A systematic dissection of the catalytic and non-catalytic functions of TET1 at the interface of epigenetic regulation

Dissertation

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München, den 17.08.2022

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Paul Stolz

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1. List of Publications

Stolz P, Salazar Mantero A, Tvardovskiy A, Ugur E, Wange LE, Mulholland CB, Cheng Y, Wierer M, Enard W, Schneider R, Bartke T, Leonhardt H, Elsässer SJ, Bultmann S (**2022**). TET1 regulates gene expression and repression of endogenous retroviruses independent of DNA demethylation, **Nucleic Acids Research**, gkac642.

Foster BM, **Stolz P**, Mulholland CB, Montoya A, Kramer H, Bultmann S and Bartke T (**2018**). Critical Role of the UBL Domain in Stimulating the E3 Ubiquitin Ligase Activity of UHRF1 toward Chromatin. **Mol Cell**, 72, 739-752.e9.

Mulholland CB, Nishiyama A, Ryan J, Nakamura R, Yiğit M, Glück IM, Trummer C, Qin W, Bartoschek MD, Traube FR, Parsa E, Ugur E, Modic M, Acharya A, **Stolz P**, Ziegenhain C, Wierer M, Enard W, Carell T, Lamb DC, Takeda H, Nakanishi M, Bultmann S and Leonhardt H (**2020**). Recent evolution of a TET-controlled and DPPA3/STELLA-driven pathway of passive DNA demethylation in mammals. **Nat Commun.**, 11, 5972.

Mulholland CB, Traube FR, Ugur E, Parsa E, Eckl EM, Schönung M, Modic M, Bartoschek MD, **Stolz P**, Ryan J, Carell T, Leonhardt H and Bultmann S (**2020)**. Distinct and stage-specific contributions of TET1 and TET2 to stepwise cytosine oxidation in the transition from naive to primed pluripotency. **Sci. Rep.**, 10, 12066.

Ignatova VV, **Stolz P**, Kaiser S, Gustafsson TH, Lastres PR, Sanz-Moreno A, Cho YL, Amarie OV, Aguilar-Pimentel A, Klein-Rodewald T, Calzada-Wack J, Becker L, Marschall S, Kraiger M, Garrett L, Seisenberger C, Hölter SM, Borland K, Van De Logt E, Jansen PWTC, Baltissen MP, Valenta M, Vermeulen M, Wurst W, Gailus-Durner V, Fuchs H, de Angelis MH, Rando OJ, Kellner SM, Bultmann S and Schneider R (**2020**). The rRNA m6A methyltransferase METTL5 is involved in pluripotency and developmental programs. **Genes Dev.**, 34, 715-729.

Ignatova VV, Kaiser S, Ho JSY, Bing X, **Stolz P**, Tan XY, Lee CL, Gay FPH, Lastres PR, Gerlini R, Rathkolb B, Aguilar-Pimentel A, Sanz-Moreno A, Klein-Rodewald T, Calzada-Wack J, Ibragimov E, Valenta M, Lukauskas S, Pavesi A , Marschall S, Leuchtenberger S, Fuchs H, Gailus-Durner V, de Angelis MH, Bultmann S, Rando OJ, Guccione E, Kellner SM and Schneider R (**2020**). METTL6 is a tRNA m3C methyltransferase that regulates pluripotency and tumor cell growth. **Sci Adv.**, 6, eaaz4551.

2. Summary

Mammalian DNA consists of millions of base pairs. To fit the DNA into the nucleus of a cell the DNA is condensed into chromosomes. The DNA is wrapped around an histone octamer forming nucleosomes which are the basis of chromatin. Chromatin is the physiological form of DNA and altered by various epigenetic mechanisms, such as DNA methylation, histone modifications and RNA modifications. During development epigenetic mechanisms drive lineage choices and cell identity. In this doctoral work we used next generation sequencing and CRISPR/Cas9 genome engineering to study different epigenetic mechanisms in mESCs. The main focus was to study the non-catalytic functions of the DNA demethylase TET1. I have found that TET1 is involved in heterochromatin formation and retroviral silencing independent of DNA demethylation (Publication I). In the scope of this doctoral work I contributed to other projects with the focus on epigenetic proteins in mESCs. First, description of the role of the UBL domain of UHRF1 in the interplay with DNMT1 and DNA maintenance methylation (Publication II). Second, identification of DPPA3 as a regulator of UHRF1 and critical for global DNA demethylation (Publication III). Third, discovery of TET1 and TET2 stage-specific roles in DNA demethylation during early embryonic development (Publication IV). Last, in the scope of this doctoral work I contributed to study the role and function of the novel RNA methyltransferases METTL5 and METTL6 in the field of epitranscriptomics. METTL5 was found to be a specific ribosomal RNA methyltransferase critical for pluripotency and differentiation (Publication V). METTL6 was discovered as a transfer RNA methyltransferase involved in cancer (Publication VI). In summary, this doctoral work investigated and described novel non-catalytic mechanisms of TET1 and studied various epigenetic modifiers and mechanisms at different epigenetic levels.

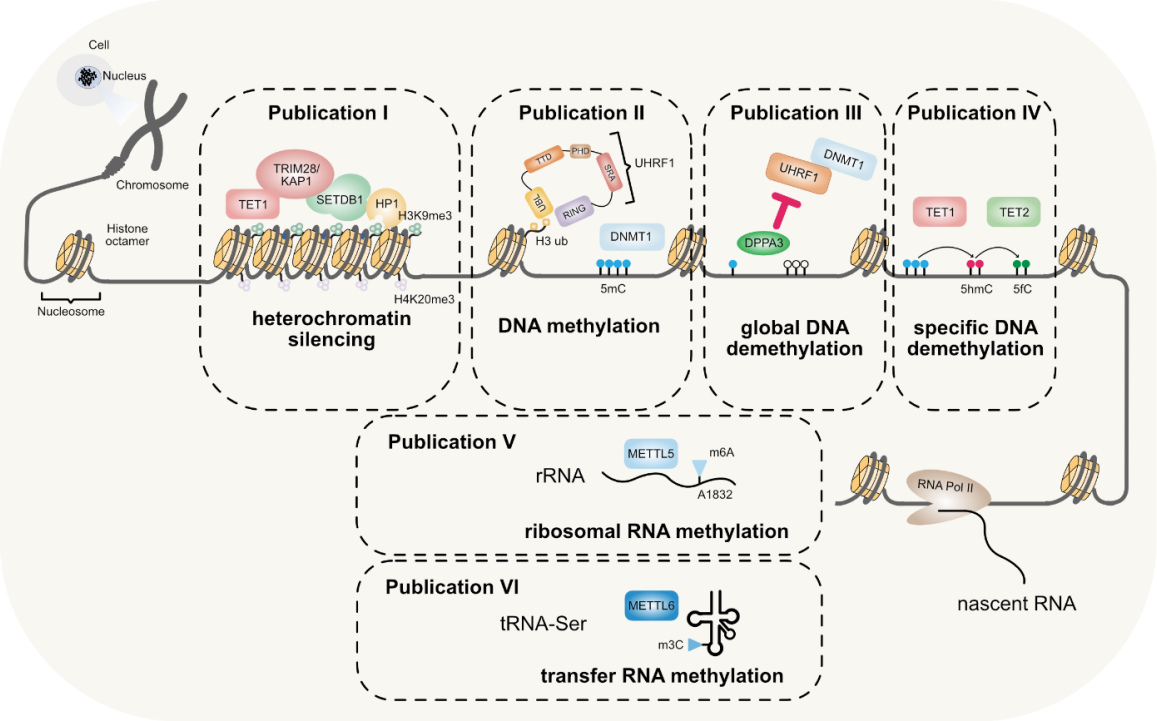


Figure 1. Graphical Summary. In eukaryotic cells, the DNA is wrapped around histones to form nucleosomes, which are the basis of chromatin and build the framework of transcriptional control. Chromatin can be regulated by various epigenetic mechanisms, like DNA and RNA methylation. Both are key epigenetic mechanisms and regulate multiple processes in a cell. In the scope of this doctoral work, functions and mechanisms of DNA and RNA methylation were investigated at different levels of chromatin regulation. TET1 mediates heterochromatin silencing independent of DNA demethylation (Publication I). UBL and RING domain of UHRF1 regulate the ubiquitination activity and therefore DNMT1 activity (Publication II). DPPA3 controls a pathway of passive global DNA demethylation (Publication III). TET1 and TET2 act distinctly and stage specific during stepwise DNA oxidation (Publication IV). The rRNA m^6A methyltransferase METTL5 regulates pluripotency and differentiation (Publication V). METTL6 is a m3C tRNA methyltransferase regulating pluripotency and tumor formation (Publication VI).

3. Introduction

3.1 Introduction into epigenetics

Already in the late 19th century, nuclear structures were described by Walther Flemming using microscopy. Thereby he coined the terms chromatin and chromosome, setting the foundation of the epigenetic field (Flemming, 1882). Remarkably, around the same time also histone proteins were discovered and described to associate with deoxyribonucleic acid (DNA) (Kossel, 1884). Following studies demonstrated that chromosomes are the carrier of genetic material (Morgan, 1911; Sturtevant, 1913) and work in maize and fruit flies provided the first important hints on non-mendelian inheritance (McCLINTOCK, 1951; Muller and Altenburg, 1930). Yet, it remained unclear how the information is transferred during cell division and how the developmental program is defined. While knowing very little about the mechanism, Conrad Hal Waddington in 1942 coined the term “epigenetics” and later also the term “epigenetic landscape” to describe phenotypic changes that are independent of genetic inheritance (Waddington, 1957, 2012). In sum, leading to the key question of the epigenetic field: How can a single fertilized egg give rise to a complex multicellular organism?

Today, epigenetic mechanisms are defined to influence gene function in a hertible fashion without altering the DNA sequence. Different epigenetic mechanisms can form chromatin, the physiological form of genetic information, to assist the DNA template and maintain or alter gene expression profiles. This way epigenetic mechanisms shape the identity of a cell. The building blocks of epigenetic mechanisms are histone variants, posttranslational modifications of amino acids (aa) of histone tails and covalent DNA modifications. Recently, also post-transcriptional modifications of ribonucleic acids (RNAs) are considered as “RNA epigenetics” or “epitranscriptomics” (He, 2010; Meyer and Jaffrey, 2014; Wiener and Schwartz, 2021).

Since Waddington introduced the term epigenetics 70 years ago, the field developed tremendously. Technology advances as next-generation sequencing (NGS) and genome engineering approaches like Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas allowed to profile the epigenome and to identify epigenetic reader, writer and eraser proteins. These proteins and epigenetic mechanisms have multiple critical roles in genome stability, cellular identity and in

development and disease throughout the animal kingdom. Only a detailed understanding of these epigenetic mechanisms will allow the development of new drugs and medicine to treat diseases.

3.2 Epigenetics and chromatin

3.2.1. Organization of DNA

In eukaryotes the DNA is compacted into chromatin. More precisely, the DNA of a single human cell entails around 3 billion base pairs (bp) and is organized in 23 pairs of chromosomes. To fit into the nucleus the approximately 2 m of double stranded DNA are tightly packed and organized in nucleosomes. The nucleosome core consists of 147 bp DNA wrapped around a histone octamer (Kornberg and Lorch, 1999; Luger et al., 1997; Richmond and Davey, 2003). Nucleosomes can be tightly placed together or arranged with greater distance to each other, which determines the accessibility of DNA. This organization of nucleosomes, also referred to as “arrays of nucleosomes” are the basic element of a chromatin structure and can form a chromatin fiber, which can condense to multiple higher order chromatin structures up to chromosomes (Luger and Hansen, 2005; Olins and Olins, 1974; Oudet et al., 1975; Woodcock et al., 1976). The most frequent state of chromatin is heterochromatin which is a condensed form of chromatin that is associated with transcriptionally inactive DNA. Heterochromatin marks repetitive regions and is known to be important for viral silencing. Accessible and transcriptionally active DNA regions on the contrary are called euchromatin and are mostly found at gene rich regions. All chromatin structures are shaped and maintained by different chromatin remodelers, transcription factors and epigenetic modifiers, like histone, DNA and RNA modifying enzymes.

3.2.2. Histone variants and modifications

Histones are fundamental proteins to build and alter chromatin structures. The four core histones are H3, H4, H2A and H2B and are highly conserved among eukaryotes. Two dimers of H2A-H2B and an H3-H4 tetramer represent the center of a nucleosome. In addition, in many eukaryotes linker Histone H1 is critical to form higher order chromatin structures (Allan et al., 1981; Fan et al., 2005; Geeven et al., 2015). Histone H1 binds and protects free linker DNA in between individual nucleosomes (Brockers

and Schneider, 2019). Histone proteins diversify into different variants, sometimes with only a few bp altered, but harboring individual functions. For example, H2A.X is involved in double-strand break (DSB) repair (Morrison and Shen, 2005), whereas H2A.Z seems to counteract heterochromatin silencing (Draker and Cheung, 2009). Another example is the H3 variant Centromere Protein A (CENP-A), which replaces canonical H3 specifically at centromeric chromatin (Palmer et al., 1991). Chromatin is not only altered by introducing different variants of histones, but histones are also decorated with post-translational modifications (PTMs) at their N-terminal tails or global core domains. The most common histone PTMs are methylation, acetylation, phosphorylation and ubiquitylation and can occur at different aa residues. Yet, even today new histone PTMs are constantly discovered, e.g. serotonylation and dopaminylation (Farrelly et al., 2019; Lepack et al., 2020). Every histone PTM is suggested to have an individual function (Jenuwein and Allis, 2001; Strahl and Allis, 2000). The interplay of histone writer, reader and eraser proteins, can shape the structure of chromatin and hence modify the activity of the underlying gene sequence. Of note, while the activity of a DNA sequence can be influenced by histones and their modifications, DNA modifications can in turn also influence the histones landscape.

3.2.3. DNA modifications

In 1948, the first chemically modified DNA base was detected in higher organisms using paper chromatography (Hotchkiss, 1948). Today, around 40 verified DNA modifications have been detected throughout the animal kingdom (Sood et al., 2019). The majority is associated with DNA damage and DNA repair pathways, but harbor the potential for additional biological functions and implications (Ito and Kuraoka, 2015; Zhu et al., 2018b). Especially, 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), 5-carboxylcytosine (5caC), 5-hydroxymethyluracil (5hmU) and N6-methyladenine (6mA) are known for their biological significance (Zhu et al., 2018b). The most prominent DNA modification is 5mC, which was already connected to gene regulation and development in the 1970’s (Holliday and Pugh, 1975). DNA modifications are known to interact with histone modifications and vice versa. In addition, DNA modifications influence genome stability, facilitate protein-DNA interactions, regulate DNA accessibility and gene expression. Similar to histone modifications, DNA modifications are installed by an interplay of writer, reader and

eraser proteins. In mammalian development DNA modifications undergo waves of remodeling and can shape chromatin structures. The predominant covalent DNA modification in mammals is 5mC, which is heavily remodeled during embryonic development and 5mC deficiencies are a hallmark of many diseases (Robertson, 2005).

