immunostainings and mean intensity values were normalized to the corresponding CID signal. Mean intensities of α-HP1 and α-H3K9me2 on whole chromosome spreads were quantified using the ImageJ/FIJI macro measureRef. Data was plotted and tested for significance (student's unpaired t test) using Prism (GraphPad).

ImageJ/FIJI macros

ImageJ/FIJI Image analysis was done with ImageJ/FIJI using available tools as well as the custom scripts spotCharacterize and measureRef. Aliakbar Jafar Pour and Holger Lorenz from the ZMBH Imaging Core Facility generated these macros. Detailed information and the source code of the macros are available upon request. In brief, for small-size spot measurements, the ImageJ plugin GaussFit OnSpot [imageJ.net] was combined with the macro spotCharacterize to facilitate a semi-automatic analysis. The approximate positions of relevant spots were manually specified and corresponding locations in multi-channel images measured GaussFit OnSpot. For region-specific mean intensity measurements in multi-channel images, measureRef assembles multi-channel images to undergo background subtraction, intensity thresholding, and mean intensity measurements in straight succession for a comparable analysis. The regions of interest for mean intensity measurements of all channels were selected from the DAPI channel. For both macros, all results were compiled in tables.

Quantification of mitotic defects

Lagging chromosomes, anaphase bridges, and chromosome fragments were counted as mitotic defects in all imaged mitotic cells unless otherwise stated. In live cell analysis, only anaphases were scored. For fixed S2 cells, larval neuroblasts, mouse ES cells, and human cancer cells, meta- and anaphases were quantified.

6.2.5 Biochemical techniques

Cell fractionation

$2x10^6$ Dnmt2-GFP cells were harvested and resuspended in 100 µl fractionation lysis buffer. Lysates were incubated for 10 min on ice and cleared by 30 min full speed centrifugation. The supernatant was collected as the cytosolic fraction. The pellet (nuclear fraction) was resuspended in lysis buffer with additional 0.3 µl/ml Benzoase (Sigma-Aldrich) and sonified with a Bioruptor (Next Gen) for 5x 30 sec on high settings. Merrit Romeike performed the fractionation.

Preparation of protein extracts from S2 cells and flies

For fly protein extracts, 3 male and female flies each were disrupted and applied for lysis in 120 µl RIPA buffer. A Bioruptor (Next Gen) was used with 10 cycles, 30 sec on-off cycle, and high power setting. Lysates were centrifuged with full speed at 4 °C for 20 min to clear the lysates, which were mixed with 1 volume 4x Laemmli sample loading buffer (SLB) and boiled at 95 °C for 5 min. For S2 cell protein extracts, $5.0\text{-}7.5x10^5$ cells were used. Lysis was performed in 0.1% SDS with 25 unit/ml BaseMuncher (Expedeon) and incubated for 10 min on ice. 1 volume 4x SLB was added and boiled at 95 °C for 5 min.

SDS PAGE and Western blot (WB) analysis

12% sodium dodecyl sulfate (SDS) polyacrylamide gels were used for gel electrophoresis (PAGE) at 100 V for 15 min and at 180 V for 45 min. To transfer the separated proteins, Western blots were performed semi-dry for fly extracts or wet for cells. Semi-dry blots were performed with a Trans-Blot Turbo Transfer System with 0.2 µm nitrocellulose for 7 min (for 2 gels). Wet blots were performed at 100 V for 2 hours using a Borate transfer buffer with 20% methanol. All PAGE and Western blot equipment used was purchased from Bio-Rad. Transfer efficiency was controlled with Ponceau staining (Applichem). Blots were washed in TBST for 10 min, blocked with 5% milk powder in PBS, and the primary antibody was incubated in blocking solution at 4 °C O/N. Following washing in PBS, blots were incubated with secondary antibodies coupled to horseradish peroxidase (HRP) at RT for 2 hours and washed again. Detection was performed using chemiluminescence HRP/ECL solution Super signal

femto (Thermo Scientific). Western blots were performed in collaboration with Andrea Bergner and Merrit Romeike.

Polysome profiling of S2 cells and *Drosophila* embryos

For polysome profiling of S2 cells, $3x10^6$ cells were harvested and washed twice in cold RNase-free PBS containing 100 µg/ml cycloheximide (CHX). Washed cells were pelleted at 800 x g and 4 °C for 5 min. Cell lysis was done in 350 µl polysome lysis buffer containing 200 µg/ml CHX, 1x Complete Protease Inhibitor (Roche), and 1 U/µl RNaseOut (Invitrogen) by thorough vortexing at 4 °C. Lysates were incubated at 4 °C for 10 min. Nuclei were subsequently pelleted with 12,000 x g at 4 °C for 10 min. Cleared lysates were transferred to fresh tubes, incubated for approximately 5 min at 4 °C and applied for ultracentrifugation. For profiling of embryos, 300-400 embryos were collected on apple juice plates with yeast paste at 25 °C O/N. Flies were removed from collection cages and embryos incubated for another two hours to obtain at least 2 hour old embryos. Embryos were washed off the plates with WEK water and washed several times with PBS to completely remove the yeast. Dechorionation was performed with 5% hydrochloride for 90 sec, slowly shaking the embryos in sieve. Dechorionated embryos were washed at least 3 times with 100 µg/ml CHX in RNase-free PBS at 4 °C, and subsequently pelleted by brief centrifugation. Pelleted embryos were lysed in 500 µl polysome lysis buffer using a pestle to disrupt the tissue. Lysed embryos were incubated for 10 min and subsequently centrifuged with 12,000 x g at 4 °C for 10 min to pellet the nuclei. The upper layer of lipids was removed, the supernatant transferred to a fresh tube and centrifuged again for approximately 5 minutes. Cleared lysates were applied to linear 17.5-50% sucrose gradient in 15 mM Tris-HCl (pH 8.0), 15 mM MgCl2, 300 mM NaCl. Ultracentrifugation was performed at 35,000 r.p.m., medium acceleration, slow deceleration, and 4 °C for 2.5 hours in a Beckman SW60 rotor. Fractionation of the gradient was performed using an ISCO UA-6 gradient fractionator, monitoring profiles continuously at 254 nm. Translationally engaged ribosomes were calculated by dividing the area under the curve of the polysomal fraction by the area under the entire curve. Three and two replicates per condition for S2 cells or embryos, respectively, were analysed. Ultracentrifugations and fractionations were performed together with

Francesca Tuorto. qPCR was used to measure the relative amounts of EndoA mRNA in selected fractions. As an internal control to normalise transcript levels to fraction volumes, a GFP-containing plasmid (pN2-EGFP) was supplemented to the fractionation buffer to a concentration of approximately 200 pg per fraction.

6.2.6 Next generation sequencing (NGS)-related techniques

RNA extraction

RNA was isolated with standard protocols for TRIzol (Ambion) RNA extraction. In brief, cells or tissue was incubated in 1 ml TRIzol at RT for 10 min. 200 µl of chloroform were added and vortexed. Following 3 min of incubation at RT, samples were centrifuged with 12,000 x g at 4 °C for 15 min. The aqueous phase was transferred to siliconised tubes and 1 volume of isopropanol and 20 µg of GlycoBlue (Ambion) were added and mixed. Following 15 min of incubation at RT, samples were centrifuged at maximum speed and 4 °C for 30 min. Supernatants were discarded and pellets washed with 600 µl of cold 75% ethanol, centrifuged again and finally the pellets were air-dried and resuspended in RNase-free water.

Quality control of RNA samples

RNA samples from S2 cells and larval tissue were quality controlled using a TapeStation (Agilent) for size distribution and integrity and a NanoDrop ND-1000 Spectrophotometer for concentration and purity. This was done for all total RNA samples, intermediate steps of NGS library preparations, and final quality controls. In addition, the TapeStation was used to determine tRNA-specific peak concentrations that were used to calculate relative tRNA levels to total RNA concentrations from ML-60218 treated S2 cells.

RNA & DNA Chromatin Immunoprecipitation (ChIP)

The ChIP protocol was adapted from Sun & Lee (Epigenesys, 2006). Non-transfected S2 cells were used for Cenp-A-ChIP using an α-CID antibody.

Cenp-C-ChIP was performed in S2 cells stably transfected with pMT-CenpC-V5-His using an α-His antibody, Expression of Cenp-C-V5-His was induced with 50 µM CuSO4 16 hours prior to harvest. $1x10^7$ cells were harvested at RT, washed and resuspended in PBS. Formaldehyde (J.T. Barker) was supplemented to a final concentration of 1% and crosslinking was performed under slow rotation at RT for 10 min. Crosslinking was stopped by the addition of glycine to a final concentration of 125 mM. From this step on, everything was performed at 4 °C. Cells were washed twice with cold PBS, pelleted and resuspended in buffer A, incubated on ice for 10 min and pelleted at 5,000 rpm for 5 min to obtain the crude nuclei fraction. The pellet was washed in buffer A lacking NP40 and resuspended in buffer B that was incubated in an ice/water bath for 10 min. Sonification was performed with a Bioruptor (Next Gen) in an ice-water bath with the following settings: 30 cycles, 30 seconds on-off cycle, and high power. Samples were cleared at full speed for 10 min. An aliquot of the cleared supernatant was stored as input. The remaining sample was diluted ten-fold in IP buffer. Binding was performed O/N with 2.5 µl antibody of α-CID (Active Motif no. 39720), α-His (Abcam ab9108) and no antibody as a control. Immunocomplexes were captured with DEPC-water washed ChIP-IT Magnetic Beads (Active Motif) and slowly rotated for 2 hours. Beads were washed five times for five minutes each in the following order: low salt wash, high salt wash, LiCl wash, and finally twice with TE (pH 8). Complexes were eluted with freshly prepared elution buffer in two rounds and corresponding eluates were pooled together. Crosslinking of IP and input samples was reversed in a final concentration of 125 mM NaCl (65 °C, 2 hours). Protein components of 500 µl eluates were digested by addition of 20 µl 1 M Tris-HCl (pH 6.5), 10 µl 0.5 M EDTA, and 20 µg of Proteinase K (Sigma-Aldrich). This was scaled accordingly for the input. Proteinase K (Sigma-Aldrich) digestion was performed at 37 °C for 30 minutes. RNA was isolated using TRIsure (Bioline), DNA using phenol/chloroform (Sigma-Aldrich) precipitation. Chromatin IPs was performed by Merrit Romeike.