3.2.4. RNA modifications

Not only DNA and histones are known to carry various modifications, but also modified RNA molecules were discovered in 1960 (Cohn, 1960). Until today more than 150 RNA modifications were identified in bacteria, eukaryotes and archaea (Boccaletto et al., 2022). Various modifications are decorating coding messenger RNA (mRNA) as well as non-coding RNAs, like transfer RNA (tRNA) and ribosomal RNA (rRNA) (Cantara et al., 2011; Höfer and Jäschke, 2018; Machnicka et al., 2013). This creates a great level of complexity in untangling the role and function of individual RNA modifications. However, in the last decade the field of “RNA epigenetics” or “epitranscriptomics” increased our understanding of individual RNA modifications and their cellular function rapidly (Schwartz, 2016). The most abundant mRNA modification is *N*6-methyladenosine (m^6A), which was detected in yeast, *Drosophila*, plants and mammals to affect folding, stability and degradation (Clancy, 2002; Frye et al., 2018; Levis and Penman, 1978; Nichols, 1979; Wei et al., 1975). tRNAs display the greatest diversity (>100) and amount of modified nucleotides among the different RNA classes. In humans single tRNAs can carry in between 11-13 different RNA modifications which can all affect translation (Jackman and Alfonzo, 2013). In comparison, rRNA only harbors a limited set of RNA modifications, but with relatively high abundance and important function for the ribosome (Sloan et al., 2017). rRNA modifications stabilize functional rRNA structures of the ribosome and are critical for high fidelity protein synthesis (Anderson et al., 2011; Polikanov et al., 2015). In summary, the various RNA modifications can impact multiple cellular functions on different levels, for example protein stability and folding, gene expression, splicing, localization and translation initiation (Decatur and Fournier, 2002; Pan, 2018; Roundtree et al., 2017).

3.3. DNA methylation

3.3.1. DNA methyltransferases

In mammals DNA methylation occurs predominantly at the 5th carbon of cytosine (5mC) and at much lower frequency also at adenine and guanine (Alderman and Xiao, 2019; Greenberg and Bourc'his, 2019) (Figure 2a). The first DNA methyltransferase to be purified and cloned was DNA methyltransferase 1 (DNMT1), which associates with the replication fork and maintains the methylation pattern during replication (Goll and Bestor, 2005a; Leonhardt et al., 1992a). Therefore, DNMT1 is historically perceived as the maintenance methyltransferase (Goll and Bestor, 2005a) (Figure 2a). Only recently, DNMT1 was shown to also have *de novo* methylation activity at intracisternal A particles (IAP) retrotransposons (Haggerty et al., 2021). The major *de novo* methyltransferases however are DNMT3A and DNMT3B (Figure 2a), which are crucial for DNA methylation remodeling during early mammalian development (Okano et al., 1998, 1999). Both *de novo* methyltransferases have an autoinhibitory domain and upon binding of unmethylated H3K4 become activated for *de novo* DNA methylation (Guo et al., 2015; Ooi et al., 2007; Otani et al., 2009; Zhang et al., 2010). In addition to the three major DNMTs, mammals express one catalytically dead DNA methyltransferase (DNMT3L), which facilitates *de novo* methylation in the germline, mouse embryonic stem cells (mESCs) and the early embryo (Bourc'his et al., 2001a; Hata et al., 2002; Ooi et al., 2007). Of note, in rodents DNMT3C evolved from a duplication of DNMT3B and methylates retrotransposons in the male germ line (Barau et al., 2016).

While in vertebrates 5mC is present in the whole genome, non-vertebrates exhibit 5mC only at certain genomic elements (Suzuki and Bird, 2008). Most eukaryotes carry methylated cytosine, some prominent exceptions are model organisms like *Saccharomyces cerevisiae*, *Drosophila melanogaster* and *Caenorhabditis elegans*, who lack DNA methylation (Goll and Bestor, 2005b; Rae and Steele, 1979; Simpson et al., 1986a). This genome-wide absence of 5mC highlights that DNA methylation is not essential to every organism. In mammals, however DNA methylation is crucial for imprinting, X inactivation and silencing of repeat elements (Greenberg and Bourc'his, 2019; Schübeler, 2015) and the loss of DNMTs causes developmental defects or embryonic lethality (Bourc'his et al., 2001b; Damelin and Bestor, 2007; Li et al., 1992; Okano et al., 1999; Takebayashi et al., 2007).

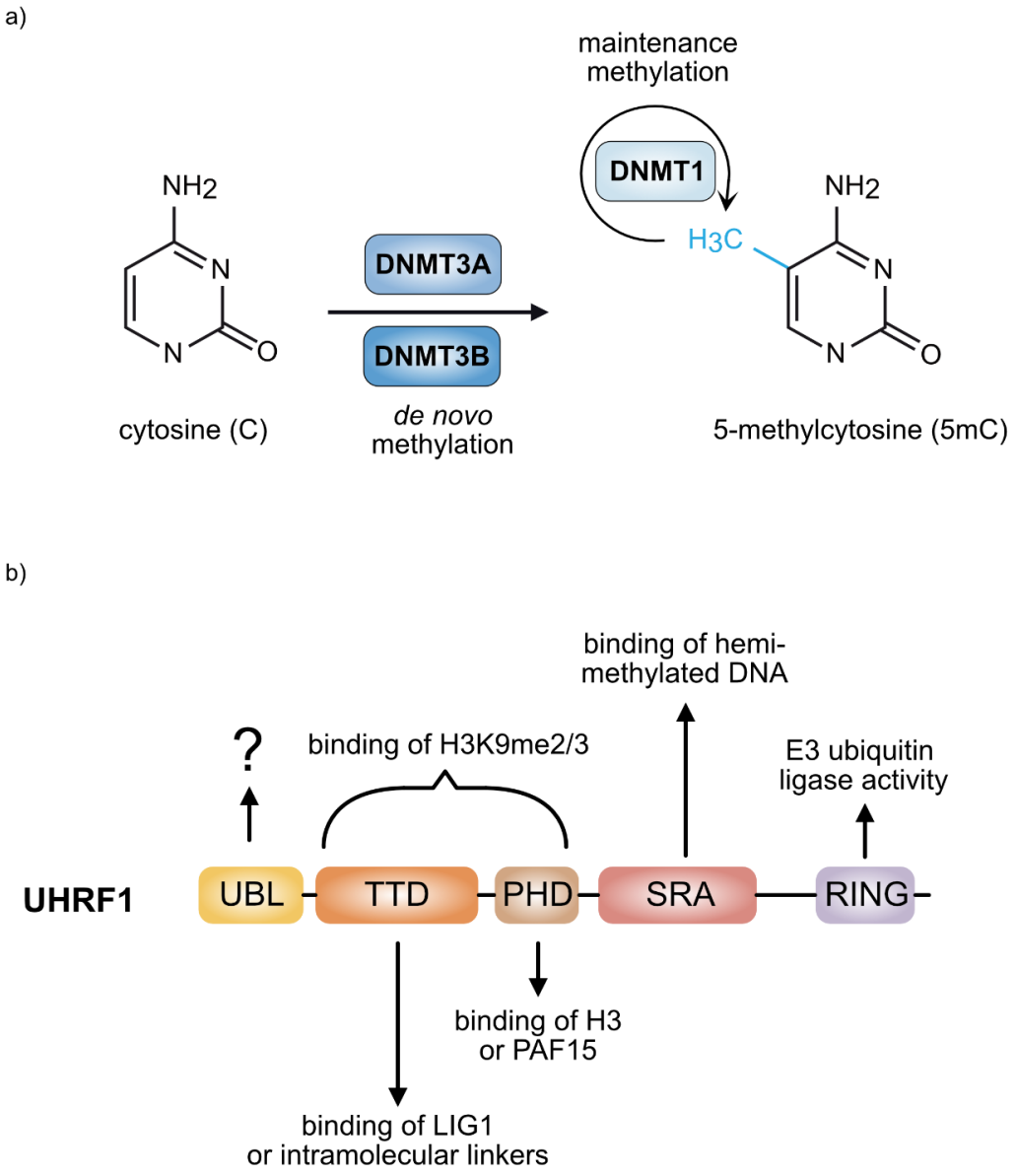


Figure 2. DNA methylation and UHRF1. a) In mammals, methylation occurs only at the 5th carbon of cytosine, converting unmodified cytosine to 5-methylcytosine. DNMT3A and DNMT3B are responsible for *de novo* methylation and DNMT1 for maintenance methylation. b) Functional description of the mouse UHRF1 domains. Ubiquitin-Like domain (UBL); Tandem Tudor Domain (TTD); Plant Homeodomain (PHD); SET and Ring-Associated domain (SRA); Really Interesting New Gene domain (RING) with E3 ligase activity.

3.3.2. UHRF1

The process of DNA methylation maintenance during replication is very complex and is enabled through an interplay of different factors. For example, various post-translational modifications of DNMT1 can regulate its enzymatic activity and protein stability (Du et al., 2010; Estève et al., 2009, 2011; Leng et al., 2018; Zhang et al., 2019). In addition, a critical regulator of DNMT1 activity at the replication fork is the multidomain protein Ubiquitin Like With PHD And Ring Finger Domains 1 (UHRF1). UHRF1 can recruit DNMT1 to ubiquitinated H3 tails and induce the methylation of the daughter DNA strand during replication (Petryk et al., 2021). In detail, the E3 ubiquitin ligase UHRF1 consists of its Ubiquitin-like (UBL) domain, the Tandem Tudor Domain (TTD), the Plant Homeo Domain (PHD) and the SET and RING finger-associated (SRA) domain (Figure 2b). The different domains of UHRF1 harbor individual and collaborative functions in mediating DNA and protein binding. The SRA domain predominantly binds hemimethylated DNA, but also methylated or unmethylated DNA, albeit with lower affinity (Bostick et al., 2007; Schneider et al., 2020; Sharif et al., 2007). The TTD and PHD cooperate to bind H3K9me2/me3 (Arita et al., 2012a; Rothbart et al., 2013). Of note, the cooperative binding of hemimethylated DNA and methylated H3K9 is required for DNA methylation maintenance (Liu et al., 2013). Furthermore, the PHD and TTD also harbor individual functions. The PHD can bind a specific peptide sequence at the amino-terminus (N-terminus) of H3 and also a very similar peptide sequence of the PCNA-associated factor 15 (PAF15). During replication UHRF1 ubiquitinates both H3 and PAF15, which is essential for DNMT1 recruitment (Nishiyama et al., 2020). However, it remains unknown how UHRF1 ubiquitinates H3 and as part of this doctoral work we addressed this question (Publication II). The TTD can bind two linker regions within UHRF1 or the methylated histone mimic DNA Ligase 1 (LIG1) (Figure 2b). While the intramolecular interaction inhibits UHRF1 recruitment, the methylation of LIG1 promotes the recruitment of UHRF1 to the replication fork (Ferry et al., 2017). Importantly, the multilayered function and regulation of UHRF1 must be tightly controlled to ensure DNA methylation maintenance. In this context, UHRF1 stability and/or activity is additionally regulated by post-translational modifications (Kori et al., 2020; Ma et al., 2012; Yang et al., 2017; Zhang et al., 2016a, 2019). An intriguing but not fully understood question is how the DNMT1-UHRF1 machinery contributes to changes in DNA methylation levels at

different stages during development. In primordial germ cells (PGCs), UHRF1 is not expressed and DNMT1 is not recruited to the replication fork, suggesting that the lack of maintenance methylation also contributes to DNA demethylation (Ohno et al., 2013). In oocytes, DNMT1 and UHRF1 are expressed and even have *de novo* methylation activity (Li et al., 2018; Maenohara et al., 2017). Intriguingly, the activity of DNMT1-UHRF1 in oocytes is controlled by Developmental pluripotency-associated protein 3 (DPPA3) via disrupting UHRF1 accumulation in the nucleus and facilitating nuclear export (Li et al., 2018). This brings up the question whether DPPA3 controls UHRF1 mediated hypomethylation also at other stages during development, like in naive mESCs (Graf et al., 2017; von Meyenn et al., 2016). As part of this doctoral work, I contributed to investigate the role of the UBL domain of UHRF1 during replication (Publication II) and the mechanisms of DPPA3 in regulating UHRF1 in naive mESCs and in ESCs of different species (Publication III).

3.3.3. DNA methylation during development

How a complex organism arises from a single fertilized cell, is one of the most fascinating questions in biology. To answer this question, it is critical to understand the basic mechanisms and processes occurring during embryonic development. In mammalian development epigenetic modifiers play a crucial role, documented by the mouse embryonic lethal phenotypes of DNA methyltransferases (DNMTs) (Li et al., 1992; Okano et al., 1999), ten-eleven translocation (TET) enzymes (Dawlaty et al., 2014; Khoueiry et al., 2017), polycomb repressive complex 2 (PRC2) (O'Carroll et al., 2001; Pasini et al., 2004; Shen et al., 2008) and histone methyltransferases (HMTs) (Bilodeau et al., 2009; Dodge et al., 2004; Tachibana, 2002; Tachibana et al., 2005). During the early development of mammals, pluripotent stem cells transition into the progenitor of a whole organism and it is critical to understand how the different epigenetic mechanisms are driving lineage choices in development. Importantly, DNA methylation dynamics are a hallmark of embryonic development and are represented by two epigenetic remodeling events and a *de novo* methylation wave in mammals.
The two remodeling events are characterized by an erasure of DNA methylation and occur at fertilization and upon PGC arrival in the gonads (Eckersley-Maslin et al., 2018) (Figure 3). In mice, X inactivation becomes initiated and completed during the transition to the zygote. From the zygote stage on, the mouse embryo undergoes

genome-wide DNA demethylation until reaching the morula stage to restore totipotency and perhaps to erase epimutations (Skvortsova et al., 2018). The second epigenetic remodeling event occurs between embryonic day 10.5 (E10.5) and E13.5 when PGCs colonize the gonads (Seisenberger et al., 2012) (Figure 3). The wave of DNA demethylation in PGCs is important to erase and restore imprints, so that later they can be established according to the sex of the germline (Smallwood et al., 2011; Wang et al., 2014a). Notably, the genome-wide DNA demethylation in PGCs seems to be absent in non-mammalian species (Macleod et al., 1999). Yet, a recent study suggests that in zebrafish embryos DNA methylation is maintained at CpG rich adult enhancers to avoid premature activation of lineage choices (Wu et al.).

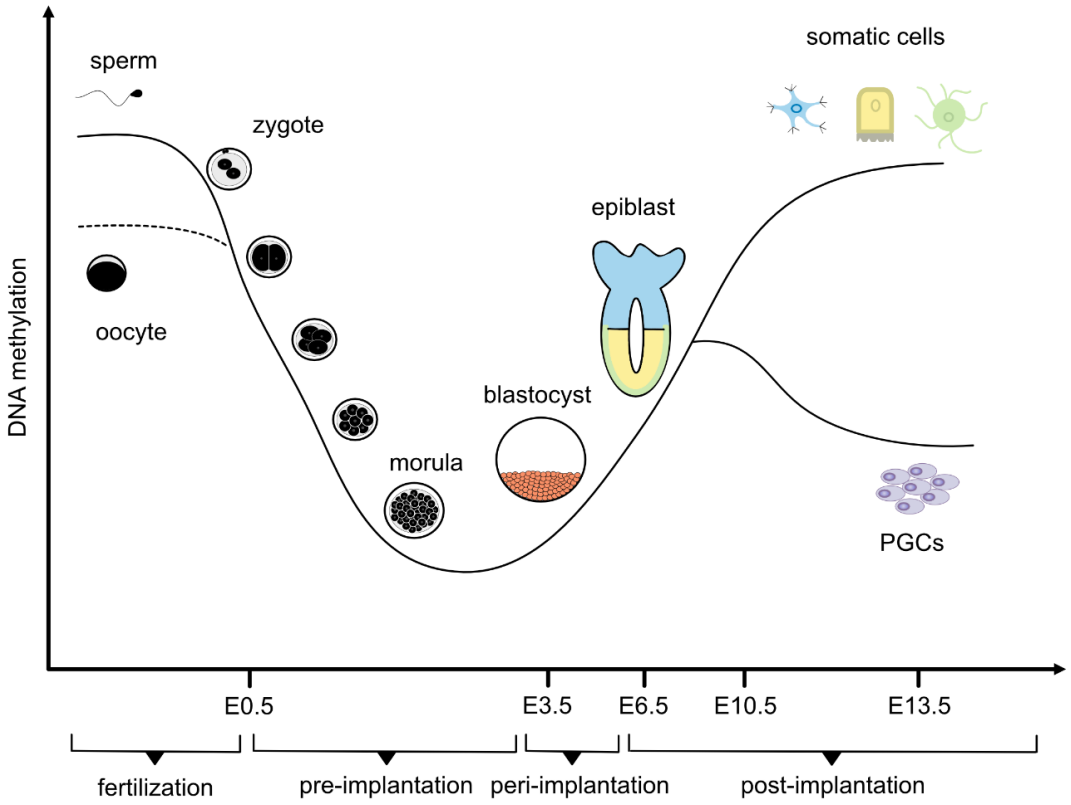


Figure 3. DNA methylation dynamics during mouse embryonic development. The fertilized zygote undergoes active and passive DNA demethylation until reaching the lowest point of DNA methylation levels at the morula stage. After blastocyst implantation the embryo gains DNA methylation until reaching a plateau in differentiated somatic cells. In contrast, primordial germ cells (PGCs) undergo a wave of DNA demethylation from E10.5 until E13.5.

Upon implantation the inner cell mass (ICM) of the blastocyst starts to undergo a wave of *de novo* DNA methylation (Borgel et al., 2010; Kafri et al., 1992; Smith et al., 2012)

(Figure 3). This marks the first differentiation event in the early embryo and is crucial for maintaining lineage stability and identity of embryonic and extraembryonic tissues. As an exception, the progenitors of the placenta, the trophoblast stem cells remain hypomethylated (Santos et al., 2002). Interestingly, in plants the endosperm also remains hypomethylated in contrast to the plant embryo (Hsieh et al., 2009), pointing towards a distinct and conserved mechanism across plants and animals. A popular system to study DNA methylation and other epigenetic mechanisms are mESCs, which cultured *in vitro* reassemble the ICM of the blastocyst and can be differentiated into various developmental stages of embryonic development. In addition, mESCs are convenient to manipulate using CRISPR-Cas and represent a well established system to study the function of the different epigenetic modifiers, as utilized in this doctoral work (Publication I - VI).

3.4. DNA demethylation

3.4.1. TET enzymes

While the role and function of DNA methylation has been intensively studied, the knowledge about DNA demethylation remained rudimentary for a long time. In 2009, TET proteins were identified as 2-oxoglutarate (2OG)- and Fe(II)-dependent enzymes, catalyzing the conversion of 5mC to 5hmC (Ito et al., 2010; Tahiliani et al., 2009). Originally, TET1 was discovered as a fusion partner of the mixed lineage leukemia (MLL) gene in acute myeloid leukemia associated with a specific translocation between chromosome 10 and 11 (Lorsbach et al., 2003; Ono et al., 2002). Subsequent studies found that TET enzymes can successively catalyze the oxidation of 5mC to 5hmC, 5fC and 5caC (He et al., 2011; Ito et al., 2011; Pfaffeneder et al., 2011). Those new cytosine derivatives have individual biological functions (Hashimoto et al., 2014; Mellén et al., 2012; Pastor et al., 2011; Raiber et al., 2015; Spruijt et al., 2013) and on the other hand can serve as an intermediate for active DNA demethylation via Thymine DNA glycosylase (TDG) and the base excision repair (BER) pathway (He et al., 2011; Maiti and Drohat, 2011) (Figure 4a). The finding of active DNA demethylation via TET enzymes revolutionized the view on DNA methylation, which was long perceived as a stable repressive mark.

In plants active DNA demethylation is mediated by repressor of silencing 1 (ROS1) and ROS1-like 5mC DNA glycosylases (DME2 and 3) (Zhu, 2009). In mammals, DNA

demethylation can be achieved actively via TET enzymes and passively by diluting 5mC during replication. Mammals harbor three TET proteins, TET1, TET2 and TET3 (Figure 4b). Their orthologues can be found throughout metazoans and homologous domains also exist in fungi and algae (Tahiliani et al., 2009). The three TET proteins are iron(II)/α-ketoglutarate (Fe(II)/α-KG)-dependent dioxygenases. They harbor a conserved catalytic domain at the carboxyl terminus which is composed of a double-stranded β-helix (DSBH) domain and a cysteine-rich domain. The DSBH contains the metal-binding residues typical for the family of Fe(II)/α-KG- dependent oxygenases, which are crucial for the hydroxylation process (Loenarz and Schofield, 2011). In contrast to the catalytic domain, the N-terminus is not conserved among the three TET proteins (Figure 4b). In vertebrates, the N-terminus of TET1 and TET3 possess a CXXC domain, whereas TET2 is lacking this part. During evolution a chromosomal inversion split the *Tet2* gene into two parts, the CXXC and the catalytic domain. The part of the *Tet2* gene encoding the CXXC domain encodes for the gene *IDAX4*, which interacts with and mediates the activity of TET2 (Ko et al., 2013).

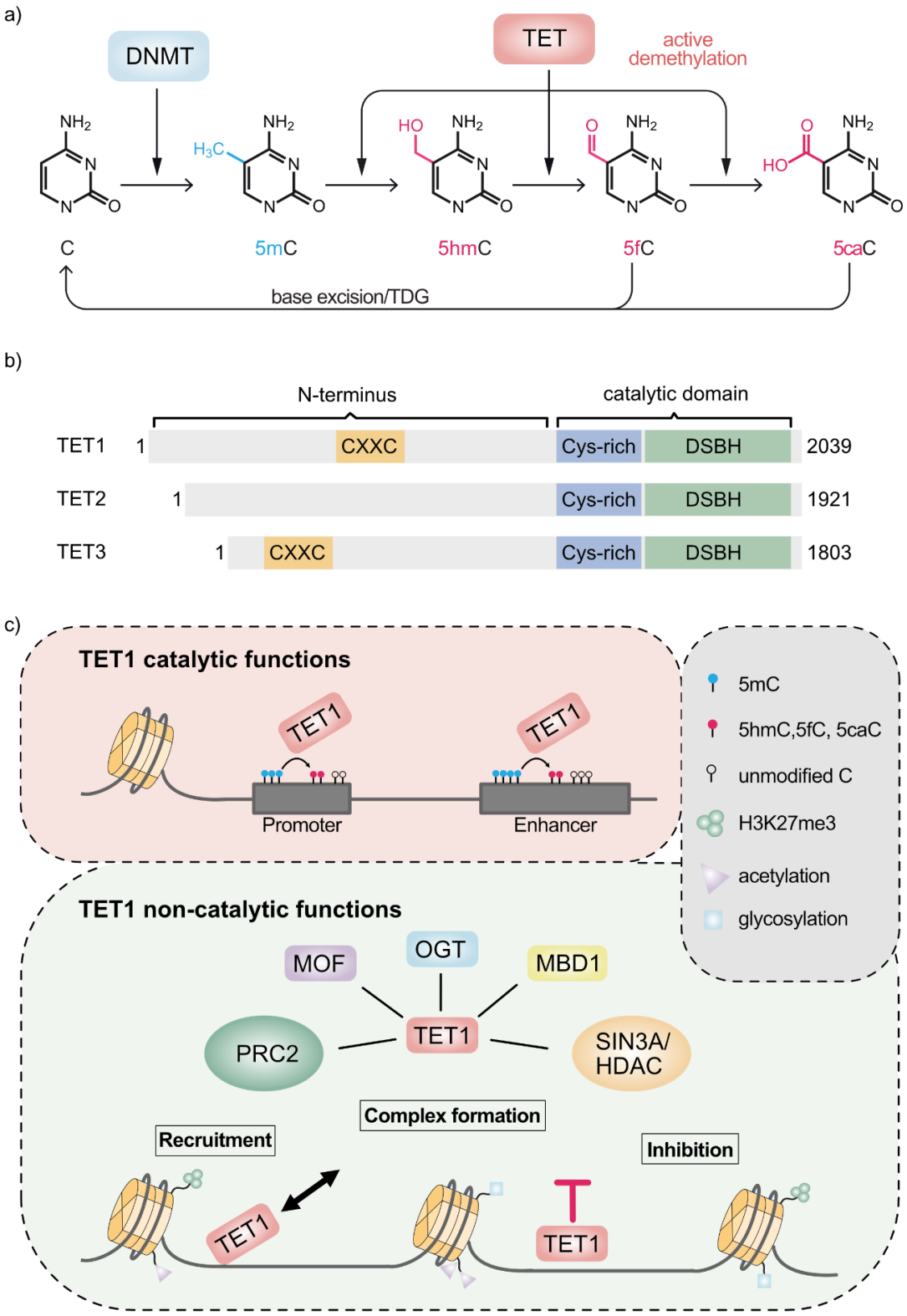


Figure 4. The TET family and TET1 catalytic and non-catalytic functions. a) DNA methyltransferase enzymes (DNMTs) install 5-methylcytosine (5mC). The family of TET enzymes promotes active DNA demethylation by successive oxidation of 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). The latter two can be excised and replaced with unmodified Cytosine (C) by

TDG and the base excision repair (BER) pathway. b) Schematic representation of the three mouse TET enzymes. The catalytic domain comprises a cysteine (Cys)-rich region and a double-stranded beta helix (DSBH) domain. The N-terminus of TET1 and TET3 harbors a CXXC zinc finger domain. Numbers indicate amino acids (aa). c) Representation of catalytic and potential non-catalytic functions of TET1 in mESCs. TET1 oxidizes 5mC to 5hmC, 5fC and 5caC mainly at promoters and enhancers. Besides its catalytic functions TET1 associates with multiple chromatin modifiers, like PRC2, MOF, OGT, MBD1 and SIN3A/HDAC. These complexes establish different chromatin marks like histone methylation, acetylation and glycosylation. TET1 might interact at different levels with the chromatin modifying proteins, e.g. recruitment to certain loci, mediating complex formation or inhibition.

3.4.2. TET enzymes during development

In vertebrates, DNA demethylation activity of TET enzymes at thousands of enhancers is crucial for body plan formation and organ development (Bogdanović et al., 2016). The three TET proteins are expressed during different stages of the embryonic development. TET1 is most abundant in primordial germ cells and the ICM of the blastocyst. Interestingly, TET proteins exist in different isoforms in mammalian development. In mESCs and PGCs, TET1 is expressed in its full form whereas in somatic cells TET1 is expressed lacking the N-terminus (Zhang et al., 2016b). TET3 is the only TET enzyme highly expressed in the zygote. In general, TET3 exists in three different isoforms, from which two variants, short TET3 (TET3s) and oocyte TET3 (TET3o), are lacking the CXXC domain. The full-length form of TET3 and TET3s, are expressed during neuronal differentiation, whereas TET3o is specifically expressed in oocytes (Jin et al., 2016). In the paternal pronucleus TET3 is crucial for the oxidation of 5mC (Gu et al., 2011; Wossidlo et al., 2011), while the maternal pronucleus exhibits much less oxidation derivatives of 5mC (Inoue et al., 2011; Iqbal et al., 2011; Wossidlo et al., 2011). In the maternal pronucleus, two DPPA3 mediated mechanisms can protect 5mC from oxidation. First, DPPA3 was shown to inhibit TET3 binding to H3K9me2 (Nakamura et al., 2012) and second, DPPA3 was suggested to inhibit the catalytic activity of TET3 (Bian and Yu, 2014). The loss of TET3 leads to embryonic defects (Gu et al., 2011) or neonatal sub-lethality (Inoue et al., 2015; Tsukada et al., 2015). However, it remains unclear if these defects are caused by reduced TET3 levels due to haploinsufficiency or the lack of TET3 mediated 5mC

oxidation (Inoue et al., 2015). In summary, the question about the important function of TET3 in preimplantation development remains elusive.

TET3 and TET1 were both reported to have important neural functions (Rudenko et al., 2013; Yu et al., 2015; Zhang et al., 2013; Zhu et al., 2016). In the brain, 5hmC, 5fC and 5caC were all found at relatively high levels (Ito et al., 2011; Kriaucionis and Heintz, 2009; Münzel et al., 2010) suggesting that the activity of TET enzymes could have critical implications for the adult brain. However, it is difficult to assign the neural defects caused by the loss of TET1 or TET3 to catalytic or non-catalytic activities (Kaas et al., 2013; Wu and Zhang, 2017). Future studies will be necessary to dissect the catalytic and non-catalytic role of TET1 and TET3 in neural function.

TET2 is expressed at the blastocyst stage and during PGC development. At both developmental stages TET1 is also expressed, yet TET2 and TET1 seem to have distinct functions (Huang et al., 2014) (Publication IV). While TET1 preferentially binds promoters in mESCs, TET2 targets gene bodies and enhancers (Hon et al., 2014; Huang et al., 2014). Here, TET2 maintains low methylation levels to keep enhancers and genes active for differentiation (Hon et al., 2014).

Whether TET enzymes are essential for mammalian development in the end is subject of controversial discussions. Different studies suggest that the loss of TET1 or TET2 does not trigger abnormalities in growth or lethality (Dawlaty et al., 2011; Li et al., 2011; Moran-Crusio et al., 2011). In contrast, TET triple knockout (TKO) mice are impared in embryonic development (Dawlaty et al., 2014). E7.5 TET TKO embryos show gastrulation defects caused by abnormal DNA methylation, directly deregulating Lefty-Nodal signaling (Dai et al., 2016).

These findings imply that TET1 and TET2 are non-essential for embryonic development, whereas the loss of TET3 causes severe embryonic defects (Gu et al., 2011). However, in 2017 the Koh lab reported post-implantation lethality of non-inbred homozygous TET1 KO mice (Khoueiry et al., 2017). Khoueiry et al. claimed that other studies worked with a mouse model expressing a hypomorphic deletion of TET1, which lacks the catalytic domain, but still expresses part of the TET1 N-terminus (Khoueiry et al., 2017). Taken together, the latest findings of Khoueiry et al. suggest that TET1, independent of its catalytic activity, is substantial for embryonic development.

3.4.3. TET non-catalytic functions

TET enzymes and their catalytic role were of great interest and have been intensively studied (Wu and Zhang, 2014). Alongside, independent studies reported non-catalytic functions of the different TET proteins in various systems (Deplus et al., 2013; Gao et al., 2016; Kaas et al., 2013; Khoueiry et al., 2017; Tsai et al., 2014; Villivalam et al., 2020; Xu et al., 2012; Zhang et al., 2015). TET1 was reported to regulate expression of the central nervous system (CNS) dependent genes and memory formation independent of DNA demethylation (Kaas et al., 2013). In adipose tissue, TET1 non-catalytically cooperates with histone deacetylase 1 (HDAC1) to regulate transcription (Kaas et al., 2013; Villivalam et al., 2020). And also during primed pluripotency, TET1 was shown to regulate transcription mostly independent of DNA demethylation (Khoueiry et al., 2017). Similarly, increased proliferation rates in *Tet2* KO immune cells can be rescued by a catalytically dead version of TET2 (Montagner et al., 2017). TET3 was reported to repress the *Snrpn* gene in neural stem cells (NSCs) in a catalytically independent way (Montalbán-Loro et al., 2019). While evidence is accumulating that non-catalytic functions of the TET enzymes might play an important role in various systems, the knowledge about the underlying mechanism remains rudimentary. Until today, TET1 was described to interact with SIN3A/HDAC, OGT, MOF, MBD1 and PRC2 (Vella et al., 2013; Williams et al., 2011; Wu et al., 2011; Zhang et al., 2017; Zhong et al., 2017), which suggests that TET1 could recruit these factors or be involved in complex formation (Figure 4c). TET2 and TET3 seem to have fewer interactors and so far were reported to interact with O-linked N-acetylglucosaminyltransferase (OGT) and SET1/COMPASS complexes (Chen et al., 2013; Deplus et al., 2013; Vella et al., 2013). TET2 was also shown to interact with HDAC1 and HDAC2 to regulate inflammatory responses (Zhang et al., 2015). The crosstalk between TET1 and most chromatin modifiers however remains elusive. Most intensive research is done on the interaction between PRC2 and TET1, which together regulate epigenetic plasticity at developmental genes in mESCs and during differentiation (Gu et al., 2018b; Neri et al., 2013; Pastor et al., 2011; Williams et al., 2011; Wu et al., 2011). However, the literature is contradictory regarding the mechanism, TET1 might facilitate the recruitment of PRC2 or the other way around (Neri et al., 2013; Wu et al., 2011)..