ChIP RNAseq library preparation

All ChIP RNA samples were tested with qPCR for SatIII levels as a positive control for centromeric enrichment. 6 corresponding samples with fold-changes

>2 were pooled together and further processed for library preparation in parallel to the corresponding input samples. First, RNA was exposed to TURBO DNase (Ambion) digestion at 37 °C for 30 min and was then phenol/chloroform extracted. The RNA was fragmented using NEBNext Magnesium RNA Fragmentation Module (New England Biolabs) at 94 °C for 3 min, and cleaned up using ethanol precipitation. The RNA was stepwise end-repaired with a T4 polynucleotide kinase (TaKaRa) at 37 °C. To dephosphorylate 3'-ends, no ATP was added to the reaction for 20 min. Subsequently, 2 µl of 10 mM dATP were supplemented, incubated for another 20 min and immediately put on ice to inhibit the enzymatic activity. Phenol/chloroform extraction was used to extract the RNA that was used immediately for library preparation using the NEBNext Small RNA Library Prep Set (New England Biolabs). Libraries were prepared according to the manufacturer's protocol applying 15 cycles of PCR amplification, and QIAquick PCR Purification Kit (Qiagen). A 6% polyacrylamide gel electrophoresis (6% TBE Gels, Novex, Invitrogen) was used to isolate the appropriate library sizes. Compatible NEBNext indices were used to multiplex the libraries and sequence them on a single lane of the Illumina HiSeq 2000 platform with 50 bp single-end chemistry. Sequencing was performed by the Deep Sequencing Core Facility of CellNetworks Exzellenzcluster (Heidelberg).

Whole-Transcriptome Bisulfite Sequencing (WTBS) sample preparation

WTBS was carried out for w^{1118} as wild type, and for $Dnmt^{99}$ and $NSun2^{ex1}$ as tRNA methyltransferase null mutant genotypes. Crawling third instar larvae were grown on standard fly food media with yeast paste in a 12 hour day/night cycle of 25 °C and 18 °C. 20 larval brains per genotype were dissected in cold DEPC-PBS in one go. The brains were slowly centrifuged at 4 °C for approximately 5 minutes and the buffer replaced by 20 µl TRIzol (Ambion). The tissue was disrupted using RNase-free pestles and the suspension was filled up to 200 µL with TRIzol. Five samples per genotype were pooled to 100 µl of overall 100 larval brains. RNA extraction was carried out and quality controlled as described above. Total RNA samples were stored at -80 °C.

WTBS library preparation

A WTBS library preparation protocol was established to perform the tRNA methylome analyses and the transcriptome-wide RNA methylation screening. The scheme of Figure 6.1 summarises the library preparation protocol. The WTBS protocol was applied to replicate mouse samples by Legrande et al. to establish a computational approach for a comprehensive transcriptome-wide methylation analysis (Legrande et al., submitted).

20 µg of total RNA were separated into a small (<200 nt) and a long (>200 nt) fraction using buffers of the mirVana Isolation Kit (Ambion) and a customised protocol from the Mello laboratory (Small RNA Cloning Protocol, Gu & Conte), briefly described in the following. 400 µl of mirVana Lysis/binding buffer were gently mixed with 48 µl of mirVana Homogenate buffer in an RNase-free siliconised tube. 80 µl of total RNA were gently mixed with the buffers and incubated at RT for 5 min. A third volume of ethanol was added, gently mixed and incubated at RT for 20 min. Subsequently, 0.8 µl of GlycoBlue (Ambion) were added and centrifuged at 2,500 x g at 21 °C for 8 min to pellet long RNAs. The supernatant contained the short fraction, which was further precipitated. In parallel, the pellet of the long fraction was washed with cold 75% ethanol, centrifuged at maximum speed and 4 °C for 20 min, air-dried and resuspended in 48 µl RNase-free H2O. The short fraction was transferred to a fresh tube, supplemented with 20 µg GlycoBlue (Ambion) and 800 µl isopropanol, and incubated at -80 °C for at least 10 min. This was centrifuged at full speed and 4 °C for at least 10 min, washed with 70% cold ethanol, centrifuged again, air-dried and resuspended in 48 µl RNase-free water. (For the tRNA methylome analysis in S.pombe the tRNAs were isolated using gel electrophoresis instead of using fractionation. Details are published in Müller et al. 2015.)

Following the ethanol fractionation, small and long RNA fractions were rRNA depleted using the RiboMinus Eukaryote System v2 (Ambion) and the manufacturer's protocol. To achieve efficient depletion of rRNA, the long fraction was depleted in 3 subsequent rounds. To concentrate the depleted RNA, an ethanol / sodium-acetate precipitation was performed.

The long fraction was fragmented with the NEBNext Magnesium RNA Frag-

mentation Module (New England Biolabs) according to the manufacturer's manual, at 94 °C for 3 min. This resulted in a suitable length distribution for sequencing with a peak at 200 nt. Ethanol/sodium-acetate precipitation with 20 µg GlycoBlue (Ambion) was used to purify and concentrate the RNA. Both fractions were Turbo DNase (Ambion) digested at 37 °C for 30 min and subsequently bisulfite treated using the EZ RNA Methylation Kit (Zymo Research) according to the manufacturer's protocol.

The converted RNA was stepwise end-repaired with a T4 polynucleotide kinase (TaKaRa) as described for the ChIP RNAseq RNA library preparation. The RNA was purified from the reaction mixture by phenol/chloroform extraction using phenol/chloroform/isoamylalcohol (Ambion) and subsequent isopropanol precipitation.

cDNA synthesis and library preparation were performed according to the NEBNext Small RNA Library Prep Set (NEB) manual. 12 cycles of PCR amplification were applied, purified with the QIAquick PCR Purification Kit (Qiagen) and size selected using a 6% polyacrylamide gel electrophoresis (6% TBE Gels, Novex, Invitrogen). All small and long fractions of the different genotypes were differently barcoded using compatible NEBNext® indices to multiplex the libraries and sequence them on a single lane of the Illumina HiSeq 2000 platform with 100 bp paired-end chemistry. Sequencing was performed by the High Throughput Sequencing Unit of the Genomics and Proteomics Core Facility of the DKFZ.

Targeted RNA bisulfite sequencing

Total RNA was Turbo DNase (Ambion) digested and directly applied to the EZ RNA Methylation Kit (Zymo). Bisulfite converted RNA was used for cDNA synthesis in two different ways. SuperScript III Reverse Transcriptase (Invitrogen) and gene-specific primers were used for specific cDNA synthesis of tRNAs, or cDNA from mRNA was synthesized using the QuantiTect Reverse Transcription Kit (QIAGEN) and random hexamers following the manufacturer's manual. tRNA-specific cDNA samples were amplified using Fire Taq blue (Steinbrenner). Random hexamer-originated cDNA samples were amplified using the PyroMark PCR Kit (QIAGEN) without Q-solution. QIAquick Gel Extraction Kit (QIAGEN) was used to isolate amplicons from 2% agarose

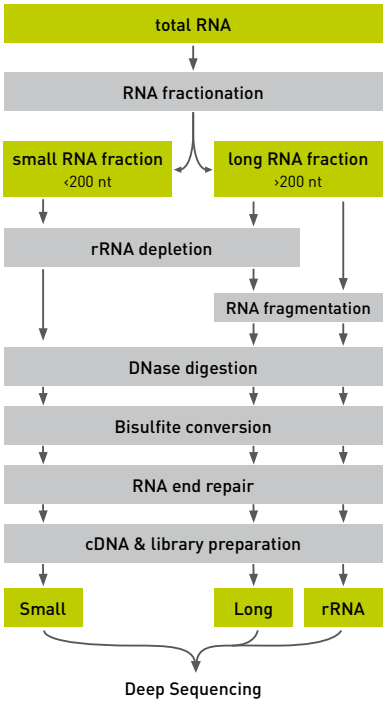


Figure 6.1: Schematic summarising the key steps of Whole-transcriptome Bisulfite Sequencing (WTBS) library preparation (Legrand et al., submitted). Total RNA from wild type (w^{1118}), Dnmt2 ($Dnmt2^{-/-}$) and NSun2 ($NSun2^{-/-}$) mutant third instar larval brains was processed according to this protocol for initial transcriptome-wide screening libraries, without replicates at this point.

gel. Concentrations of samples were determined with Quant-iT PicoGreen (Invitrogen) reagent measured with the FLUOstar OPTIMA (BMG Labtech) plate reader. All amplicons were prepared for sequencing according to the Roche 454 Junior protocol and sequenced in an equimolar pool on a Roche 454 Junior platform. Sequencing data was analysed using BiSQuID, an internal bisulfite sequencing alignment tool, which utilizes ClustalW2 and was programmed by Cassandra Falckenhayn.

6.2.7 Computational analyses

Genomic tRNA analysis in *Drosophila*

To display tRNA genes in the *Drosophila* genome, all tRNA genes annotated in the genomic tRNA database [Chan and Lowe, 2009] were loaded into the

Drosophila genome dm6 (BDGP Release 6, 2014) using the UCSC genome browser [Kent et al., 2002].

ChIP-RNAseq analysis

Raw data from ChIP RNA sequencing was quality controlled with FastQC (0.11.5) and sequences were trimmed from 3' adaptors (AGATCGGAAGAGCACACGTCT) using cutadapt (1.8.1). A whole transcriptome analysis was performed using STAR (2.4.0.h1) to align all reads to the Ensembl fly genome (Dmel84) with applied options "–outFilter MatchNmin 12 –outFilter MatchNminOverLread 0 –outFilter MismatchNoverLmax 0.1 –outFilter ScoreMinOverLread 0 –limitOut SJcollapsed 5000000 –outFilter MultimapNmax 14". Read counts were generated by Rsubread with fractional counting. 1/n was used for each alignment of multi-mapping reads, where n was the total number of alignments. To categorise the read distribution of different RNA types within the IP and input libraries, R was used to generate boxplots with counts per million (CPM). To quantitatively estimate the expression of tRNAs, a customized tRNA reference was generated based on the D. melanogaster tRNA database (FlyBase 6.10) [Gramates et al., 2016]. Redundant sequences were removed and CCA tails were added to the 3' ends of all remaining unique sequences. Reads were trimmed as before and mapped to the customised tRNA reference using Bowtie (0.12.8) with the options "–best –strata -a". Zero, one, or two mismatches were allowed and further analysed via "-v". Rsubread was used to calculate the read counts of each feature, while reads mapped to multiple references were counted as fractional counts. Mapping, read counts calling, and Quantimap analysis of ChIP-RNAseq was performed by Chunxuan Shao from the Höfer laboratory.

To calculate the specific centromeric distribution of tRNAs, CPM values of ChIP libraries were divided by the corresponding input CPM. tRNAs were arranged by the sum of Cenp-A and Cenp-C fold changes in the heatmap. Particularly tRNAs were further analysed for potential single nucleotide polymorphisms (SNPs) or single nucleotide variants (SNVs, editing sites) using the customised reference with the Integrative Genomics Viewer (IGV). IGV tracks were generated in collaboration with Merrit Romeike from the Erhardt laboratory.

Transcriptome-wide and tRNA methylome analysis

FastQC (0.11.5) was used to quality control the sequencing data of WTBS libraries. The tRNA methylome analysis of yeast data has previously been described [Müller et al., 2015]. This pipeline was applied to *Drosophila* data for a tRNA methylome analysis and a transcriptome-wide screen for non-tRNA methylation candidate sites.