Besides PRC2, the SIN3A/HDAC complex is a prominent interactor of TET1 (Chandru et al., 2018; Williams et al., 2011; Zhu et al., 2018a). Interestingly, knockdown experiments of SIN3 Transcription Regulator Family Member A (SIN3A) caused an upregulation of long interspersed nuclear elements 1 (LINE1) in a TET1 dependent manner (de la Rica et al., 2016). However, the loss of TET1 alone does not lead to an upregulation of LINE1 elements (de la Rica et al., 2016), whereas the loss of all three TET proteins triggers a wide range of transposable element (TE) activation. TET TKO mESCs exhibit an upregulation of LINEs, short interspersed nuclear elements (SINEs) and in particular ERVs (Lu et al., 2014). The most significant upregulation was observed at ERVL and correlated with Tripartite Motif Containing 28 (TRIM28/KAP1) binding and activity (Lu et al., 2014).

While two studies so far described a connection between TET enzymes and TE regulation (Lu et al., 2014; de la Rica et al., 2016), the underlying mechanism remains widely unclear. The catalytic and non-catalytic role of the single TET enzymes, as well as the interplay with SIN3A and TRIM28/KAP1 in TE regulation is not understood. In addition, it is unclear if the catalytic activity of the TET enzymes is important for the functional interaction with different chromatin modifying complexes or if TET1 recruits/interacts with those complexes independent of DNA demethylation. In this doctoral work we addressed the non-catalytic role of TET1 in gene and TE regulation in mESCs (Publication I). In the future, it will be an intriguing task to investigate the non-catalytic roles of TET enzymes in development and diseases.

3.5. Heterochromatin & retroviruses

3.5.1. Retroviruses and their classification

Heterochromatin is a condensed and inactive form of chromatin and can be subdivided into constitutive and facultative types (Grewal and Jia, 2007). Facultative heterochromatin is marked by H3K27me3, can change upon external stimuli and acts dynamically in a developmental context. Constitutive heterochromatin marks regions with a high density of repetitive DNA elements such as TEs and is maintained throughout the cell cycle. It is believed that silencing of parasitic mobile TEs is the main reason why heterochromatin was established during evolution. TEs are subdivided into two groups of DNA transposons and retrotransposons (Figure 5a). In mammals, retrotransposons are characterized by the presence or absence of long

terminal repeats (LTRs) flanking the coding region and can thereby be divided into non-LTR and LTR containing retrotransposons. The most well known non-LTR retrotransposons are SINEs and LINEs. The LTR-retrotransposons have a high similarity to proviral elements and are also called endogenous retroviruses (ERVs) (Groh and Schotta, 2017). There are three sub-classes of ERVs (I-III), based on the sequence homology of the reverse transcriptase (RT) of exogenous and endogenous retroviruses (Gifford and Tristem, 2003; Johnson, 2015). Class I (ERV1) includes gammaretroviruses and epsilonretroviruses, class II (ERVK) alpharetroviruses, betaretroviruses, and lentiviruses and class III (ERVL) spumaretroviruses (Figure 5a). Around 10% of the mouse genome and 8% of the human genome originates from ERV insertions (Consortium and Mouse Genome Sequencing Consortium, 2002; Lander et al., 2001). ERVs are the most active TEs and account for 10% of germline mutations in laboratory mouse strains (Nellåker et al., 2012). To ensure genome stability ERVs need to be tightly controlled. Over time, the ongoing battle between viruses and the defense mechanisms of the host also served as a driver of genetic variation and genome evolution. For example, the *syncytin* gene derived from an retroviral envelope protein, got integrated into the human genome and is involved in the development of the placenta (Dupressoir et al., 2012; Mi et al., 2000). TEs can serve as promoters or enhancers to regulate gene expression in mice and humans (Faulkner et al., 2009; Gifford et al., 2013). Today, it is accepted that ERVs are a driver of mammalian evolution (Bourque et al., 2008; Chuong et al., 2013; Cordaux and Batzer, 2009; Lynch et al., 2011). In mice some ERVs are still functional, like IAPs and therefore need to be tightly controlled (Dewannieux et al., 2004). Deficient ERV silencing may cause infertility (Bourc'his and Bestor, 2004), embryonic lethality (Walsh et al., 1998), autoimmune responses (Chiappinelli et al., 2017; Roulois et al., 2015) or apoptosis (Pasquarella et al., 2016). At the same time the activation of ERVs is crucial at defined developmental stages in humans and mice. ERVL are expressed in the early embryo to regulate genome plasticity (Göke et al., 2015; Macfarlan et al., 2012; Peaston et al., 2004). At the zygote stage, MERVL retrotransposons are de-repressed before any other gene is transcribed and account for ~3% of the produced mRNA (Kigami et al., 2003; Peaston et al., 2004). After the 2C stage MERVL expression is silenced again (Svoboda et al., 2004). During this defined time frame, MERVL virus-derived promoters serve as transcriptional initiators of 2C stage specific gene expression (Macfarlan et al., 2012). In addition, ERV1 and ERVK are expressed in mouse and

human ESCs and suggested to be under the control of core pluripotency factors (Fort et al., 2014). However, in most cell types ERVs remain silenced which is controlled by various mechanisms.

3.5.2. Silencing mechanisms of viruses

3.5.2.1. RNA based silencing

Retrotransposons can be silenced by various RNA-mediated mechanisms. Three well studied RNA silencing mechanisms are the generation of small interfering RNAs (siRNAs), production of antisense RNA and piwi-interacting RNA (piRNA) mediated silencing (Groh and Schotta, 2017). siRNA are known to silence human LINE1 elements (Watanabe et al., 2008; Yang and Kazazian, 2006) and upon DNA hypomethylation endogenous siRNAs were shown to restrict TE activation in mESCs (Berrens et al., 2017). In general antisense transcripts seem to have multiple roles in regulating viral expression (Werner, 2013). LINE1 and IAP retrotransposons were shown to be silenced or inhibited by their corresponding antisense transcript (Bierhoff et al., 2014; Li et al., 2014b). In addition, in preimplantation stem cells tRNA-derived fragments (tRFs) silence ERVs, most likely during phases of epigenetic remodeling (Schorn et al., 2017). However, the predominant RNA-based silencing mechanisms in heterochromatin formation seems to be mediated by piRNA (Aravin et al., 2007; Groh and Schotta, 2017). Mechanistically, piRNAs can induce degradation of retroviruses and also influence the chromatin structure. In *drosophila,* piRNAs were discovered to regulate H3K9me3 mediated ERV silencing (Pal-Bhadra et al., 2004). and in mice to induce targeted DNA methylation (Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008).

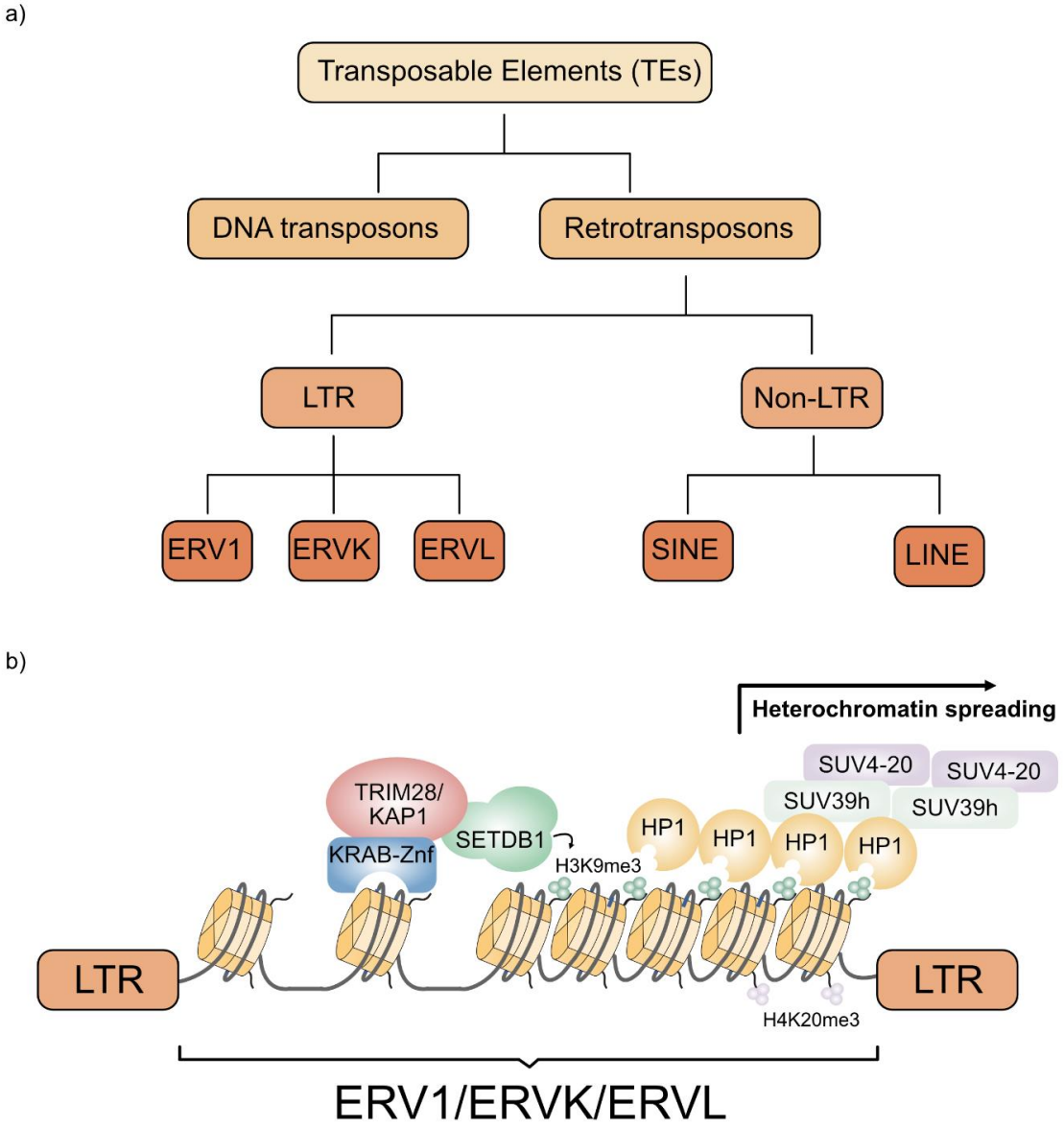


Figure 5. Transposable Elements and ERV silencing. a) Classification of Transposable Elements (TEs). TEs can be divided into DNA transposons and retrotransposons. Retrotransposons are either classified as long terminal repeats (LTR) or as non-LTRs. LTRs are endogenous retroviruses (ERVs) and can be separated into ERV1, ERVK and ERVL. Non-LTRs can be divided into viral elements, like long interspersed elements (LINE) or short interspersed elements (SINE). Figure adapted from (Geis and Goff 2020). b) Model figure illustrating the epigenetic silencing mechanism of ERVs in mESCs. KRAB-Znf recruits TRIM28/KAP1 and subsequently the H3K9me3 methyltransferase SETDB1 to ERV1, ERVK and ERVL elements. HP1 proteins bind H3K9me3, recruit the histone methyltransferases SUV39H and SUV4-20H for the establishment of H3K9me3 and H4K20me3 domains, causing heterochromatin (HC) spreading.

3.5.2.2. Epigenetic ERV silencing

The establishment and maintenance of ERV silencing is an interplay of different factors and closely linked to specific epigenetic marks. The crucial epigenetic hallmarks of constitutive heterochromatin are DNA methylation and histone based post translational modifications, such as H3K9me3 and H4K20me3 (Groh and Schotta, 2017). In mESCs, the establishment of H3K9me3 is crucial for repressing ERVs (Matsui et al., 2010; Mikkelsen et al., 2007) (Figure 5b). The current model suggests that Krüppel-associated box domain zinc finger (KRAB-Znf) proteins recruit TRIM28/KAP1 to specific loci (Friedman et al., 1996; Jacobs et al., 2014; Tan et al., 2013; Wolf and Goff, 2009; Wolf et al., 2015, 2020). TRIM28/KAP1 is one of the master regulators of ERV silencing (Rowe et al., 2010) and interacts with SET Domain Bifurcated Histone Lysine Methyltransferase 1 (SETDB1) (Schultz et al., 2002), the H3K9 methyltransferase for H3K9me3 installation (Matsui et al., 2010). Next, heterochromatin proteins (HP1α, HP1β and HP1γ, also called CBX1, CBX3 and CBX5) bind H3K9me3 (Jacobs and Khorasanizadeh, 2002; Jacobs et al., 2001; Lachner et al., 2001) and recruit suppressor of variegation 3-9 homolog (SUV39H) and suppressor of variegation 4-20 (SUV4-20) for subsequent spreading of heterochromatin (Bulut-Karslioglu et al., 2014). SUV39H is the methyltransferase for H3K9 methylation and SUV4-20 methylates H4K20 (Schotta et al., 2004). While the establishment of H3K9me3 is crucial for silencing of ERVs in mESCs, it seems that the interplay of both H3K9me3 and DNA methylation has the greatest impact on ERV silencing (Sharif et al., 2016). DNA methylation alone is not sufficient to silence ERVs (Karimi et al., 2011) and seems to be more important for ERV repression during differentiation and in somatic cells (Sharif et al., 2016). While the role for DNA methylation in ERV silencing is well studied, the role of DNA demethylation and TET enzymes remains unclear. For example, LINE1 elements are decorated with 5hmC and not deregulated in TET TKO mESCs, whereas ERVs are activated (Lu et al., 2014; de la Rica et al., 2016). In summary leading to the question of the underlying mechanism in TE regulation and the individual contributions of the three TET enzymes. In this doctoral work we investigated the role of TET1 in ERV silencing and found that TET1 is crucial for H3K9me3 establishment at specific ERVs independent of DNA demethylation (Publication I).

3.6. RNA modifications in development and disease

3.6.1 METTL enzymes and other RNA modifiers

In eukaryotes m^{6}A is the most abundant messenger RNA modification and is involved in various cellular, developmental, and disease processes (Roundtree et al., 2017). The last decade was marked with the discoveries of m^{6}A writers (e.g. METTL14, WTAP, and KIAA1429) (Liu et al., 2014a; Ping et al., 2014; Wang et al., 2014c), m^{6}A readers (YTHDF1, YTHDF2, and YTHDF3) (Li et al., 2014a; Wang et al., 2014b, 2015; Zhu et al., 2014) and m^{6}A erasers (FTO and ALKBH5) (Jia et al., 2011; Zheng et al., 2013) (Figure 6). Among human and mouse m^{6}A is strongly conserved (Dominissini et al., 2012) and the loss of critical m^{6}A enzymes causes severe defects in human, mouse, drosophila, yeast and plant (Batista et al., 2014; Clancy, 2002; Hongay and Orr-Weaver, 2011; Zhong et al., 2008).