In brief, reads were trimmed with a Galaxy-integrated fastq quality trimmer to a minimum aggregate score of 30 and further adaptor-trimmed using cutadapt 1.8.1 [Martin, 2011]. A two-step sequence alignment was performed using the aligner BSMAP version 2.74 [Xi and Li, 2009]. BSMAP was run with options "-s 12 –v 0.1,-g 0 –w 1000 –S 0 –p 1 –V 1 –n 0 –r 2 –m 15 –x 1000", allowing a 10% mismatch rate and disregarding reverse complemented reads. In a first step, reads were aligned to a custom tRNA reference based on the genomic tRNA database [Chan and Lowe, 2009]. Duplicate sequences were removed and 3' CCA tails were added to each reference sequence. In a second step, reads were aligned to a transcriptome-wide reference, which was assembled from FlyBase Dmel_Release_6.01 [Gramates et al., 2016], pruned of duplicate sequences. Finally, reads that mapped to both, the transcriptome and tRNA reference, were filtered out.

For methylation calling, internal Python scripts were used. Tested candidate sites originated from multiple analysis rounds with differently stringent settings. As a result, final selected cutoffs were $\geq$ 20 nt for the read length and $\geq$ 20 reads for the coverage. Methylation candidates were chosen by additionally filtering for sites with wild type cytosine ratios >0.8 and mutant ratios $<$ 0.2 at the same position. The deamination efficiency was calculated at selected known non-methylated sites of tRNAs. Sebastian Bender from the Lyko laboratory performed mappings, methylation callings, and calculation of the deamination efficiency.

To display the tRNA methylation analysis, one representative sequence per isoacceptor family was chosen. All positions that contain $\geq$ 0.6 methylation ratios of at least one cytosine in one tRNA are shown. Dnmt2- and NSun2-dependent methylation was calculated as the absolute value of the difference of wild type and mutant ratios. Heatmaps summarise all wild type methylations, and Dnmt2- and NSun2-dependent methylation sites. Calculation of the enzy-

matic activity of MTases was done using an internal script that counts reads of every library, which cover positions that contain minimal 60% cytosines in both control and mutant libraries with a minimal coverage of 5. This was normalized to all cytosine-containing reads with a minimal coverage of 5 of the corresponding library. The difference between wild type and mutant libraries reflects the Dnmt2- or NSun2-dependent methylation sites, respectively, and thus the computational enzymatic activity. Cassandra Falckenhayn wrote the script to count the cytosine-containing reads.

Statistical Analyses

Prism (GraphPad) was used to display all quantifications and to apply chi-square or Student's t-test, and to calculate p-values. The required level of significance of differences was defined as 5% ($p \leq 0.05$). The type of test, sample sizes (n), and p-values are stated in the respective figure legends.

Design and Illustration

InDesign (Adobe) was used to design all figures and to illustrate and modify graphical schemes.

This thesis was written in LaTeX.

A

Perspectives

In this doctoral thesis, three main aspects remain open regarding their exact role at centromeres. At least partially, they can all together be examined by use of centromeric ChIP-RNAseq, a powerful tool to study centromere identity. First, to answer the question of RNAPIII-mediated transcription of centromeric tRNA genes, ChIP-RNAseq data should be extended to the analysis of nascent or pre-tRNA transcripts and could be combined with use of the RNAPIII inhibitor. Second, the role of methylation-dependent tRNA fragmentation can be analysed by quantifying tRNA fragments in MTase-mutant conditions. Third, the ChIP-RNAseq protocol provides a method to comprehensively analyse all centromeric RNA (cenRNA) in all kind of functional studies such as cell cycle- or stress-dependence, various knock outs, overexpressions, and drug treatments.

A.1 ChIP-RNAseq enables comprehensive analysis of centromere-associated RNA.

The functionality and applicability of centromeric ChIP-RNAseq was demonstrated here and the experiences made in this study provide important information to further improve this method. First of all, use of two different pull-downs and respective input libraries provided important reproducibility of the results and should be used in every condition to be analysed. Secondly, analysis should be expanded to at least two or better three replicates per condition to gain further confidence and allow quantitative transcriptomic analysis

at transcript resolution. ChIP-RNAseq was establishmed as a method suitable to identify annotated and novel centromere-associated transcripts, and to analyse their differential abundance in different conditions. This needs a certain confidence in terms of statistical power, coverage and overlapping reads, respectively. Centromeric ChIP-RNAseq enables a comprehensive analysis of centromere-associated RNA in practically all model organisms and a variety of conditions.

A.1.1 Analysis of the differential composition of cenRNA in different conditions.

Cell cycle dependence can easily be analysed applying the colcemid block used in this study for ChIP-BS-RNAseq. Likewise, the influence of active transcription of different polymerases on the centromeric transcriptome can be examined by applying corresponding transcription inhibitors to the cells, which also improves the possibility to discriminate between specific and unspecific effects. The influence of RNA MTases (and other RNA processing proteins) can be analysed by depletion, overexpression, and catalytically inactive constructs or model organisms, respectively. Practically all centromeric factors of interest can be analysed for potential roles in centromeric RNA regulation. Moreover, model systems can be exposed to stress prior to chromatin IP.

A.2 In depth analysis of Dnmt2-mediated methylation.

As part of this doctoral thesis, a library preparation protocol for whole transcriptome bisulfite sequencing (WTBS) was successfully established. In an initial transcriptome-wide screen for Dnmt2 and NSun2 targets, no other substrates than the known tRNAs could be detected for Dnmt2. The mRNA of Endophilin A (EndoA) was the only confirmed non-tRNA substrate positively validated for NSun2. As discussed in detail in the supplements, this finding reflects the highly conserved substrate-specificity of Dnmt2 as a three-tRNA methyltransferase, which has previously been confirmed in mouse (Reinhard Liebers, PhD thesis, 2015). The WTBS protocol should be performed in repli-

cates to allow a statistically confident analysis of the entire transcriptome. In this way Dnmt2 can be further confirmed as a specific tRNA-methyltransferase.

As presented for an amplicon-based approach, centromeric ChIP can be combined with bisulfite sequencing to analyse the methylation of centromeric transcripts. This can in theory be expanded to transcriptome-wide sequencing of all centromeric transcripts, introducing an additional bisulfite step into the protocol to perform ChIP-BS-RNAseq. Such an experiment could provide important information whether methylation activities of RNA MTases are dynamic, and whether other transcripts than tRNAs are methylated at centromeres. The ChIP protocol can additionally be applied to other modifications, such as hydroxymethylation or adenosine methylation to further examine the centromeric epitranscriptome.

Drosophila as a model organism to examine RNA methylation provides the advantage that the DNA methyltransferases Dnmt1/3 and therefore distinct DNA methylation patterns are missing (Raddatz et al. 2013). This facilitates a more specific use of azacytidine, a cytosine analogue that traps Dnmt proteins to nucleic acids and thereby blocks their activity. Previously, azacytidine was successfully used to specifically inhibit Dnmt2 but not NSun2 (Schaefer et al. 2009), likely due to the different enzymatic mechanism employed by NSun2, which enables a more detailed study of Dnmt2 at centromeres applying the drug to mitotic spreads or ChIP-RNAseq.

A.3 In-depth analysis of tRNA transcription and fragmentation at centromeres.

The findings that centromere-associated tRNAs are edited and methylated, that tRNA methyltransferases co-localise these tRNAs, and that impairment of the catalytic activity of Dnmt2 leads to chromosome segregation defects strongly suggest a regulatory role of RNA modification in mitosis. The question which one of the proposed molecular mechanisms discussed here is true requires further investigation.

The observed mature centromeric tRNAs may in theory be independent of

centromeric transcription, associating in *trans* from a nuclear pool or from retrograde imported cytosolic tRNAs (Huynh et al. 2010; Quan et al. 2007). Therefore, the analysis of ChIP-RNAseq data should be expanded to elucidate whether pre-tRNAs, mature full length or processed tRNA fragments are present at centromeres. This requires advanced bioinformatics analysis. For example, introns present in some tRNAs could be used to quantify pre-tRNAs. Trailer sequences of centromeric pre-tRNAs can currently not be used because of the lack of information of centromeric DNA sequences (supplements 1). However, an indirect way to answer whether the enriched tRNAs are centromere-encoded can be performed. Trailer sequences could be mapped to annotated non-centromeric sequences and remaining reads with trailers of unidentified origin were potentially (peri-) centromeric encoded.

Better proof for ongoing transcription could be provided by neusRNA-seq (or GRO-seq; Gardini 2017), which is an NGS method using ethylene uridine (EU)-labelling to specifically sequence nascent RNA and has successfully been applied to analyse RNAPIII transcription (Orioli et al. 2016). Analysis of reads assigned uniquely to pre-tRNAs have been quantified and directly correlated to active transcription. For potentially centromere-encoded tRNA within the *Drosophila* genome, the lack of centromeric sequence information hinders approaches that make use of pre-tRNA sequences, because no up- or downstream sequence information is available for large (peri-) centromeric domains. Emerging long-read sequencing techniques may soon provide enough information to assemble highly repetitive pericentromeric and centromeric DNA sequences. Together with the ChIP-RNAseq approach presented here, analysis of nascent RNA at centromeres might become possible soon.

Pipelines for tRNA fragment analysis of NGS data have previously been published (Xu et al. 2017; Keam et al. 2014; Soares et al. 2015). The differential distribution of read coverage over the length of a given tRNA transcript reveals differences that could originate from tRNA fragments. However, such information needs to be taken cautiously as RNA modifications are known to cause PCR and reverse transcription artefacts. One way to address this would be to analyse the dynamic differences of coverage upon Dnmt2 depletion that is known to increase tRNA fragmentation at defined sites (Zeljko Durdevic,PhD thesis, 2013; Durdevic et al. 2013).

To continue with functional investigations, a good starting point would be the question whether tRNAs directly affect chromosome segregation or whether they are simply by-products of the transcriptional process required at centromeres. Live cell imaging rescue experiments with full length or fragmented, and methylated or unmethylated tRNAs, respectively, could provide information whether the methylation sites per se or fragmentation of tRNAs are critical, or if active RNA methyltransferases but not the transcripts themselves are needed for centromeric regulation.

B

Supplements

B.1 tRNA genes in the Drosophila genome

304 tRNA genes are annotated for the Drosophila genome (tRNAscan-SE Analysis of Drosophila melanogaster, release 5 April 2006; [Chan and Lowe, 2009]). Figure B.1 illustrates all known tRNA gene loci on all chromosomes together with repetitive sequence elements. The major chromosomes (chr2+3) are separated into chromosome arms. The chromosome sequences are not completely assembled due to the highly repetitive nature of pericentromeric domains, which leads to a lack of information including centromeres.

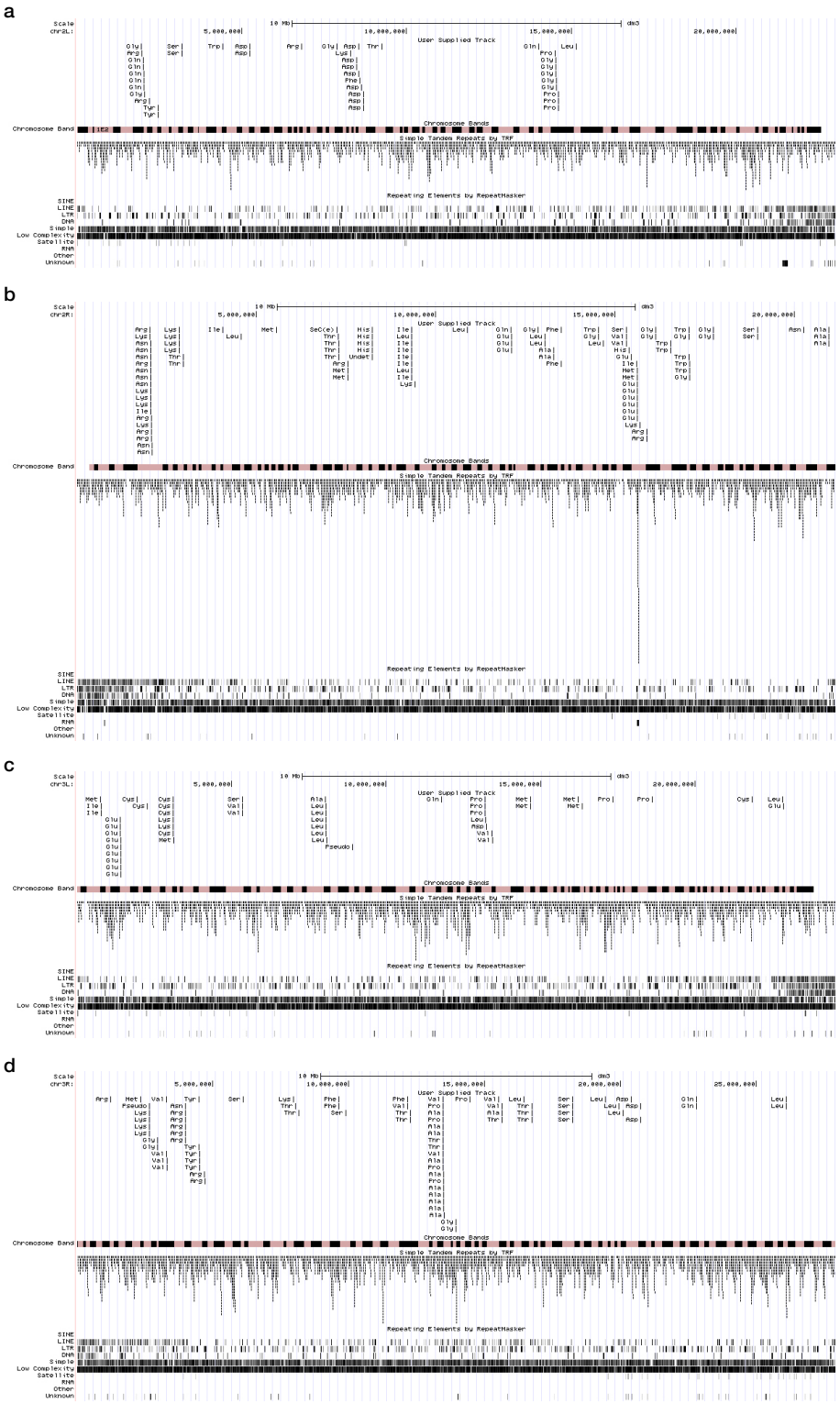


Figure B.1: Genome Browser: tRNA genes in Drosophila. See below.

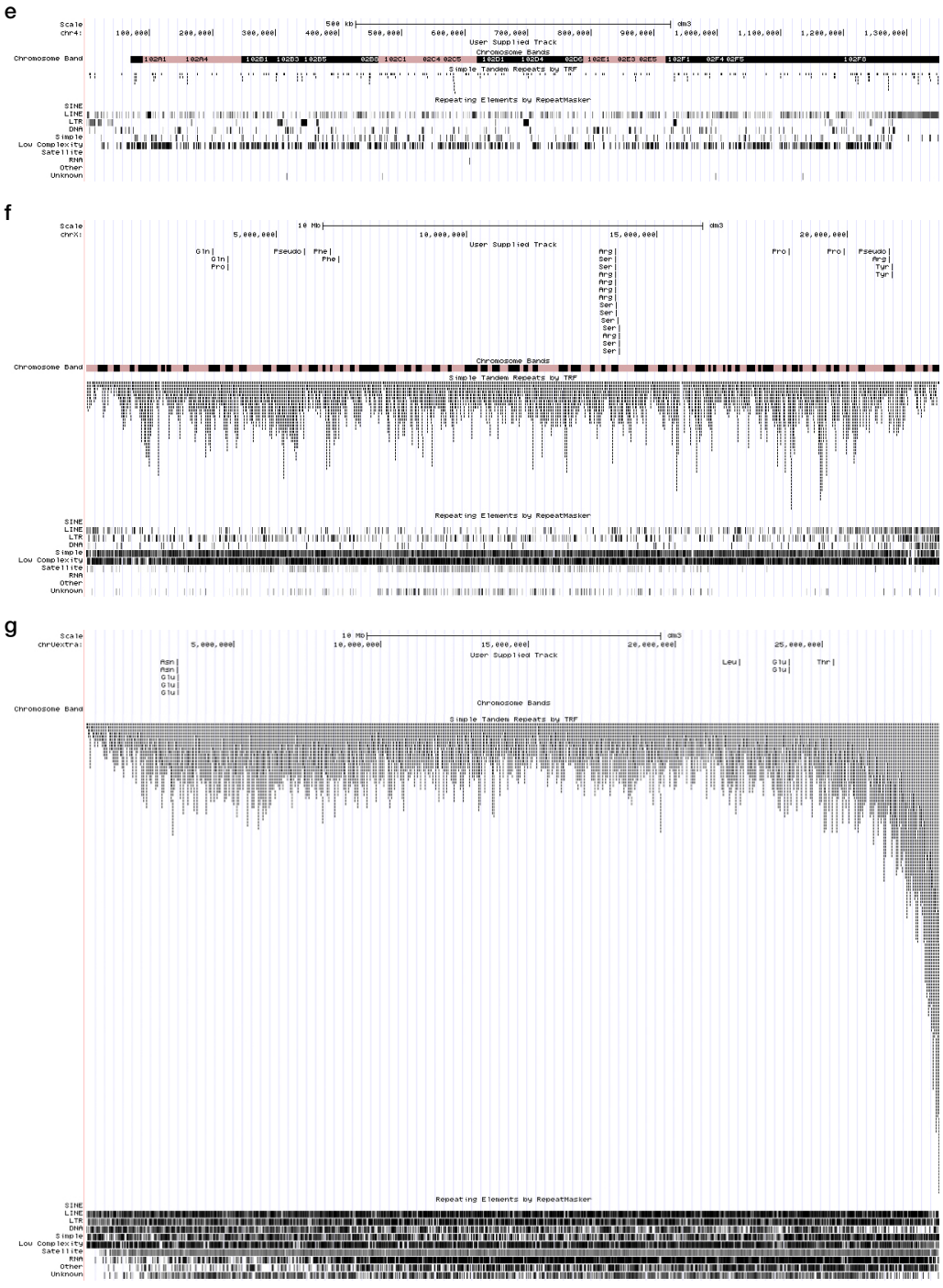


Figure B.1: Genome Browser: tRNA genes in Drosophila. a-g, The UCSC genome browser was used to assign all annotated tRNA genes in GtRNAdb (Chan & Lowe). Depicted are chromosomes **(a)** 2L, **(b)** 2R, **(c)** 3L, **(d)** 3R, **(e)** 4, **(f)** X, and **(g)** Uextra. Chromosome Uextra represents an artificial chromosome assembly of all not assembled sequencing reads. Depicted are also chromosome banding patterns and repetitive sequences.

B.2 NGS quality control

In this PhD two next generation sequencing (NGS)-based RNA analysis methods were established. Figures B.2-B.5 summarise library size distributions and quality controls of NGS experiments using Illumina technology performed in this thesis. Figures B.2-B.3 refer to the input or IP libraries, respectively, of the ChIP-RNAseq experiment. Figures B.4-B.5 refer to the short or long fractions of transcriptome-wide bisulfite libraries of wild type (w^{1118}), $Dnmt2^{-/-}$, or $NSun2^{-/-}$ genotypes. The short fractions were used for the tRNA methylome analysis, both the short and the long fraction for the transcriptome-wide screen (supplements 5). Figure B.6 displays the base distributions for the sequences from the bisulfite experiment, which demonstrate the efficient conversion of cytosines to thymines using bisulfite chemistry. The short fractions that contain the tRNAs display defined peaks that correspond to the Dnmt2- and NSun2-dependent methylation sites described in the results. In summary, the established ChIP-RNAseq protocol provides a method to comprehensively analyse centromere-associated RNA. The protocol for transcriptome-wide bisulfite sequencing provides a base for Whole-Transcriptome Bisulfite Sequencing (WTBS) analysis as applied for mouse by Legrand et al. (submitted).

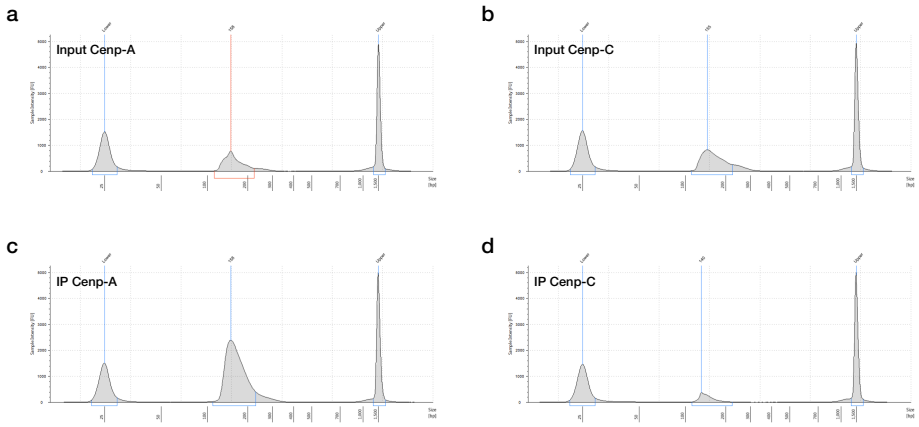


Figure B.2: ChIP-RNAseq: Size distribution of adaptor-ligated NGS libraries. Agilent TapeStation electropherograms of **(a)** Cenp-A input, **(b)** Cenp-C input, **(c)** Cenp-A IP, and **(d)** Cenp-C IP.