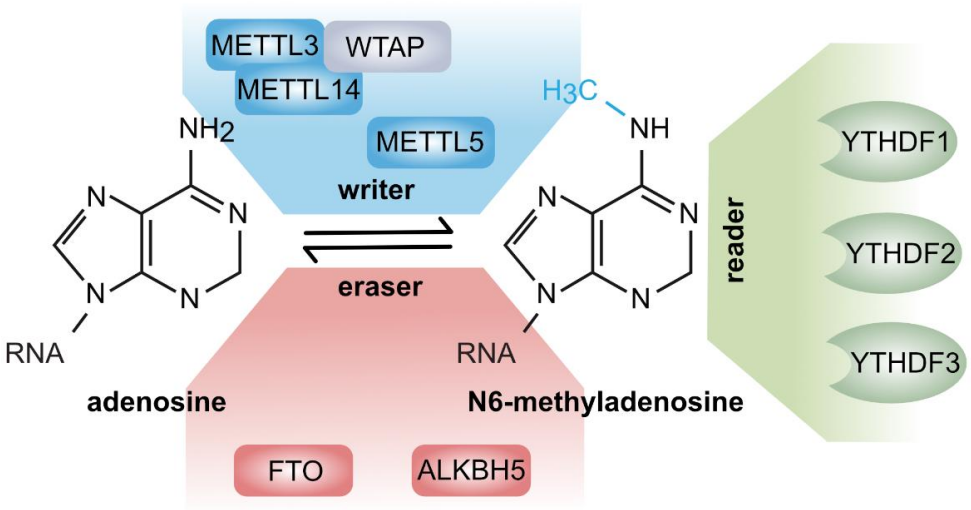


Figure 6. m6A writers, readers and erasers. *N*6-methyladenosine (m^{6}A) is primarily installed by the METTL3/METTL14 writer complex but also by other enzymes like METTL5. The main m^{6}A readers are YTHDF1, YTHDF2 and YTHDF3. The two major m^{6}A erasers are FTO and ALKBH5. The figure is adapted from (Shi et al. 2019).

In mammals, Methyltransferase-like 3 (METTL3) and METTL14 are the core of a multi-unit m^{6}A RNA methyltransferase complex and crucial for placing m^{6}A on mRNA. During mouse embryonic development, the loss of either METTL3 or METTL14 reduces m^{6}A and promotes transcript stability of pluripotency genes (Wang et al.,

2014c). In addition, *Mettl3* knock-out mice are not viable and *Mettl3* knock-out mESCs are unable to exit naive pluripotency (Geula et al., 2015). Besides METTL3 and METTL14, the family of METTL proteins includes a variety of enzymes targeting different kinds of RNA and even protein, thereby regulating various cellular processes (Wong and Eirin-Lopez, 2021). In general, the METTL enzymes are present in most metazoan phyla and represent a group of seven-beta-strand methyltransferases with *S*-adenosyl methionine-binding domains that can potentially modify DNA, RNA, and proteins (Wong and Eirin-Lopez, 2021). For example, METTL21D catalyzes the methylation of the chaperon VCP at lysine312 and can promote tumor formation (Kernstock et al., 2012; Thiele et al., 2011). Interestingly, in *C.elegans* DAMT-1, a protein similar to METTL4 in humans acts as a m^6A DNA methyltransferase (Greer et al., 2015). In total, 33 METTL proteins were identified that are either DNA/RNA or protein methyltransferases or still of unknown function (Wong and Eirin-Lopez, 2021). However, compared to DNMT enzymes, the family of METTL enzymes remains understudied. Besides mRNA methylation, METTL proteins are also involved in the methylation of rRNA, tRNA and small nuclear RNA (snRNA) and therefore influencing cellular processes on multiple levels.

3.6.2. METTL enzymes in development and disease

Human rRNA contains multiple modifications, including 2'-*O*-methyls, pseudouridines, and base methylations. However, only two m^6A sites exist on rRNA, one at position A1832 of 18S rRNA and one at position A4220a on 28S rRNA (Maden, 1986, 1988; Natchiar et al., 2017). Both m^6A rRNA sites are located at functionally important sites and have potential implications for human health (Natchiar et al., 2017). Only in recent years, METTL5 and ZCCHC4 were discovered as the responsible m^6A rRNA methyltransferases of A1832 and A4220 (Ma et al., 2019; van Tran et al., 2019). However, the role and function of METTL5 and ZCCHC4 in development and disease remains elusive.

tRNA are subject to a wide range of modifications, which can impact tRNA stability, folding and translation efficiency of mRNA (Delaunay and Frye, 2019). Today, more than 610 tRNAs are identified and most are individually modified and expressed in a tissue specific manner. This versatility is required for effective interaction between the

ribosome and the translation factors (Kuhn, 2016). Loss of tRNA modifications is tightly connected to cancer progression and disease. In humans for instance, tRNA enzymes that catalyze the modification of wobble uridine 34 (U34) are essential for the survival of melanoma cells via regulating translation efficiency of *HIF1A* mRNA (Rapino et al., 2021). Deleterious mutations in the tRNA methyltransferase NOP2/Sun RNA methyltransferase 2 (NSUN2) cause growth retardation and neurodevelopmental deficits in humans and mice (Abbasi-Moheb et al., 2012; Khan et al., 2012; Sun et al., 2020). Re-expression of the tRNA methyltransferase 9-like (hTRM9L/KIAA1456) suppresses tumor growth *in vivo* (Begley et al., 2013). Further, the establishment of the tRNA N^7-methylguanosine (m^7G) methylome by the METTL1/WDR4 complex is essential for mRNA translation in mammals and the loss of METTL1 causes defects during embryonic development (Lin et al., 2018). Importantly, tRNA modifications and their multiple functions in various cellular and developmental processes are potential drug targets. This creates the need to better understand tRNA modifying enzymes and their mechanism for effective drug development in the future. As part of this doctoral work, I investigated novel functions of the RNA methyltransferases METTL5 and METTL6 in development and disease (Publication V and VI).

3.7. Technological milestones

3.7.1. Next generation sequencing

The decision to sequence the entire human genome marked a milestone in the development of NGS techniques. Since the human genome project in 2003 was completed, the costs for sequencing the human genome dropped from 150 mio to below 1000 dollars (Goodwin et al., 2016; Roberts et al.). This advancement was triggered by great innovations in the field of sequencing and data analysis and made NGS more accessible for basic research and medicine. NGS can be divided into short- and long-read sequencing. Many short-read sequencing platforms are based on sequencing by synthesis. Primer-directed polymerase extension uses deoxynucleotide triphosphate (dNTPs) coupled to fluorescent markers allowing base calling while DNA synthesis (Ronaghi et al., 1998). Today, short-read sequencing has the greatest market share and Illumina is the dominating provider of a short-read sequencing platform (Goodwin et al., 2016). For the Illumina workflow, the DNA is

fragmented into small pieces and ligated with an adaptor sequence. The adaptors are used for clonal PCR amplification in which the signal during the sequencing process is enriched. The DNA library is then applied to a flow cell which is covered with adaptors that match the adaptors of the DNA library. The mobile DNA fragments attach to the flow cell and are amplified by bridge PCR using dNTPs with base-specific fluorophores. The fluorescent signal is excited by a laser and converted into sequencing reads for computational analysis. The development of NGS allowed the concurrent development of a wide-range of applications like chromatin immunoprecipitation sequencing (ChIP-seq), RNA-seq and whole genome bisulfite sequencing (WGBS). This allowed for genome-wide chromatin profiling during development and disease. While the mapping of epigenetic marks was a major breakthrough, the technological advancements are ongoing. Single-cell sequencing of the transcriptome and epigenome provides crucial information to understand the complex cellular interplay in organs, tissues and embryonic development (Buenrostro et al., 2015; Nagano et al., 2013; Patel et al., 2014; Rotem et al., 2015; Smallwood et al., 2014; Tirosh et al., 2016; Venteicher et al., 2017; Zheng et al., 2017). Despite the rapid development and great benefits, short-read sequencing harbors some limitations. The mapping of short reads to the genome can be computationally challenging, the PCR amplification can introduce sequencing errors and some repetitive regions remain inaccessible due to the PCR amplification step (Chaisson et al., 2019). To avoid these difficulties, the development of long-read sequencing approaches offers an attractive solution. The two upfront long-read sequencing platforms are Oxford Nanopore Technologies and PacBio (Logsdon et al., 2020). Both platforms create thousands of kilobases (kb) in read length directly from native DNA. PacBio uses hairpin adapters on both sides of an up to 100 kb big DNA insert to create circular DNA. This allows the amplification of long reads by DNA Polymerase. To discriminate between individual DNA bases, fluorescently labeled dNTPs are used to detect and record fluorescent emission (Logsdon et al., 2020). Oxford Nanopore Technologies uses linear, up to several mega bases long DNA fragments attached to an adapter sequence and a motor protein. This DNA fragment is loaded onto a flow cell covered with nanopores and the negatively charged DNA is moved through the nanopore by the motor protein and an electric current. While the DNA is passing through the pore the current changes characteristically for each DNA base and is measured in real-time (Logsdon et al., 2020). Long-read sequencing offers chromatin

profiling methods without chemical treatments during library preparation and can be used to detect epigenetic marks on DNA like 5mC (Feng et al., 2013; Flusberg et al., 2010; Rand et al., 2017), 5hmC (Laszlo et al., 2013; Wescoe et al., 2014), 6mA (McIntyre et al., 2019) and 8-oxoguanine (An et al., 2015), but also modifications on RNA (Leger et al., 2021; Liu et al., 2019). Today, short-read sequencing is still the gold standard, but long-read sequencing is developing and improving rapidly. Taken together NGS approaches have revolutionized basic research, clinical diagnostics and medicine.

3.7.2. CRISPR/Cas9

3.7.2.1. Brief history and basic principle

The utilization of homology directed repair (HDR) to modify the genome allowed the shutdown, mutation or tagging of genes of interest and was a great improvement for many biological fields (Capecchi, 1989). Subsequently, several nuclease based genome editing technologies have been developed. The first two were meganucleases derived from microbial mobile genetic elements and zinc-finger nucleases (ZFNs) based on eukaryotic transcription factors (TFs) (Miller et al., 2007; Smith et al., 2006; Urnov et al., 2005). Shortly after, transcription activator-like effectors (TALENS) from *Xanthomonas* bacteria were developed for targeted genome engineering (Boch et al., 2009; Christian et al., 2010). Nevertheless, precise and efficient modulation of the large and complex eukaryotic genome remained very challenging. TALENS and ZFNs are difficult to design and clone and new TALENS and ZNF proteins need to be validated for new target sites (2017). The discovery of CRISPR-Cas9 however marks a turning point in genome engineering and shaped the last decade in fundamental research, biotechnology and medicine.

Clustered regularly interspaced short palindromic repeats (CRISPR) together with the associated *Cas* gene was first described in 2007 as a defense mechanism of prokaryotes against viruses (Barrangou et al., 2007). The CRISPR system was known as a defense mechanism of bacteria and archaea (Bhaya et al., 2011; Terns and Terns, 2011; Wiedenheft et al., 2012). In 2012 the labs of Charpentier and Doudna discovered that double stranded RNA can target Cas9 to specific DNA sites to create double strand breaks (Jinek et al., 2012). This finding simplified the gene editing process significantly. Before, genome editing systems relied on expertise in

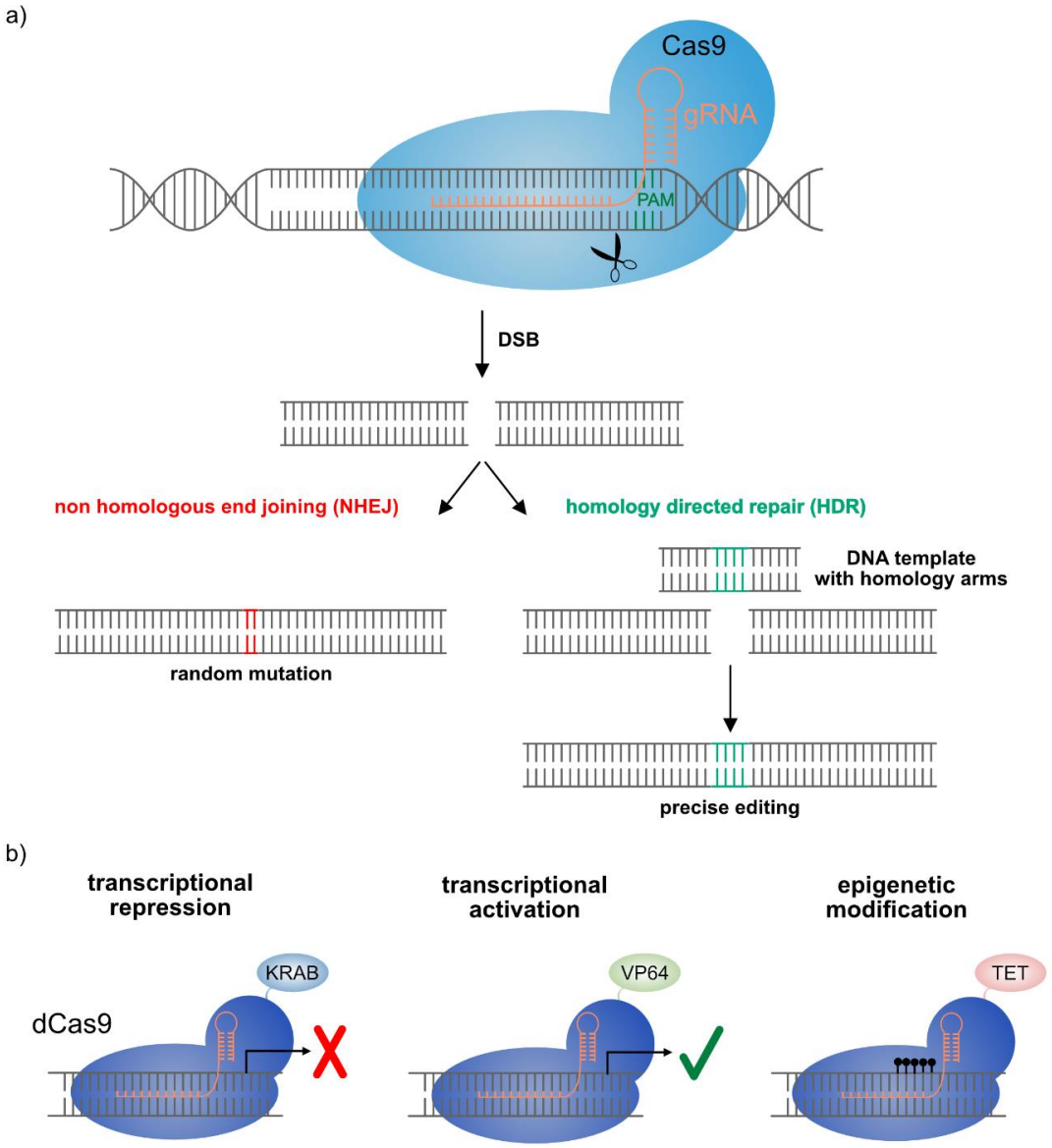
engineering customized DNA-binding proteins, whereas CRISPR-Cas9 genome engineering relies on base pairing of nucleic acids (Pickar-Oliver and Gersbach, 2019).

In more detail, Cas9 endonuclease directed genome engineering depends on the protospacer adjacent motifs (PAM) and a short RNA which guides the Cas9 endonuclease. In nature, the guide RNA of Cas9 consists of CRISPR-RNA (crRNA) and a complementary *trans*-activating crRNA, which can when merged guide Cas9 to the PAM (Deltcheva et al., 2011). For genome engineering approaches, single-guide RNAs have been developed which can be easily synthesized. The guide RNA and the Cas9 nuclease associate with the DNA by base pairing of the guide RNA and the recognition of the PAM. Upon successful alignment, Cas9 creates a blunt-ended double strand break (DSB) 3 bp upstream of the PAM (Garneau et al., 2010). The DSB can be repaired by two different DNA repair mechanisms, either non homologous end joining (NHEJ) or HDR. NHEJ creates random insertions or deletions useful for gene disruptions. HDR uses a DNA template for repair of the DSB, which entails a homologous sequence of the target site and can be utilized to incorporate a tag, fluorophore or mutation (Figure 7a).

3.7.2.2. Epigenetic toolbox

Shortly after its discovery, the CRISPR-Cas9 system was successfully utilized for genome engineering in mammalian cells (Cong et al., 2013; Mali et al., 2013). The engineering strategies can be planned using different softwares and algorithms (e.g. www.benchling.com). Cas9 created double strand breaks can be used to either screen for gene deletions or knockouts or used to insert a desired protein tag for subsequent experiments. Meanwhile, there are countless examples where CRISPR-Cas9 created double strand breaks were successfully used for genome engineering in different biological systems (Cho et al., 2013; Dever et al., 2016; Jinek et al., 2012; Min et al., 2019; Shan et al., 2014; Shimatani et al., 2017). The great success is based on easy design and cloning, high targeting efficiency and the great diversity to utilize the CRISPR-Cas system. For example, mutating the RuvC (D10A) and HNH (H840A) nuclease domains of Cas9 disrupts its catalytic activity, but conserves the ability to bind DNA in an RNA-guided manner (Jinek et al., 2012; Qi et al., 2013). This catalytically dead version of Cas9 (dCas9) can be fused to a diverse range of effectors

like fluorophores, activators, repressors and epigenetic modifiers (Figure 7b) (Anton et al., 2018). In eukaryotic cells, the fusion of Krüppell-associated box (KRAB) to dCas9 can effectively repress transcription of targeted regions (Gilbert et al., 2013; Thakore et al., 2015). On the other side, genes can be activated by fusing transcription activation domains to dCas9, like four repeats of the herpes simplex VP16 activation domain (VP64) (Maeder et al., 2013; Perez-Pinera et al., 2013). The dCas9 driven transcriptional activation was already successfully used for reprogramming cell fate or pluripotency (Black et al., 2016; Liu et al., 2018). Further, dCas9 can be also used for site-directed acetylation or methylation of histones and methylation or demethylation of DNA (Choudhury et al., 2016; Hilton et al., 2015). Hence, the recruitment of epigenetic modifiers by Cas9 can induce activation, repression or remodeling of DNA, offering the intriguing possibility to thoroughly dissect epigenetic mechanisms in development and disease.



F**igure 7. CRISPR/Cas gene and epigenome editing toolbox.** a) Cas9 nuclease is directed to a specific loci by the gRNA and PAM and creates double strand breaks (DSB). The repair pathways of the cell are either non homologous end joining (NHEJ) or homology directed repair (HDR), which can be utilized to create random mutations respectively for precise editing using DNA templates with homology arms. b) The catalytic dead version of Cas9 (dCas9) can be directly fused to different epigenetic effectors for the recruitment to a specific site in the genome. The Krüppel-associated box domain (KRAB) induces transcriptional repression, VP64 acts as a transcriptional activator and the catalytic domain of TET enzymes catalyzes DNA demethylation.

3.7.2.3. Applications in development and disease

The field of applications for CRISPR-Cas seems infinite and multiple strategies have been applied to study biological processes and disease. Besides binding and cleaving DNA, CRISPR-Cas was also utilized to target RNA in living cells (O'Connell et al., 2014). This strategy was already successfully used for live tracking of mRNAs (Nelles et al., 2016) and to specifically eliminate toxic RNAs in patient derived cells (Batra et al., 2017). These studies highlight the potential of Cas9 for fundamental research and future human therapeutics.

Recently, Cas13a was discovered to be a naturally occurring RNA-targeting endonuclease (Abudayyeh et al., 2016). The Zhang lab developed a Cas13-based molecular detection platform, called Specific High Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK), which utilizes the Cas13 RNA sensitivity (Gootenberg et al., 2017). More precisely, Cas13 is coupled to disease specific gRNA and quenched fluorescent RNA for fluorescent readout and can be used for detecting Zika, Dengue virus and Covid-19 (Kellner et al., 2019). Until today, new CRISPR-Cas family variants are being discovered in many different organisms (Edraki et al., 2019; Esvelt et al., 2013; Hou et al., 2013; Müller et al., 2016). The different variants can bring distinct advantages, like smaller protein size (Ran et al., 2015) or different PAM sequences (Miller et al., 2020), holding great potential for new applications in research and medicine.

Diseases caused by gene mutations generally harbor great potential for CRISPR-Cas based therapies, like retinitis pigmentosa (RP), which can result in blindness (Daiger et al., 2013). First experiments in animal models suggest that CRISPR-Cas based *in vivo* knockdown of the RP critical *Nrl* gene leads to preserved cone function and improved survival in three independent mouse models (Yu et al., 2017). Another example for a disease caused by a gene defect is Duchenne muscular dystrophy (DMD). Here, a mutation in the *DMD* gene leads to the absence of dystrophin causing a neuromuscular disorder (Hoffman et al., 1987). Different studies already showed that CRISPR-Cas gene editing enables the restoration of dystrophin expression and normal muscle formation in different animal models (Amoasii et al., 2018; Hakim et al., 2018; Long et al., 2016; Nelson et al., 2016; Tabebordbar et al., 2016; Young et al., 2016).

This shows that CRISPR-Cas based therapies harbor great potential for clinical applications, even though some bottlenecks still have to be overcome. New vectors have to be found as an expression system to solve limited DNA capacities, control the expression of Cas9 and reduce the risk of integration into DSB (Nelson et al., 2019). First achievements in clinical trials could be already celebrated in 2021 (Gillmore et al., 2021). Using a lipid nanoparticle carrier system to transport mRNA of Cas9 and gRNA to knock-out transthyretin (TTR) and to cure the life-threatening disease Transthyretin amyloidosis (ATTR amyloidosis) offered promising results. After 28 days TTR protein concentration was reduced up to 87% (Gillmore et al., 2021). Although, lipid nanoparticles as a carrier system for CRISPR-Cas might offer a solution to treat so far incurable diseases, long-term studies and increased patient numbers are needed to confirm the first positive results

While CRISPR-Cas has already revolutionized basic research, some hurdles still need to be passed before it becomes a standardized medical application. Fortunately however, several trials are already ongoing using CRISPR-Cas to repair gene defects in blood disorders, cancers, eye disease, chronic infections and protein-folding disorders. In summary, it might still take years until first CRISPR-Cas therapies are approved by the FDA. However, with the potential for engineering DNA, RNA and the epigenome, CRISPR-Cas already today has an enormous benefit and impact on research, drug and therapy development and the work presented in this doctoral work (Publication I - VI).

4. Results

4.1. Publication I: TET1 regulates gene expression and repression of endogenous retroviruses independent of DNA demethylation

Paul Stolz, Angelo Salazar Mantero, Andrey Tvardovskiy, Enes Ugur, Lucas E Wange, Christopher B Mulholland, Yuying Cheng, Michael Wierer, Wolfgang Enard, Robert Schneider, Till Bartke, Heinrich Leonhardt, Simon J Elsässer, Sebastian Bultmann, *Nucleic Acids Research*, Volume 50, Issue 15, 26 August 2022, Pages 8491–8511
doi: org/10.1093/nar/gkac642

4.2. Publication II: Critical Role of the UBL Domain in Stimulating the E3 Ubiquitin Ligase Activity of UHRF1 toward Chromatin

Foster BM, Stolz P, Mulholland CB, Montoya A, Kramer H, Bultmann S, Bartke T. *Mol Cell*. 2018 Nov 15;72(4):739-752.e9. Epub 2018 Nov 1
doi: 10.1016/j.molcel.2018.09.028.

4.3. Publication III: Recent evolution of a TET-controlled and DPPA3/STELLA-driven pathway of passive DNA demethylation in mammals

Mulholland CB, Nishiyama A, Ryan J, Nakamura R, Yiğit M, Glück IM, Trummer C, Qin W, Bartoschek MD, Traube FR, Parsa E, Ugur E, Modic M, Acharya A, Stolz P, Ziegenhain C, Wierer M, Enard W, Carell T, Lamb DC, Takeda H, Nakanishi M, Bultmann S, Leonhardt H. *Nat Commun.* 2020 Nov 24;11(1):5972. Erratum in: Nat Commun. 2020 Dec 17;11(1):6443.
doi: 10.1038/s41467-020-19603-1.

4.4. Publication IV: Distinct and stage-specific contributions of TET1 and TET2 to stepwise cytosine oxidation in the transition from naive to primed pluripotency

Mulholland CB, Traube FR, Ugur E, Parsa E, Eckl EM, Schönung M, Modic M, Bartoschek MD, Stolz P, Ryan J, Carell T, Leonhardt H, Bultmann S. *Sci Rep.* 2020 Jul 21;10(1):12066.
doi: 10.1038/s41598-020-68600-3.

4.5. Publication V: The rRNA m 6 A methyltransferase METTL5 is involved in pluripotency and developmental programs

Ignatova VV, Stolz P, Kaiser S, Gustafsson TH, Lastres PR, Sanz-Moreno A, Cho YL, Amarie OV, Aguilar-Pimentel A, Klein-Rodewald T, Calzada-Wack J, Becker L, Marschall S, Kraiger M, Garrett L, Seisenberger C, Hölter SM, Borland K, Van De Logt E, Jansen PWTC, Baltissen MP, Valenta M, Vermeulen M, Wurst W, Gailus-Durner V, Fuchs H, Hrabe de Angelis M, Rando OJ, Kellner SM, Bultmann S, Schneider R. *Genes Dev.* 2020 May 1;34(9-10):715-729. Epub 2020 Mar 26.
doi: 10.1101/gad.333369.119.

4.6. Publication VI: METTL6 is a tRNA m3C methyltransferase that regulates pluripotency and tumor cell growth

Ignatova VV, Kaiser S, Ho JSY, Bing X, Stolz P, Tan YX, Lee CL, Gay FPH, Lastres PR, Gerlini R, Rathkolb B, Aguilar-Pimentel A, Sanz-Moreno A, Klein-Rodewald T, Calzada-Wack J, Ibragimov E, Valenta M, Lukauskas S, Pavesi A, Marschall S, Leuchtenberger S, Fuchs H, Gailus-Durner V, de Angelis MH, Bultmann S, Rando OJ, Guccione E, Kellner SM, Schneider R. *Sci Adv.* 2020 Aug 26;6(35):eaaz4551.
doi: 10.1126/sciadv.aaz4551.

5. Discussion

5.1. DNA methylation

5.1.1. UHRF1 and DNA methylation

The DNA methyltransferase DNMT1 was described early on as a maintenance methyltransferase engaging with replicating protein proliferating cell nuclear antigen (PCNA) (Leonhardt et al., 1992b). Subsequent studies showed that the interaction of DNMT1 with PCNA is not crucial for maintenance methylation (Schermelleh et al., 2007; Spada et al., 2007), but that DNMT1 is regulated by intramolecular events (Jeltsch and Jurkowska, 2016), PTMs (Du et al., 2010; Estève et al., 2009, 2011; Zhang et al., 2019) and UHRF1 (Bostick et al., 2007; Sharif et al., 2007). Today, the multidomain protein UHRF1 is well characterized as a crucial player in regulating DNMT1 and passive DNA demethylation in mammalian development (von Meyenn et al., 2016). However, the mechanisms by which UHRF1 recruits DNMT1 to chromatin are numerous and complex and are still a subject of research. While, the E3 ubiquitination ligase activity of UHRF1 towards H3 is critical for recruiting DNMT1 to replicating chromatin (Ishiyama et al., 2017; Nishiyama et al., 2013; Qin et al., 2015), the mechanism regulating the ubiquitination activity of UHRF1 itself remained unknown. In the scope of this doctoral work I collaborated with the group of Till Bartke to identify a hydrophobic patch in the UBL domain which is critical for E2 ligase (UbcH5a) targeted ubiquitination of H3 and which stabilizes the complex formation with the RING-domain of UHRF1 for E3 ligase activity (Publication II) (DaRosa et al., 2018). Further, using CRISPR/Cas in mESCs, I contributed to the finding that a single F46A point mutation in the hydrophobic patch of the UBL domain disturbs the E3 ligase activity of UHRF1, which ultimately impedes DNA methylation maintenance (Figure 8, Publication II).

The necessity to understand the ubiquitination mechanism of UHRF1 was again highlighted by a recent study of Nishiyama and colleagues (Nishiyama et al., 2020). Besides its known ubiquitination sites at Lysine 14, 18 and 23 of H3, UHRF1 also ubiquitinates PAF15 at two lysine residues (Nishiyama et al., 2020). Both

ubiquitination events lead to the recruitment of DNMT1 to chromatin and are therefore critical for DNA methylation maintenance (Nishiyama et al., 2020). Intriguingly, these two ways of DNMT1 recruitment are most likely separated in time and space. PAF15 is suggested to compensate for the lack of H3 ubiquitination at early S-phase, whereas H3 ubiquitination could be the critical factor in late S-phase (Nishiyama et al., 2020). However, it remains unclear how this switch between PAF15 and H3 ubiquitination is regulated. One hypothesis is that PAF15 is degraded in late S-phase to ensure proper H3 ubiquitination. *Paf15* knock-out mice are viable, whereas *Dnmt1* and *Uhrf1* knockouts are embryonic lethal. This suggests that either H3 ubiquitination compensates for PAF15 or another not identified protein can substitute PAF15. Considering the multiple intramolecular interactions among the different domains of UHRF1, another possibility would be a change in the intramolecular states or folding of UHRF1 to switch between different ubiquitination targets. Thus, future studies of a full length UHRF1 protein, in particular harboring the UBL and RING domain, are needed to completely understand the function and mechanism of the UHRF1 ubiquitination activity.

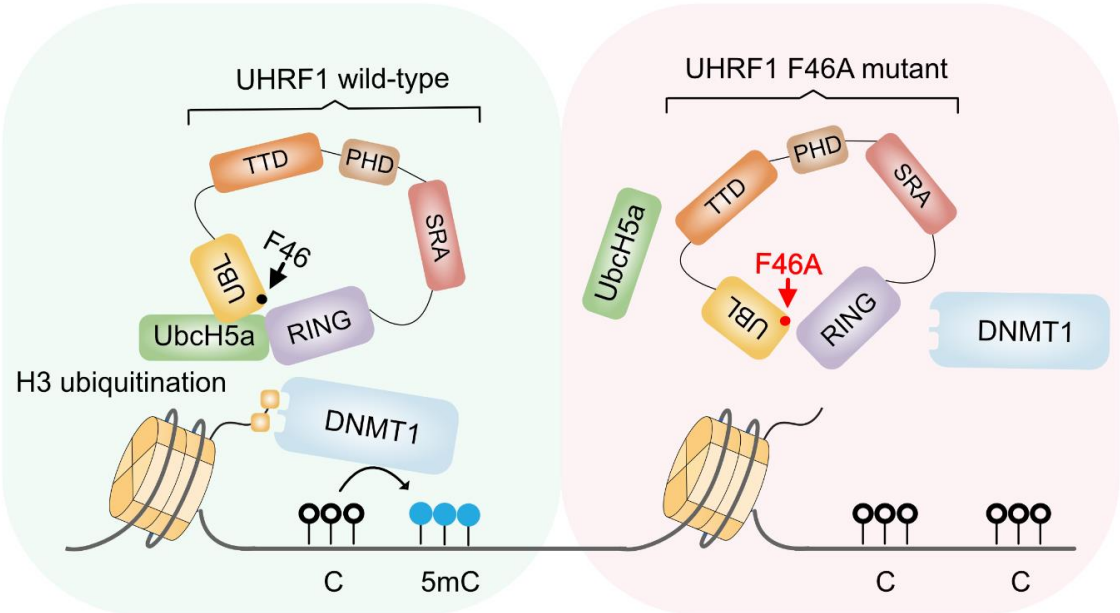


Figure 8. The UBL domain of UHRF1 is essential for H3 ubiquitination and subsequent DNA methylation maintenance. Schematic representation of UHRF1 impacting H3 ubiquitination and DNA methylation maintenance. The point mutation F46A in the UBL domain destabilizes the binding of the E2 ligase (UbcH5a) to UHRF1, which impairs H3 ubiquitination, inhibits the recruitment of DNMT1 and causes a reduction in DNA methylation levels.