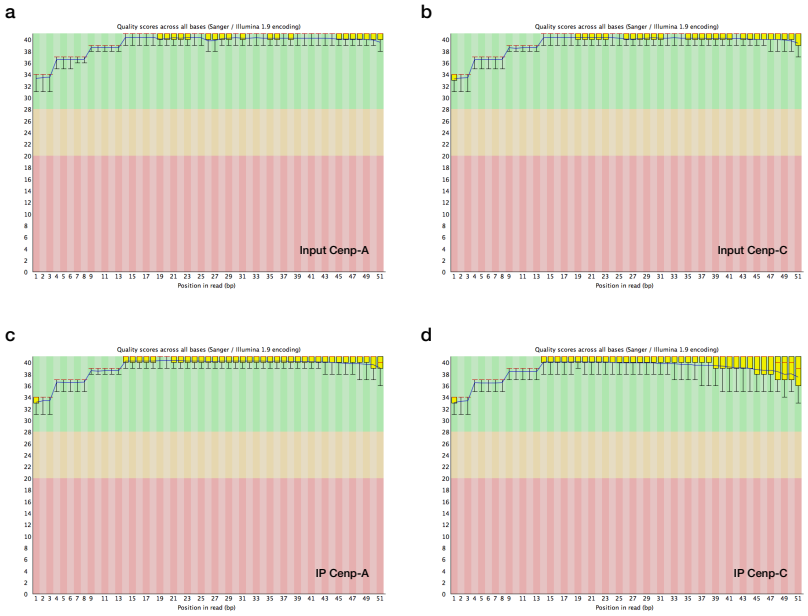


Figure B.3: ChIP-RNAseq: ChIP-RNAseq: FastQC report. Per base sequence quality plots generated using FastQC for **(a)** Cenp-A input, **(b)** Cenp-C input, **(c)** Cenp-A IP, and **(d)** Cenp-C IP (by Chunxuan Shao).

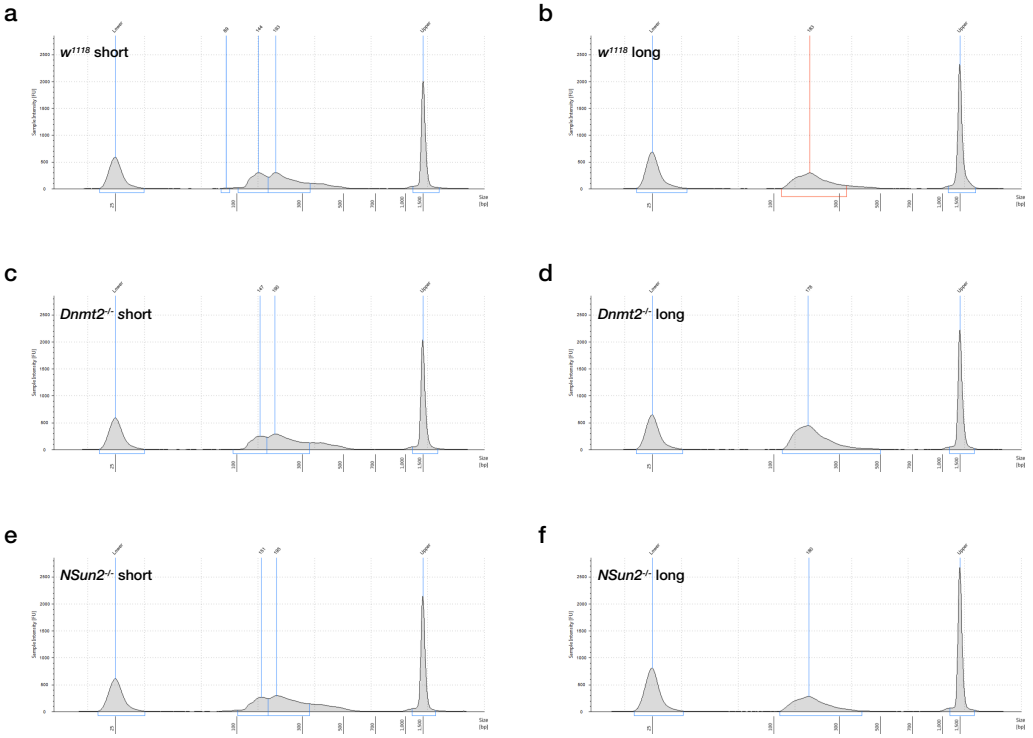


Figure B.4: WTBS: Size distribution of adaptor-ligated NGS libraries. Agilent TapeStation electropherograms of **(a)** w^{1118} short, **(b)** w^{1118} long, (c) *Dnmt2*$^{-/-}$ short, (d) *Dnmt2*$^{-/-}$ long, **(e)** *NSun2*$^{-/-}$ short, and **(f)** *NSun2*$^{-/-}$ long. Short and long refer to the short (<200 nt) and long (>200 nt) fractions of total RNA.

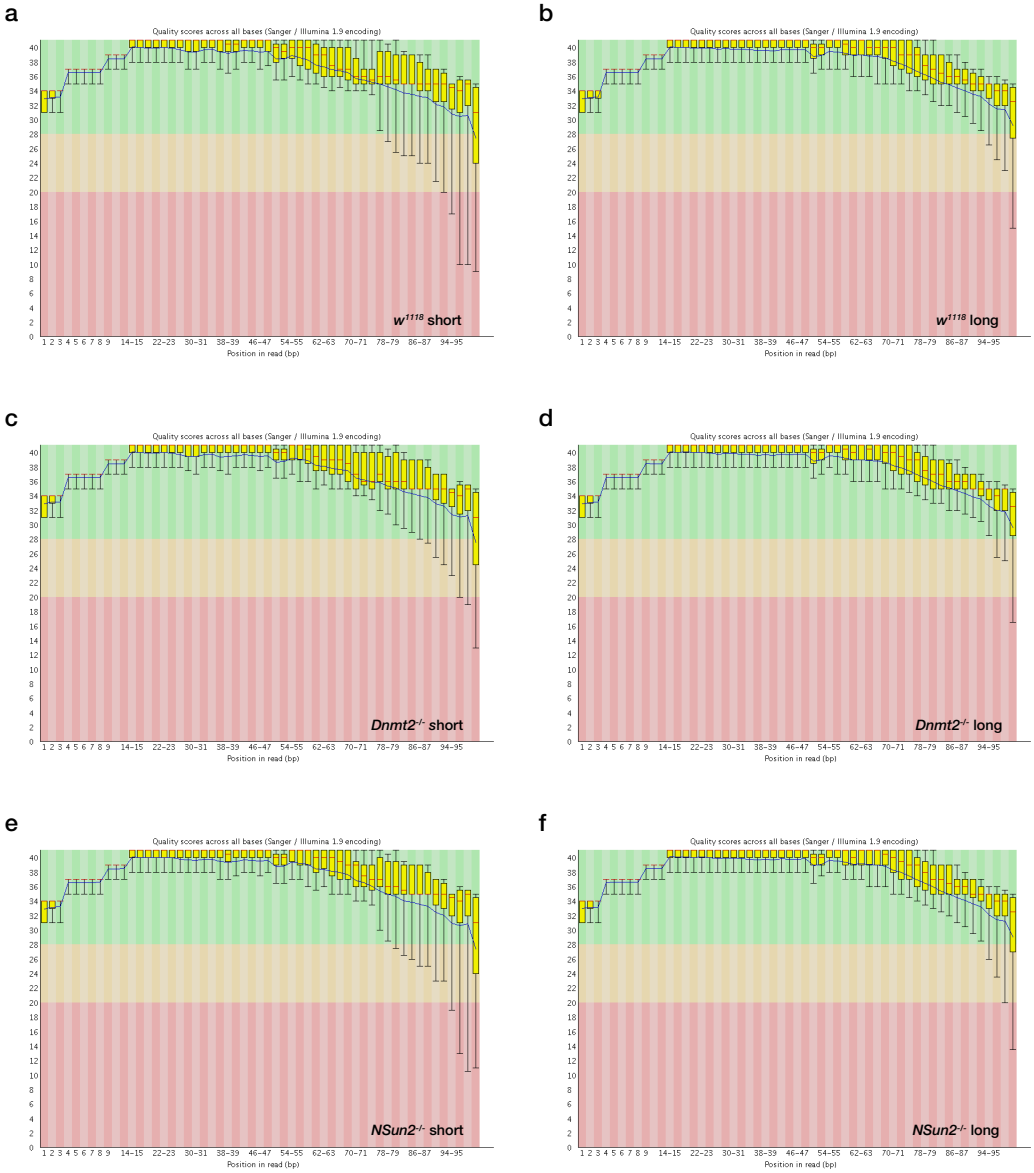


Figure B.5: WTBS: FastQC report. Per base sequence quality plots generated using FastQC for **(a)** w^{1118} short, **(b)** w^{1118} long, **(c)** *Dnmt2*$^{-/-}$ short, **(d)** *Dnmt2*$^{-/-}$ long, **(e)** *NSun2*$^{-/-}$ short, and **(f)** *NSun2*$^{-/-}$ long. Short and long refer to the short (<200 nt) and long (>200 nt) fractions of total RNA.