Besides the ubiquitination activity of UHRF1, various other mechanisms are involved in regulating the UHRF1-DNMT1 interaction and activity. The PHD and TTD domain cooperate in regulating the binding of UHRF1 to H3K9me2/3 (Arita et al., 2012b; Qin et al., 2015; Rothbart et al., 2012). This is controlled by intramolecular contacts of the TTD domain and binding of histone mimic protein LIG1 (Ferry et al., 2017; Gao et al., 2018). Moreover, the binding of methylated, hemi-methylated and unmethylated DNA depends on the SRA domain of UHRF1, which is also critical for DNA methylation maintenance (Arita et al., 2008). In the scope of this doctoral work I contributed to identify DPPA3 as a direct binding partner of UHRF1 and a significant regulator of DNA methylation maintenance (Publication III). DPPA3 can bind and remove UHRF1 from chromatin, thereby inducing global hypomethylation by passive DNA demethylation (Publication III). Interestingly however, the expression of DPPA3 is regulated by active DNA demethylation of the *Dppa3* locus by TET1 and TET2 (Publication III).

In addition to intramolecular regulation and the interaction with different proteins, UHRF1 can be regulated by PTMs, highlighting the multilayered complexity of UHRF1-DNMT1 activity regulation. However, it remains unclear how the different modes of DNMT1 recruitment by UHRF1 are coordinated. In colorectal cancer for instance the PHD and SRA domain are crucial for cancer-specific DNA methylation patterns, but not the E3 ubiquitination activity of UHRF1 (Kong et al., 2019). This finding raises the question whether the E3 ubiquitination activity of UHRF1 is required for DNA methylation only in specific cell types or defined stages of embryonic development. One experimental approach to address this question, could be to induce degradation of UHRF1 and other proteins involved at different stages during development using the auxin-inducible degron (AID)-Auxin (Nishimura et al., 2009) or degron Tag (dTag) (Nabet et al., 2018) system. This would allow the measurement of epigenetic marks, like ubiquitination, upon UHRF1 loss at different timepoints during development. Another approach could be the mutation or deletion of the UBL domain in different cell types or using a Cre-lox system to delete the UBL domain at different timepoints during mouse development. This experimental setup could answer when and if the E3 ubiquitination activity of UHRF1 is required for DNA methylation.

This complex regulation of UHRF1-DNMT1 shows that the interplay needs to be tightly controlled to act at different genomic regions at different timepoints. In addition,

DNMT1 was suggested to associate with non-replicating chromatin (Easwaran et al., 2004), but it remained unclear whether the UHRF1-DNMT1 tandem is also involved in *de novo* DNA methylation activity. Recently however, Haggerty and colleagues showed DNMT1 *de novo* activity at specific TEs, namely IAP elements, in a UHRF1-dependent manner (Haggerty et al., 2021). This fascinating finding brings up the following question: Which regulatory mechanisms of UHRF1-DNMT1 control the switch between maintenance and *de novo* methylation? UHRF1 seems to be recruited to TEs in a TRIM28 and H3K9me3 dependent manner (Haggerty et al., 2021). However, H3K9me3 is a pervasive mark at TEs and it remains elusive how UHRF1 is specifically targeted to IAP elements. It is intriguing to speculate, that zinc finger proteins, UHRF1 folding, PTMs or another epigenetic mechanism might individually or in combination control *de novo* methylation by DNMT1-UHRF1, to in the end silence ERVs at certain time points during embryonic development.

5.2. DNA demethylation

Originally, prokaryotes used DNA methylation as a defense mechanism against viruses (Blow et al., 2016). The host genome becomes targeted by DNMTs to protect it from restriction enzymes, whereas foreign viral DNA is cleaved and destroyed by restriction enzymes (Bickle and Krüger, 1993). During evolution viruses adapted methylation such as 5mC to hide their invasive DNA from restriction enzymes, which in turn triggered the emergence of 5mC sensitive restriction enzymes in bacteria. In this ongoing battle between hosts and viruses new strategies and DNA modifications evolved. Bacteriophages were first to decorate their DNA with 5hmC to protect it from enzymatic digestion by the host's defense system (Bickle and Krüger, 1993). Today, we know that in eukaryotes 5hmC, 5fC and 5caC are part of an active DNA demethylation pathway by TET enzymes, TDG and BER (He et al., 2011; Ito et al., 2011; Tahiliani et al., 2009). At the same time, it is intriguing to speculate that 5hmC, 5fC and 5caC also evolved as a part of viral defense mechanisms (Deniz et al., 2019), similarly to 5mC, which is clearly linked to silencing parasitic viral elements (Walsh et al., 1998; Yoder et al., 1997), However, it remains widely elusive how and if the different steps during DNA demethylation are involved in the regulation of viral

elements. This idea brings up some important questions: Why do mammals have two waves of DNA demethylation during early embryonic development? Does DNA demethylation harbor a risk for deficient virus activation, while ensuring genome plasticity? How did active and passive DNA demethylation evolve in mammals? Can the highly mutagenic nature of 5mC and other DNA modifications (Kamiya et al., 2002; Mahfoudhi et al., 2016; Supek et al., 2014; Tomkova and Schuster-Böckler, 2018; Xing et al., 2013) be a risk for a developing organism and could this be the reason for DNA hypomethylation during early embryonic development?

While germline mutations caused by DNA methylation can serve as an important driver of evolution, the deposition of 5mC generally harbors also a high mutagenic risk for harmful mutations (Tomkova and Schuster-Böckler, 2018). For example, heterozygous *Tet1* mutant mice, that are hampered in DNA demethylation, accumulate mutations and face genome instability (Zhong et al., 2017). To avoid such instabilities, it is beneficial for an organism to control 5mC and 5hmC (Lindahl, 1993). Some organisms like *Drosophila melanogaster* and *Caenorhabditis elegans* even lost the DNA methylation machinery during evolution, most likely because of its high mutagenic cost (Goll and Bestor, 2005a; Simpson et al., 1986b; Urieli-Shoval et al., 1982). In pluripotent stem cells, mutagenesis can have severe consequences for body plan formation and lead to disease. In cancer, formerly methylated cytosine residues are one of the most common mutated sites in the genome (Tubbs and Nussenzweig, 2017). This might offer an explanation why methylated plant and animal genomes tend to show a depletion of CG sites (Tran et al., 2005; Zemach et al., 2010). Altogether, this suggests that to avoid mutagenesis, the zygotic embryo reduces the amount of DNA methylation and maintains a hypomethylated state until implantation and lineage specification.

In support of this hypothesis, mESCs are viable despite losing all DNMTs and TETs (Dawlaty et al., 2014; Li et al., 1992). This suggests that in a pluripotent state mammals reduce the risk of mutagenesis and genome instability by relying on other mechanisms to silence viral elements (Matsui et al., 2010; Rowe et al., 2010). In mESCs and PGCs, repressive histone modifications, mainly H3K9me3, are essential for TE silencing and mESCs viability (Liu et al., 2014b; Matsui et al., 2010; Rowe et al., 2010), whereas DNA methylation is dispensable for TE suppression in mESCs (Hutnick et al., 2010; Karimi et al., 2011; Matsui et al., 2010). A silencing pathway based on modified proteins might be beneficial in mESCs, because the DNA is not exposed to the

mutagenic instability of 5mC. Interestingly, upon differentiation, the mechanism of regulation switches and silencing of TE is regulated by DNA methylation (Bulut-Karslioglu et al., 2014; Karimi et al., 2011). At the same time, DNMT TKO and TET TKO mESCs face severe embryonic defects at later stages during development, which correlates with TE activation (Dawlaty et al., 2013, 2014; Lei et al., 1996; Li et al., 1992; Sakaue et al., 2010; Walsh et al., 1998). In summary, it is plausible that DNA demethylation evolved to reduce the risk of mutagenesis during critical steps of embryonic development, such as pluripotency and PGC development and at the same time caused the development of other mechanisms to silence TEs ensuring genome stability.

However, it remains elusive why DNA methylation is dispensable during pluripotency, but essential during embryogenesis. Why can histone based silencing mechanisms compensate for DNA methylation during pluripotency, but not during differentiation and in somatic tissue? And how is this mechanistic switch regulated? The genome of a pluripotent cell requires both at the same time, high plasticity and strict control to regulate the activation of essential viral elements (Fadloun et al., 2013; Macfarlan et al., 2012). In pluripotent stem cells and during PGC development, histone based silencing mechanisms control virus activation and genome plasticity, while the genome remains globally hypomethylated (Karimi et al., 2011; Macfarlan et al., 2012; Matsui et al., 2010). But again, we do not know why other mechanisms than DNA methylation are the main regulator of TEs during toti- and pluripotency. One reason for this could be that histone based silencing might allow a more flexible and beneficial regulation of the genome then DNA methylation. However, we are currently lacking a comprehensive and systematic comparison of DNA methylation and histone based silencing.

One possibility to answer some open questions would be a CRISPR screen in TET TKO or DNMT TKO for novel proteins that affect self-renewal or viability. The functional switch between DNA methylation and histone based silencing might be explained by 5mC, 5hmC, 5fC and 5caC-sensitive or -insensitive proteins. TFs for example could maintain the pluripotency network to great parts without TETs and DNMTs at the blastocyst stage. Collectively, epigenetic regulation during embryonic development is very complex and while it is intriguing to speculate that DNA demethylation evolved to reduce the risk of mutagenesis at critical developmental timepoints, DNA demethylation and TET enzymes also acquired other important

functions during evolution. Studies describing the catalytic and non-catalytic functions of TET enzymes are numerous and diverse and will be discussed in the next chapter.

5.2.1. TET enzymes - catalytic function

The discovery of active DNA demethylation by TET enzymes in mammals triggered a multitude of studies focusing on the role of DNA demethylation in development and disease (Wu and Zhang, 2014). TET-driven oxidation of 5mC can either lead to replication-dependent passive DNA demethylation (Inoue and Zhang, 2011) or 5fC and 5caC can be excised via TDG and BER, known as active DNA demethylation (Wu and Zhang, 2014). While 5fC (0.06 - 0.6% of 5mC) and 5caC (0.01% of 5mC) represent only a small fraction of chromatin, 5hmC (~5% of 5mC) makes up a significant proportion of chromatin (Ito et al., 2011; Pfaffeneder et al., 2011). The highest levels of 5hmC were found in mESC and in the brain (Szwagierczak et al., 2010). Besides being intermediates of active DNA demethylation, 5hmC and 5fC are also stable epigenetic marks (Bachman et al., 2014, 2015) and have individual reader proteins (Spruijt et al., 2013). At E3.5 the ICM of the blastocyst undergoes massive re-methylation, but only little was known about the 5mC oxidation dynamics in this developmental time frame (Smith et al., 2012; Wang et al., 2014a). I have contributed to a study of our lab that identified increasing levels of 5mC, 5hmC, 5fC and 5caC during the transition from naive mESCs to mouse epiblast-like cells (mEpiLC) (Figure 9, Publication IV). In addition, the study discovered stage-specific contributions of individual TET enzymes. Whereas TET2 is required for large parts of the 5hmC production in naive mESCs and accounts for the majority of 5fC in both stages of pluripotency, TET1 is mainly responsible for 5hmC in mEpiLCs (Figure 9, Publication IV). These findings bring up two main questions. First, why do the levels of 5hmC, 5fC and 5caC increase in the ESC to EpiLCs transition? Second, how is the distinct oxidation preference of TET1 and TET2 regulated?

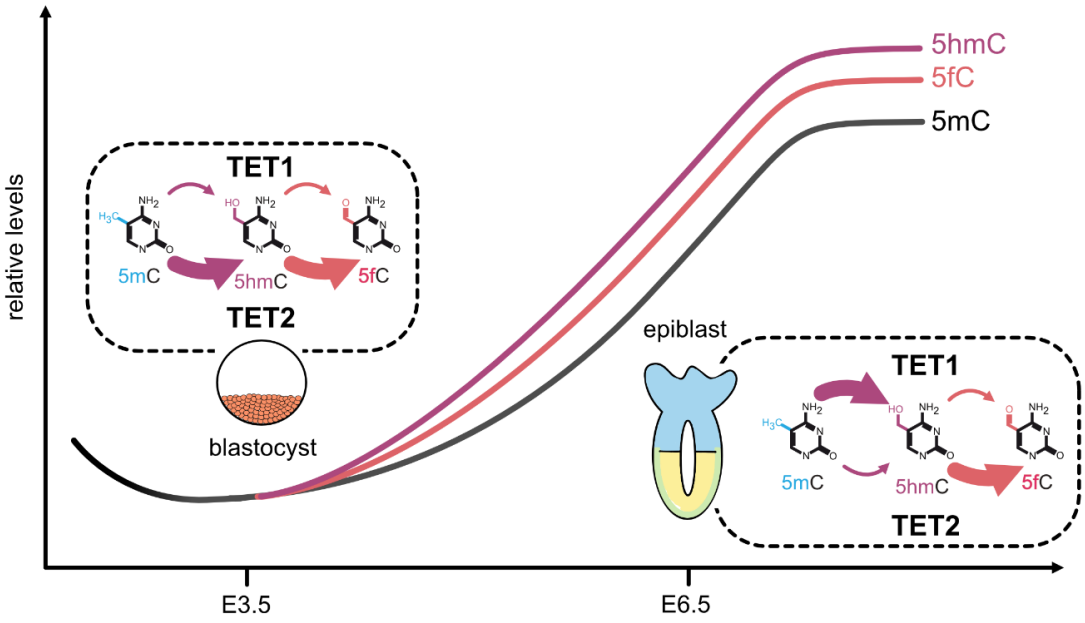


Figure 9. Dynamics of DNA modifications and stage specific contributions of TET1 and TET2 in naive mESCs and primed mEpiLCs. Illustration of relative levels of 5mC, 5hmC and 5fC in the transition from naive mESCs to primed mEpiLCs and the stage specific contributions of TET1 and TET2 to the oxidation of the individual DNA modifications. In naive mESCs TET2 is responsible for the majority of the 5hmC and 5fC production, whereas in primed mEpiLCs TET1 is the main driver of 5hmC production and TET2 remains critical for 5fC levels.