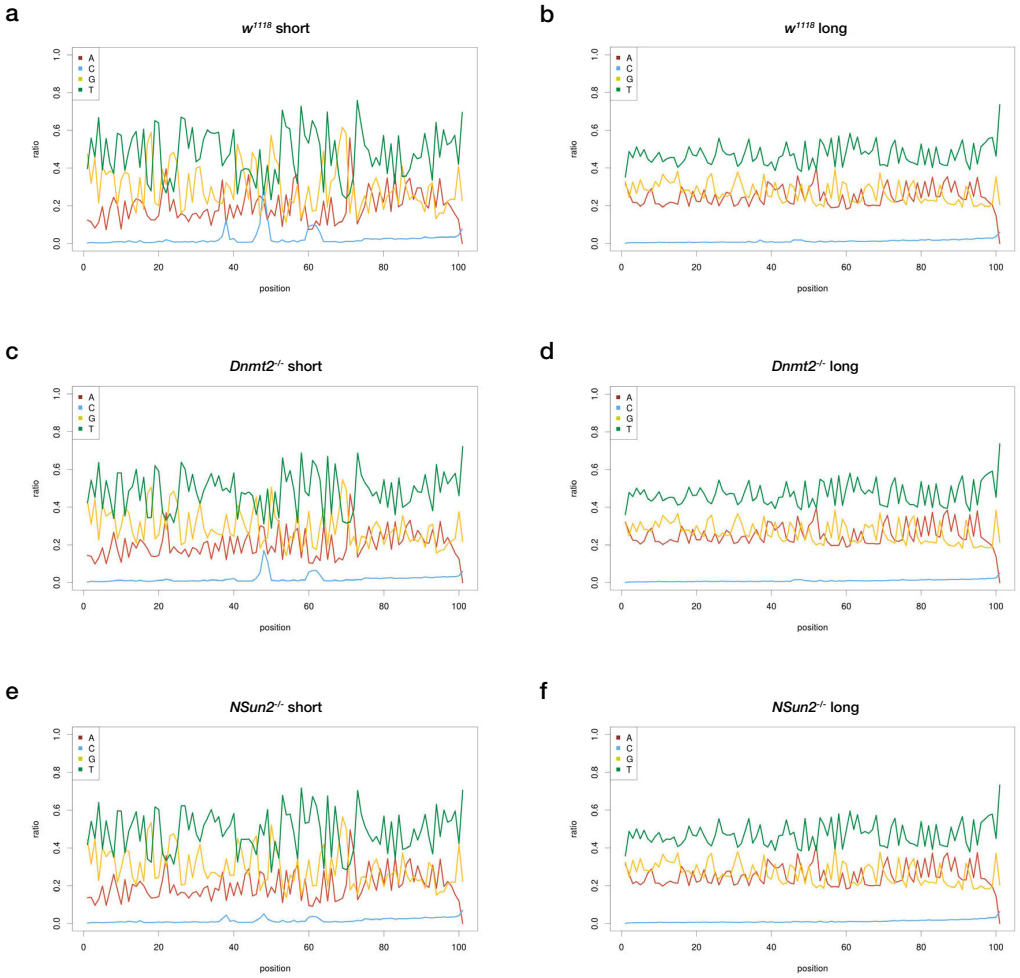


Figure B.6: WTBS: Base distribution Base distribution using FastQ base fraction for **(a)** w^{1118} short, **(b)** w^{1118} long, **(c)** *Dnmt2*$^{-/-}$ short, **(d)** *Dnmt2*$^{-/-}$ long, **(e)** *NSun2*$^{-/-}$ short, and **(f)** *NSun2*$^{-/-}$ long. Short and long refer to the short (<200 nt) and long (>200 nt) fractions of total RNA.

B.3 tRNA methylome analysis in yeast reveals a micronutrient-dependent single substrate-specificity of Pmt1

Analogous to the tRNA methylome analysis in Drosophila, a comprehensive cytosine-5 methylation analysis of tRNAs in S.pombe was done using bisulfite sequencing (Figure B.7). This data has previously been published and a detailed description and discussion can be found in Müller et al. (2015). In brief, 90% (18 out of 20) of all isoform classes were methylated on at least one isoacceptor. 87% (30 out of 46) of isoacceptor families were methylated. The Dnmt2 homolog Pmt1 was found to methylate only $tRNA^{Asp(GTC)}$ and this methylation activity was dependent on the presence of the micronutrient queuosine (Q) in the medium. C38 methylation of $tRNA^{Asp(GTC)}$ was the only Q-dependent methylation within all tRNAs. Thus, 90% of all isoforms carry at least one methylation mark, however only one of these marks (1 of 47) is Pmt1- and Q-dependent. Q is a micronutrient that is obtained by higher eukaryotes from the diet and gut microflora. According to that, this finding demonstrates an impact of the environment on tRNA modification.

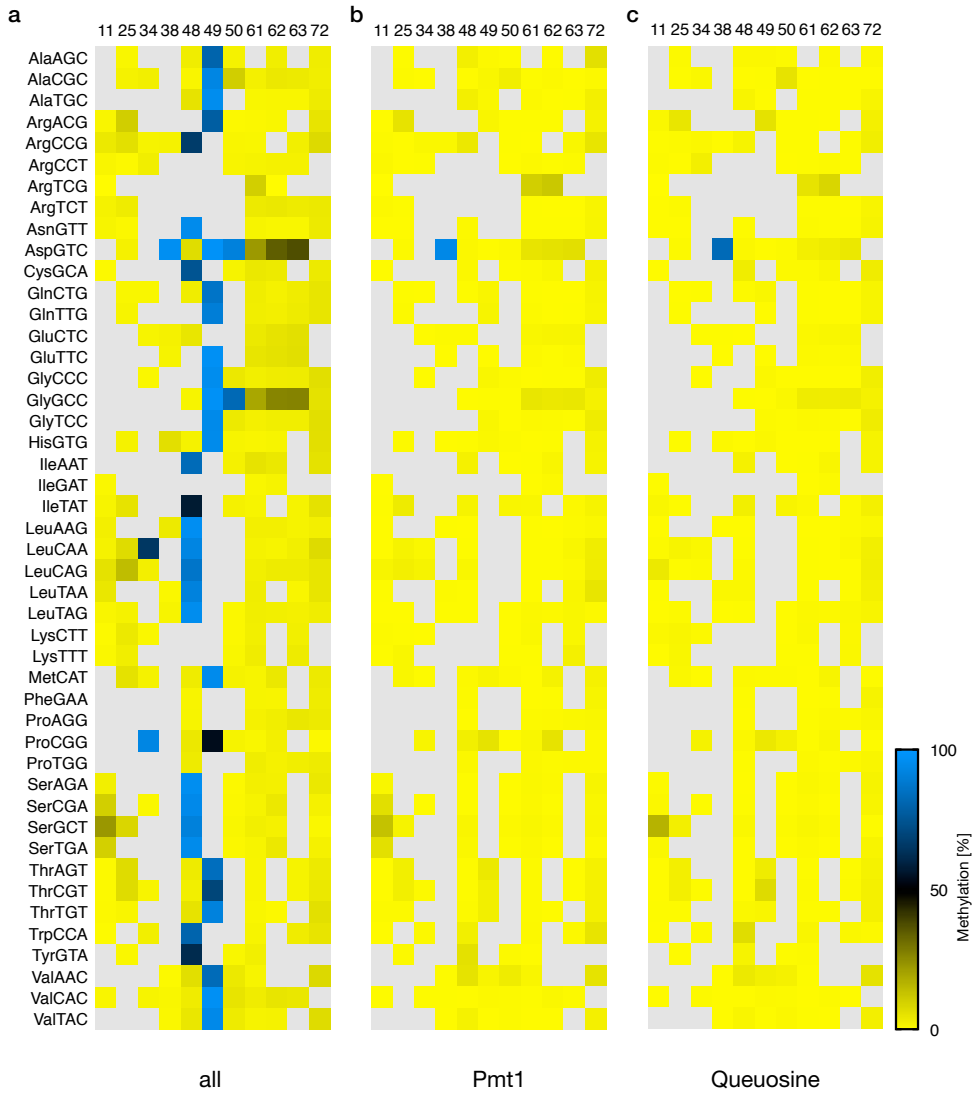


Figure B.7: tRNA methylome analysis of S.pombe reveals a comprehensive map of cytosine-5 methylation and tRNA substrates of Pmt1 in dependency of queuosine (Q). a-c, Genome-wide tRNA methylome analysis of S.pombe. The colour gradient displays the amount of unconverted cytosines at the indicated position. Data has been published by Müller et al. (2014). **a**, All positions with unconverted cytosines >15% plus position 72 are depected. **b**, pmt1-dependent methylation sites. **c**, Q-dependent methylation sites.

B.4 Insulator factors associate with mitotic centromeres

As part of this doctoral thesis further factors have been found to associate with centromeres during mitosis. CTCF and CP190 are insulator factors functioning at chromatin boundaries and probably additionally as facilitators of higher-order chromatin organisation [Moon et al., 2005, Mohan et al., 2007, Schoborg and Labrador, 2014]. Immunofluorescence analysis of mitotic chromosome spreads revealed that both factors remained chromatin-bound including centromeres (Figure B.8 a-b). Remarkably, centromeric localisation of both insulator factors was restricted to the autosomes. Neither of the two proteins could be detected within pericentric chromatin or at centromeres of the X chromosome. This restriction is a remarkable difference to the localisation of RNA polymerase III, the RNAPIII-specific transcription factor TRF1, the tRNA methyltransferases Dnmt2 and NSun2, and the RNAi components Ago2 and Dcr2, which associated with all centromeres of all chromosomes (Figure 3.16.c; Figure 3.5.c; Figure 3.14). This discrepancy indicates an independent centromeric function of the RNAPIII machinery and the RNA-processing enzymes from CTCF and CP190.

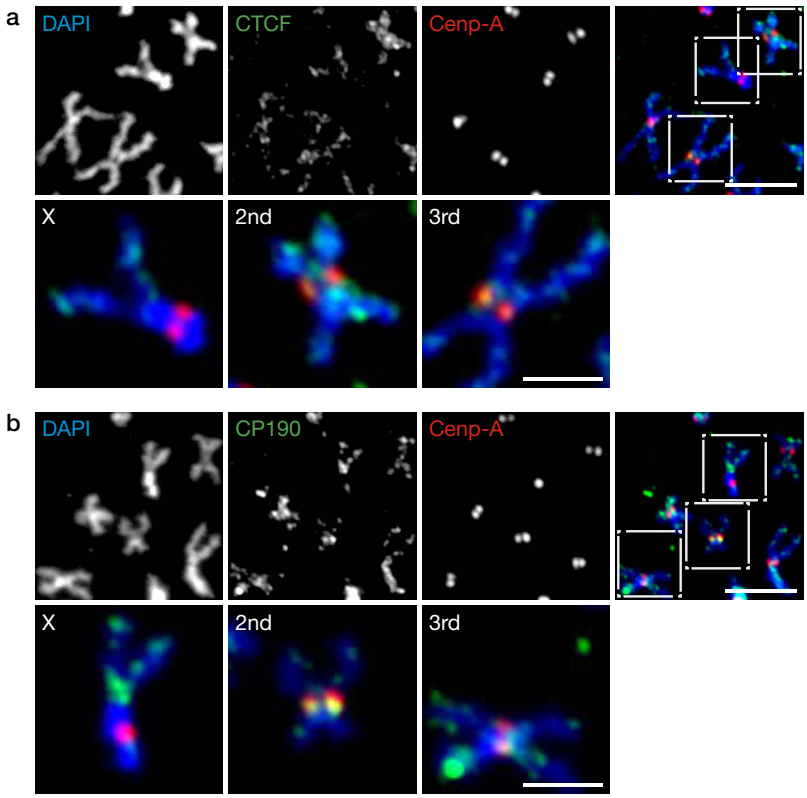


Figure B.8: Insulator factors CTCF and CP190 stay chromatin-associated and localise to autosomal centromeres during mitosis. **a-b**, Immunofluorescence on mitotic chromosome spreads of S2 cells, stained with DAPI (blue), **(a)** anti-CTCF or **(b)** anti-CP190 (green), and anti-Cenp-A (red). Zoom images show chromosomes X, 2, and 3. Scale bars, 5 µm and 2 µm (zoom).

B.5 The coding region of EndoA mRNA is methylated by NSun2

The amount and distribution of RNA cytosine-5 methylation (5mC) within the transcriptome is controversially discussed [Blanco and Frye, 2014]. The existence of this modification in tRNA as well as rRNA has been confirmed repeatedly with different methods [Motorin Y, 1999, Schaefer et al., 2009, Schaefer et al., 2010, Tuorto et al., 2012, Edelheit et al., 2013, Khoddami and Cairns, 2013, Blanco et al., 2014, Müller et al., 2015, Tuorto et al., 2015, Metodiev et al., 2009, Machnicka et al., 2013, Sharma et al., 2013, Bourgeois et al., 2015, Schosserer et al., 2015]. This doctoral thesis provides further proof of widespread tRNA methylation and demonstrates its high degree of conservation (Figure 3.7; Figure B.7). Although some publications have reported 5mC on mRNA and (non-tRNA and non-rRNA) ncRNA, the global dimension, the enzymatic dependence, and especially potential functions mostly remain elusive [Squires et al., 2012, Edelheit et al., 2013, Hussain et al., 2013b, Hussain et al., 2013a, Khoddami and Cairns, 2013, Shafik et al., 2016, David et al., 2017, Amort et al., 2017].

As part of this thesis a protocol for whole transcriptome bisulfite sequencing (WTBS) library preparation was established and applied to wild type (w^{1118}), Dnmt2 ($Dnmt2^{-/-}$) and NSun2 ($NSun2^{-/-}$) null mutant Drosophila third instar larval brain tissue (Figure B.9). Calculating the enzymatic activity by counting the number of reads containing cytosines resistant to bisulfite conversion, both Dnmt2 and NSun2 experiments revealed a predominantly enzymatic activity for tRNAs (Figure B.9.a) (in collaboration with Cassandra Falckenhayn). Even though Dnmt2 has repeatedly been described as a tRNA-specific methyltransferase, a small subset of potential non-tRNA substrates remained after transcriptome-wide mapping. Remarkably, the range of 5mC candidate sites on mRNA has been reported from a few to tens of thousands of transcripts within different transcriptomes [Legrand et al., , Squires et al., 2012, Edelheit et al., 2013, Hussain et al., 2013b, Khoddami and Cairns, 2013, David et al., 2017, Amort et al., 2017]. The direct NSun2-dependency could only be shown for some transcripts, most of the predicted methylation sites, however, re-

mained unconfirmed [Squires et al., 2012, Hussain et al., 2013b, Hussain et al., 2013a, Khoddami and Cairns, 2013, David et al., 2017]. Here only a small number of non-tRNA NSun2-dependent candidate sites were predicted (Figure B.9.a).

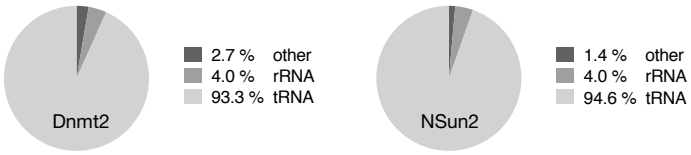
The number of predicted candidate sites for Dnmt2- and NSun2-dependent non-tRNA and non-rRNA methylation varied from 63 to over 20,000, depending on the mapping and methylation calling parameters and the applied filters. This observation stressed the need for a solid replicate- and statistics-based analysis pipeline for WTBS data.

WTBS validations were performed using amplicon-based bisulfite sequencing. One single site out of 36 tested methylation candidate sites was positively validated using amplicon-based bisulfite Sanger sequencing on an ABI machine (Figure B.9.b). Thus, the presence of tens of thousands of methylated mRNAs appears unlikely in Drosophila third instar larval brain tissue.

However, the presence of the single bisulfite-resistant cytosine in EndoA mRNA was demonstrated in wild type (w^{1118}) and $Dnmt2^{-/-}$ mutant tissue and disappeared in $NSun2^{-/-}$ as well as in $Dnmt2^{-/-}$;$NSun2^{-/-}$ double mutant samples (Figure B.9 d). The methylation and the NSun2-dependency were confirmed using quantitative Roche 454 bisulfite sequencing (Figure B.9 c). Sequencing of genomic DNA revealed no polymorphism at the methylation site, indicating that the detected methylation sites are not artefacts caused by underlying DNA polymorphisms (Figure B.9 e).

About 16,000 genes can be found in Drosophila, but only a subset is expressed in every cell type and developmental stage, for example about 2,500 genes in the third instar larval central nervous system [Graveley et al., 2011]. Assuming a maximal 3% rate of methylated transcripts as shown by WTBS validations, less than 100 methylated gene products would theoretically be present in the larval brain. This hypothetical calculation contradicts publications suggesting thousands of potentially methylated mRNAs in the human transcriptome [Squires et al., 2012, Khoddami and Cairns, 2013] and high levels of 5-hydroxymethylcytosine (5hmC) in the Drosophila transcriptome [Delatte et al., 2016], since bisulfite-based methods cannot discriminate between 5mC and 5hmC. However, a replicate-based WTBS analysis needs to be done to confirm these numbers.

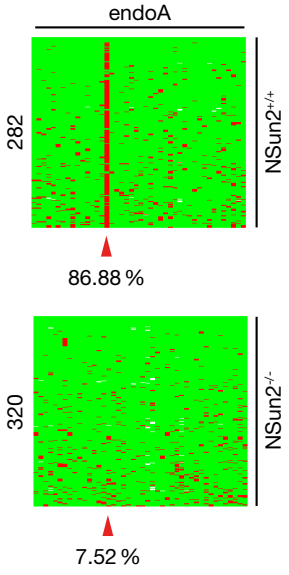
a



b

				Whole Transcriptome Bisulfite Sequencing						Sanger
				wildtype		*Dnmt2-/-*		*NSun2-/-*		
ID	Symbol	Annotation	Position	Ratio	Cov	Ratio	Cov	Ratio	Cov	Base at position
FBti0019644	NOFb		103	1.00	20	0.18	34	0.83	36	T
FBtr0070106		CG13377	893	0.95	39	0.04	47	1.00	20	T
FBtr0075614	Toll-6	CG7250	4411	0.97	356	0.02	359	0.95	95	T
FBtr0078121	spen	CG18497	583	1.00	1,796	0.23	74	1.00	1,312	T
FBtr0082001	neur	CG11988	133	1.00	32	0.20	20	0.71	21	T
FBtr0273225	shot	CG18076	15385	0.92	120	0.00	92	0.89	57	T
FBtr0336736	sick	CG43720	3192	1.00	766	0.06	80	1.00	531	T
FBtr0337028	cow	CG13830	1363	0.88	24	0.07	28	0.88	41	T
FBti0019042		opus	633	1.00	20	0.83	12	0.18	22	T
FBtr0072114	wmd	CG3957	1506	0.81	32	0.68	19	0.00	24	T
FBtr0079607	Mur29B	CG31901	1425	0.88	50	0.63	32	0.00	22	T
FBtr0081193	ssp3	CG18397	5218	0.91	121	0.89	166	0.10	199	T
FBtr0081621	Dfd	CG2189	488	0.89	287	0.96	464	0.02	4,721	T
FBtr0082704	ry	CG7642	1263	0.87	31	0.83	29	0.03	97	T
FBtr0083698	EndoA	CG14296	1101	0.90	41	0.70	30	0.00	35	C
FBtr0085718	CG15546	CG15546	1235	1.00	49	0.67	12	0.14	29	T
FBtr0339671		CG8668	742	0.92	24	0.75	16	0.01	82	T
FBtr0082929	CG3307	pr-set7	1443	1.00	9,341	0.08	26	0.11	36	T
FBtr0082929	CG3307	pr-set7	1451	1.00	9,335	0.11	27	0.11	38	T
FBtr0082929	CG3307	pr-set7	1448	1.00	9,335	0.07	28	0.10	40	T
FBtr0082929	CG3307	pr-set7	1437	0.99	9,263	0.00	28	0.04	28	T
FBtr0083400	cal1	CG5148	2503	0.93	217	0.00	20	0.36	28	T
FBtr0083400	cal1	CG5148	2508	0.89	225	0.00	30	0.25	32	T
FBtr0083400	cal1	CG5148	2511	0.90	225	0.00	34	0.00	26	T
FBtr0083400	cal1	CG5148	2515	0.89	225	0.00	36	0.08	24	T
FBtr0083400	cal1	CG5148	2518	0.89	225	0.00	36	0.00	20	T
FBtr0083400	cal1	CG5148	2519	0.89	225	0.03	38	0.00	20	T
FBtr0083400	cal1	CG5148	2521	0.88	229	0.05	38	0.00	22	T
FBtr0304604	pros	CG17228	7059	0.81	32	0.00	44	0.33	18	T
FBtr0304604	pros	CG17228	7060	0.87	30	0.00	42	0.33	18	T
FBtr0304604	pros	CG17228	7062	0.88	34	0.00	40	0.33	18	T
FBtr0304604	pros	CG17228	7063	0.89	36	0.00	38	0.38	16	T
FBtr0304604	pros	CG17228	7065	0.89	36	0.00	32	0.50	20	T
FBtr0304604	pros	CG17228	7066	0.86	37	0.06	32	0.50	20	T
FBtr0304604	pros	CG17228	7068	0.89	37	0.00	32	0.56	18	T
FBtr0304604	pros	CG17228	7069	0.89	35	0.06	32	0.44	18	T

c



d e

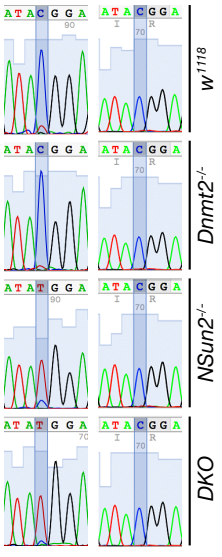


Figure B.9: Transcriptome-wide methylome analysis reveals a single mRNA methylation site within the CDS of endoA mRNA. **a**, Calculation of Dnmt2- and NSun2-dependent bisulfite-resistant cytosine-containing reads in different RNA classes of WTBS samples summarising methylation candidate sites. Calculation was done by Cassandra Falckenhayn. **b**, WTBS validation using Sanger bisulfite sequencing: Tested methylation candidate sites predicted from single WTBS libraries per genotype. Transcripts with IDs, names (Symbol) and Annotations are listed with predicted candidate sites (position), corresponding amount of sequenced cytosines (Ratio) and coverage (Cov) at the respective position for wild type, *Dnmt2*$^{-/-}$ and *NSun2*$^{-/-}$. Thymines (T) at the annotated position reflects false positive and cytosines (C) true positive predictions. **c**, WTBS validation using 454 bisulfite sequencing: Analysis of the only positively validated methylated cytosine from **(b)**. m5C heatmaps of endoA mRNA in wild type (NSun2+/+) and mutant (*NSun2*$^{-/-}$) third instar larval brains. Columns indicate cytosine residues (Cs), rows single sequencing reads, numbers on the left side represent the coverage. Converted Cs are shown in green and unconverted Cs in red. Arrowheads mark the predicted NSun2-dependent methylation site and the corresponding non-conversion levels [%] reflecting the degree of methylation. **d**, Analysis of the enzyme-specificity of endoA mRNA methylation examining wild type (w^{1118}) two single mutant RNA methyltransferases (*NSun2*$^{-/-}$, *Dnmt2*$^{-/-}$) and the corresponding double mutant (DKO) using Sanger bisulfite sequencing. **e**, Analysis of endoA genomic sequences of genotypes used in **(d)** to exclude polymorphism using Sanger sequencing.

Nonetheless, the existence of the modification site in EndoA mRNA is unambiguous. Regardless whether mRNA methylation is a canonical mechanism or a biological artefact (e.g. caused by structural similarities to confirmed methylation targets), the question arises whether loss of this mRNA methylation has biological consequences.

Endophillin A (EndoA) is an essential presynaptic factor at neuromuscular junctions (NMJ), and the absence of EndoA impairs endocytosis [Verstreken et al., 2002]. Three transcripts of different lengths are annotated and all of them are methylated at the same site (data not shown).

In collaboration with Ine Maes and Patrik Verstreken from Leuven, Belgium, wild type (w^{1118}) and RNA methyltransferase null mutants Dnmt2 (*Dnmt2*$^{-/-}$) and NSun2 (*NSun2*$^{-/-}$) were examined for known EndoA-related phenotypes. (Ine Maes performed all experiments and analyses shown in Figure B.10). Synaptic satellite boutons at the NMJ are a characteristic phenotype observed in EndoA mutant larvae. These small evaginations at the NMJ appear more frequently when EndoA is depleted [Dickman et al., 2006]. Strikingly, significantly increased amounts of satellite boutons were found in NSun2 mutant but not Dnmt2 mutant larvae (Figure B.10 a-b). This phenotype is accompanied

by a significant reduction of EndoA protein levels at the NMJ as quantified by immunofluorescence staining of larval filets (Figure B.10 c-d).

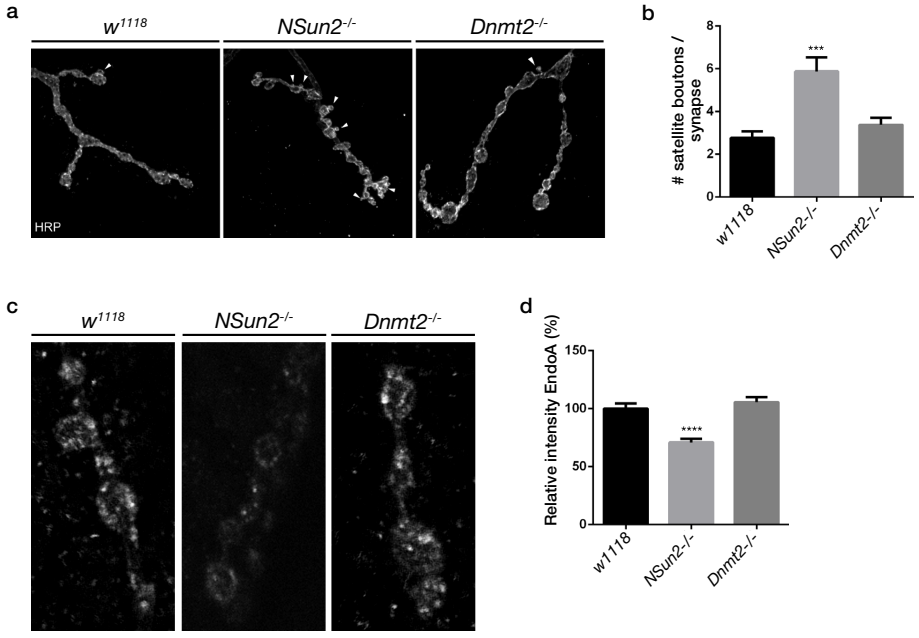


Figure B.10: NSun2 mutant larvae have decreased endoA expression levels, which coincides with endoA phenotypes at neuromuscular junctions (by Ine Maes & Patrik Verstreken). **a-d**, Analysis of neuromuscular junctions (NMJs) from wild type (w^{1118}), NSun2 ($NSun2^{-/-}$) and Dnmt2 ($Dnmt2^{-/-}$) mutant larval filets. **a**, HRP staining of NMJs, arrowheads mark satellite boutons at synapses. **b**, Quantification of number of satellite boutons (one-way Anova, n=24, p>0.05 (ns), p<0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001 (****)). **c**, Immunofluorescence using anti-endoA staining of NMJs. **d**, Quantification of endoA mean intensities reflecting expression levels. 24 images from six larvae were examined (one-way Anova, n=24, p>0.05 (ns), p<0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001 (****)).

The observed phenotype is probably caused by reduced protein levels at NMJs [Dickman et al., 2006]. Possible reasons for this can be altered transcription, transcript processing or stability, disturbed subcellular mRNA localisation, or affected translation. Investigation of polysome profiles revealed that global translation was not affected in NSun2-depleted S2 cells compared to control (Figure B.11 a, c). The fractions of the profiling were collected and the distribution of EndoA mRNA was examined using qPCR. The amount of polysome-associated mRNA remains highly similar from wild type to NSun2 depletion (it may be argued that there is a marginal, and insignificant decrease) (Figure B.11 b). Of note, polysome profiles and qPCR analysis of the EndoA

mRNA distribution are preliminary results from a single experiment and need to be confirmed by repetition.

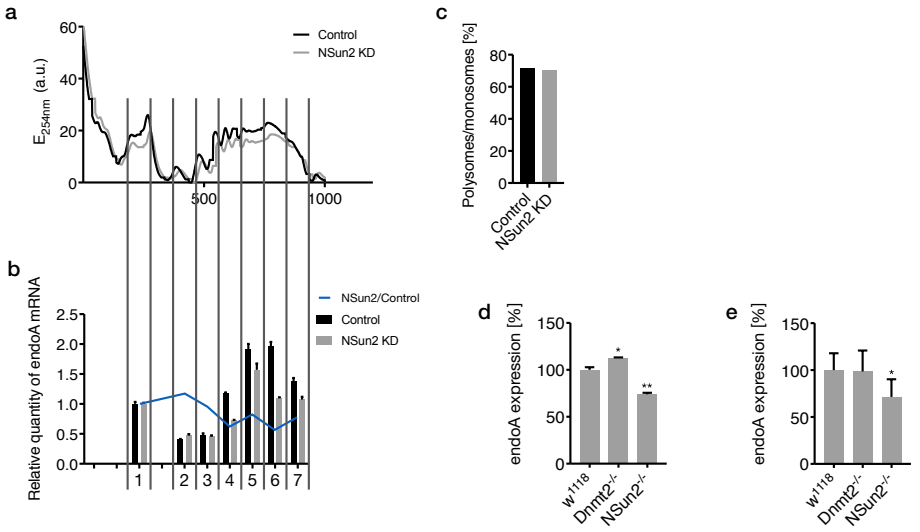


Figure B.11: Overall endoA mRNA levels are reduced in NSun2 mutant larvae. **a-c**, Examination of NSun2-dependent endoA translation in NSun2 knock down (KD) or control Drosophila S2 cells. **a**, Representative polysome profiles. **b**, qPCR analysis of endoA mRNA distribution in selected fractions from polysome profiling. Dashed lines mark used fractions. **c**, Polysome to monosome quantification of shown polysome profiles. **d-e**, qPCR analysis of endoA mRNA levels in wild type (w^{1118}), Dnmt2 ($Dnmt2^{-/-}$), and NSun2 ($NSun2^{-/-}$) mutant third instar larval brains. **d**, Total RNA of 20 pooled larval brains per genotype were analysed each in technical triplicates. (n=3, $Dnmt2^{-/-}$ (p=0.0156), $NSun2^{-/-}$ (p=0.0016), Student's t-test). **e**, Total RNA of five individual brains per genotype were analysed each in technical triplicates. (n=3, $Dnmt2^{-/-}$ (p=0.9102), $NSun2^{-/-}$ (p=0.0117), Student's t-test).

In agreement with reduced protein levels, EndoA mRNA levels were also globally reduced in third instar larval brains. Pools of 20 brains or five individual brains per genotype were analysed using qPCR and revealed a decrease to 71% and 74% of EndoA mRNA in $NSun2^{-/-}$ (Figure B.11 d-e). Whether the reduction is caused by less efficient transcription or decreased transcript stability needs to be examined with further experiments like pre-mRNA qPCR analysis. Single molecule FISH on larval filets may answer whether the subcellular localisation of EndoA mRNA is affected or not.

The Nsun2-dependent methylation of EndoA mRNA appears to be reliable. Further confirmation should be achieved using a rescue fly with transgenic NSun2 in the mutant background. Strikingly, EndoA mRNA remained the

only identified non-tRNA methylation site in this study, which may be very informative for further and prior RNA methylation studies. Molecular biology methods and bioinformatics analyses require positive controls and validations. For bioinformatics approaches, a known methylation site can contribute to find the highest stringency settings without filtering out true positive hits. From a biological point of view, in depth analysis of this particular mRNA methylation may give insights whether 5mC in coding regions influences mRNA transcription, processing, stability, structure, subcellular localisation, protein interactions, or translation. Currently it remains to be determined whether mRNA methylation is a ubiquitous artefact or a rare, tissue- and/or time-dependent phenomenon [Amort et al., 2017].

EndoA may be a highly specific target, i.e. the exception that proves the rule, widespread mRNA methylation however, as for tRNAs, is unlikely. Nevertheless, even if mRNA methylation is a biological artefact, there can be cellular impacts as demonstrated here where NSun2 mutant larvae mimic the EndoA phenotype.

In conclusion, the postulated thousands of 5mC sites within mRNA could not be confirmed here. 5mC mRNA methylation appears to be a very rare event in Drosophila larval brain tissue. Advanced bioinformatic analyses of WTBS data based on replicates and with high statistical power (as described by Legrand et al., submitted), accurate validation including identification of the responsible RNA methyltransferases, and detailed investigation of the molecular function of putative 5mC sites are necessary to reliably evaluate the role of cytosine-5 methylation of mRNA.

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List of Publications

Martin Müller, Mark Hartmann, Isabelle Schuster, Sebastian Bender, Kathrin L. Thüring, Mark Helm, Jon R. Katze, Wolfgang Nellen, Frank Lyko and Ann E. Ehrenhofer-Murray (2015) Dynamic modulation of Dnmt2-dependent tRNA methylation by the micronutrient queuine. Nucleic Acids Res. 43(22):10952-62

Carine Legrand, Francesca Tuorto, Mark Hartmann, Reinhard Liebers, Dominik Jacob, Mark Helm and Frank Lyko (2017) Statistically robust methylation calling for whole-transcriptome bisulfite sequencing reveals distinct methylation patterns for mouse RNAs (submitted)

Mark Hartmann, Merrit Romeike, Sarah Doppler, Chunxuan Shao, Andrea Bergner, Bianca Genenncher, Holger Lorenz, Matthias R. Schäfer, Thomas Höfer, Frank Lyko and Sylvia Erhardt (2017) Centromeric tRNA methylation by Dnmt2 is required for chromosome segregation in mitosis (manuscript in preparation)

Sreemukta Acharya, Mark Hartmann, Sylvia Erhardt (2017) Chromatin-associated non-coding RNA in development and inheritance (working title). WIREs RNA (manuscript in preparation)

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