While TET enzymes are not essential for naive mESCs (Dai et al., 2016; Dawlaty et al., 2014), DNA demethylation activity of TETs plays an essential role in controlling Nodal signaling pathways during gastrulation (Dai et al., 2016; Gu et al., 2018b). Further, the loss of TET enzymes causes severe defects and lethality of postimplantation embryos (Dai et al., 2016; Dawlaty et al., 2014; Khoueiry et al., 2017). Yet, it remains unknown to what extent individual 5hmC, 5fC and 5caC levels contribute to the developing embryo upon implantation. Until today, numerous methods were developed to map 5hmC, 5fC and 5caC, which help to understand their function and role (Hon et al., 2014; Schutsky et al., 2018; Song et al., 2011; Sun et al., 2015; Wu and Zhang, 2015; Wu et al., 2014; Zeng et al., 2019; Zhu et al., 2017). 5hmC is mostly enriched at gene bodies and enhancers of active genes (Tsagaratou et al., 2014) and 5fC is enriched at promoters and exons (Raiber et al., 2012). Several studies suggest that 5hmC levels correlate with chromatin accessibility and gene activity (Ficz et al., 2011; Mellén et al., 2012; Wu et al., 2014). In contrast, the genomic

abundance of 5caC is very low and even though there are methods to map 5caC (Lu et al., 2015; Wu et al., 2016), mapping remains technically challenging and genome-wide studies are rare. Like 5hmC, 5fC also correlates with promoters of transcribed genes and active histone marks (He et al., 2021; Raiber et al., 2012; Zhu et al., 2017). However, it is not fully understood how 5hmC and 5fC dynamis contribute to differentiation. The discovery of an intermediate rosette-like cell state with elevated 5hmC levels that is present before the primed mEpiLC state is reached, further increases the complexity (Neagu et al., 2020). A recent study suggests that the oxidation to 5hmC is not sufficient for the transition to another cell state and that TET enzymes mainly drive oxidation towards 5fC/5caC and promote rapid DNA demethylation (Caldwell et al., 2021). In addition, several studies reported that TET driven DNA demethylation is important to counteract DNMT3A/B *de novo* methylation (Dai et al., 2016; Ginno et al., 2020; Gu et al., 2018b; Manzo et al., 2017). These findings suggest that 5hmC, 5fC and 5caC are in the first place intermediates for active DNA demethylation. However, the question remains whether the DNA demethylation via TDB/BER is essential or whether 5hmC and 5fC execute an important function upon implantation and lineage specification. For example, 5hmC is found at high levels in the brain and imbalances of 5hmC are closely linked to brain disorders (Azizgolshani et al., 2021; Globisch et al., 2010; Mellén et al., 2012; Song et al., 2011; Szulwach et al., 2011; Wang et al., 2012). It will be an fascinating task to study whether 5hmC patterns set during implantation are defining neuronal cell identity. Here the recent development of *in vitro* cell culturing systems (Aguilera-Castrejon et al., 2021; Beccari et al., 2018; Liu et al., 2021b), together with imaging and single cell technology (Stuart and Satija, 2019) will allow the study of those DNA modifications and enables the tracking of respective cell identities in the early embryo.

The second question arising from our data is, why TET1 is critical for 5hmC production in primed mEpiLCs, whereas TET2 is responsible in naive mESCs and how this switch is regulated. The N-terminus of TET2 lacks the chromatin binding parts of TET1 and its chromatin affinity and oxidation activity seems to be regulated by IDAX4 (Ko et al., 2013). Interestingly, the catalytic activity of TET1 does not correlate with chromatin binding (Zhang et al., 2016b). This indicates that other factors regulate the stage specific contribution of TET1 and TET2 towards 5mC oxidation, like Spalt Like Transcription Factor 4 (SALL4) (Xiong et al., 2016) or 5hmC reader 5-Hydroxymethylcytosine Binding, ES Cell Specific (HMCES) for example (Spruijt et al.,

2013). HMCES associates with TET1 (Publication I) and is proposed to be a regulator of different repair pathways (Halabelian et al., 2019). Besides the association with different proteins, the supposedly greater catalytic activity of TET2 might be explained by a higher mobility of TET2 compared to TET1 in mESCs (Ryan, 2020). However, detailed structural analysis and dissecting the interaction networks of TET1 and TET2 in the transition from naive mESCs to mEpiLC are needed in order to explain TET1 and TET2 oxidation preferences.

5.2.2. TET enzymes - non-catalytic function

Since their discovery the role and significance of TET enzymes for embryonic development remain contradictory. While Dawlaty et al. reported embryonic viability upon loss of TET1 or TET2 (Dawlaty et al., 2011, 2013), Khoueiry et al. described severe embryonic defects for Tet1 KO mice (Khoueiry et al., 2017). Interestingly, Khoueiry et al. explain the difference to previous mouse models with a hypomorphic deletion of the *Tet1* gene, which still allows the expression of the TET1 N-terminus. Further, they demonstrate that non-catalytic functions of TET1 are essential for embryonic development. However, in the last decade the majority of studies focused on describing the catalytic activity of TET enzymes and a detailed analysis of non-catalytic functions was lacking. This doctoral work describes novel non-catalytic functions and mechanisms of TET1 in mESCs (Publication I). I found that TET1 regulates gene and ERV expression independent of DNA demethylation. Further, I identified TET1 as a regulator of various histone modifications and as an interaction hub of multiple chromatin modifying complexes (Publication I). While the interaction with SIN3A and PRC2 accounts for most of the transcriptional changes observed in Tet1 KO mESCs (Publication I) (Chrysanthou et al., 2022a), it cannot explain all transcriptional and histone modification changes observed. We found that, besides SIN3A and PRC2, TET1 associates with multiple chromatin modifying complexes. Some were already described to interact with TET1, like Glutamine And Serine Rich 1 (QSER1) (Dixon et al., 2021) and Non-POU Domain Containing Octamer Binding (NONO) (Li et al., 2020), but others are unknown and interesting targets to further dissect non-catalytic mechanisms of TET1.

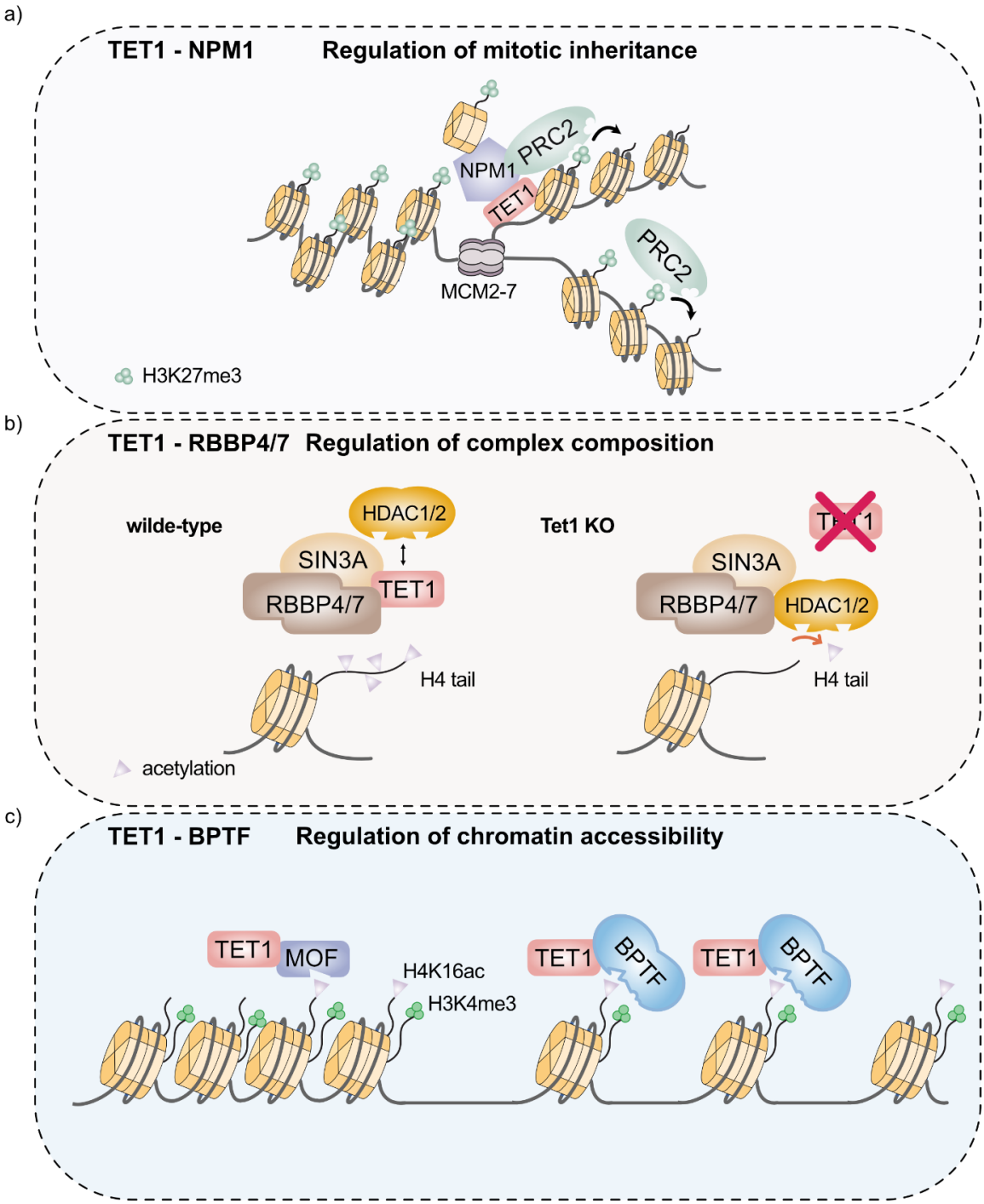


Figure 10. Potential non-catalytic mechanisms of TET1 with associated proteins from a TET1 chromatin IP (Publication I). a) Proposed model of TET1 regulating mitotic inheritance of repressive histone marks. At the DNA replication fork, TET1 might facilitate the inheritance of H3K27me3 by the histone chaperone NPM1 and thereby foster the propagation of H3K27me3 to naive nucleosomes. b) Proposed model of TET1 regulating the complex composition of SIN3A-RBBP4/7-HDAC1/2. TET1 might compete with HDAC1/2 for the binding of RBBP4/7 and controls acetylation of the H4 tail. c) Proposed model of TET1 regulating chromatin accessibility. The interaction of TET1 with the H4K16 acetyltransferase MOF might

directly or indirectly recruit BPTF to doubly-modified nucleosomes and might regulate chromatin accessibility.

TET1 associates with core proteins (EED, SUZ12, EZH2) of the PRC2 complex (Publication I). The interaction and interplay of TET1 with PRC2 regulates developmental genes in mESCs (Publication I) (Chrysanthou et al., 2022a; Gu et al., 2018b). Different studies suggest that TET1 counteracts the methylation activity of DNMT3A/B to regulate PRC2 at developmental genes (Ginno et al., 2020; Gu et al., 2018b; Manzo et al., 2017). In contrast, this doctoral work and the work of others now shows that PRC2 and the deposition of H3K27me3 is controlled by catalytically independent mechanisms of TET1 (Publication I) (Chrysanthou et al., 2022a; Wu et al., 2011). Through its interaction with the core components of the PRC2 complex (SUZ12/EZH2/EED), TET1 facilitates the recruitment of PRC2 directly to target genes (Publication I) (Chrysanthou et al., 2022a; Wu et al., 2011). However, TET1 binds multiple genomic sites (Williams et al., 2011) and it remains elusive how TET1 recruits PRC2 specifically only to developmental genes. The chromatin IP data of TET1 revealed that TET1 associates with the histone chaperone Nucleophosmin 1 (NPM1), which was recently shown to regulate H3K27me3 at PRC2 target genes across replication (Escobar et al., 2022). At the same time the chromatin IP data identified minichromosome maintenance complex component 3 (MCM3) and origin recognition complex subunit 2 (ORC2) to associate with TET1 (Publication I). Both are essential components of the DNA replication machinery (Bleichert et al., 2015, 2017; Madine et al., 1995) and this suggests that TET1 might localize to the replication fork. In addition, the loss of NPM1 and TET1 in mESCs triggers a delay in G1 phase (Chrysanthou et al., 2022b; Escobar et al., 2022). Every round of DNA replication also entails the maintenance of repressive chromatin marks and can have a huge impact on the transcriptional program and cell identity (Escobar et al., 2021). Together, these findings propose that TET1 cooperates with NPM1 in regulating the inheritance of H3K27me3 during S-phase at developmental genes (Figure 10a). To test this hypothesis, TET1 could be degraded using the AID-Auxin (Nishimura et al., 2009) or dTag (Nabet et al., 2018) system to compare the direct effects of TET1 on H3K27me3. Using the AID-Auxin system, NPM1 is completely degraded after 4-6h and H3K27me3 levels are significantly reduced (Escobar et al., 2022). Genome-wide H3K27me3 analysis and cell cycle measurements at different timepoints after degradation could

be used to explore the functional relationship between NPM1, TET1 and chromatin replication.

The association with the histone chaperone NPM1 during replication offers the possibility of a striking mechanism for the regulation of the repressive chromatin mark H3K27me3. However, the loss of TET1 also leads to a global reduction of pH4Kac, a chromatin mark associated with transcriptional activation. Currently, we assume that only repressive and not active histone marks can be maintained throughout mitosis (Escobar et al., 2019). This indicates that the global acetylation dynamics and pH4Kac loss in Tet1 KO mESCs are controlled by mechanisms independent of replication. In the TET1 chromatin IP data, TET1 also associates with the histone chaperones Retinoblastoma-Binding Protein 4 (RBBP4) and RBBP7. In mESCs RBBP4/7 form a repressive deacetylation complex together with SIN3A, HDAC1 and HDAC2 (Silverstein and Ekwall, 2005). Interestingly in iPSCs, TET1 and HDAC2 are competing for the binding of RBBP4 and the loss of TET1 triggers global H4 deacetylation (Wei et al., 2015). This proposes that upon loss of TET1, HDAC2 is enriched in RBBP4 containing complexes and induces global deacetylation of pH4Kac. In mESCs the competition between TET1 and HDAC1/2 might regulate the complex composition and therefore the deacetylation activity of the SIN3A/HDAC complex (Figure 10b). To test this hypothesis, RBBP4/7 could be tagged and immunoprecipitated in WT and Tet1 KO mESCs to study whether RBBP4/7 are enriched for HDAC1/2, explaining the global deacetylation phenotype.

Besides the association with different histone chaperones, TET1 also associates with the chromatin remodeler Bromodomain PHD-finger Transcription Factor (BPTF) (Publication I). BPTF is the largest subunit of the NURF chromatin remodeling complex (Barak et al., 2003) and *Bptf* KO mice die shortly after implantation (Landry et al., 2008). Similar to TET1, BPTF is required for the trophectoderm specification (Goller et al., 2008) and the loss causes the differentiation to mesoderm and endoderm lineages (Landry et al., 2008). BPTF can specifically bind double modified nucleosomes carrying H3K4me3 and H4K16ac (Ruthenburg et al., 2011). Strikingly, TET1 is described to regulate H4K16ac in mESCs by controlling the auto-acetylation activity of the acetyltransferase Males absent on the first (MOF) (Zhong et al., 2017). TET1 regulating H4K16ac is in accordance with the global loss of pH4Kac and offers a potential pathway for a functional interplay with BPTF (Publication 10c). However, Zhong et al. used solely *in vitro* studies to investigate the functional relationship

between MOF and TET1. Further, MOF was not identified to associate with TET1 in the chromatin IP data (Publication I). This could have several reasons, but suggests that the interaction between TET1 and MOF is more complex. To test whether TET1 regulates MOF, H4K16ac and thereby BPTF/NURF activity, quantitative ChIP-seq and mass spectrometry approaches could be used to study H4K16ac levels in Tet1 KO mESCs. To further dissect the potential crosstalk between BPTF, MOF and TET1, a detailed analysis of the H4K16ac and BPTF binding patterns in WT and Tet1 KO mESCs would give first correlative information. Next, fluorescent mESC lines of BPTF and TET1 in combination with high resolution live cell imaging could provide information about colocalization dynamics in mESCs and during differentiation. The tagged cell lines could be further used for mass spectrometry to study the NURF complex composition in a Tet1 KO background.

In summary, it became clear that TET1 has important non-catalytic functions in mESCs, yet the potential mechanisms seem to be numerous and diverse. Future systematic studies will be needed to dissect the non-catalytic mechanisms of TET1. In addition, the primary use of TET knock-outs and the lack of TET catalytic mutant studies bears the need to revisit the already described functions of TET enzymes in development and disease. It will be an intriguing task for the future to decipher the non-catalytic functions of TET enzymes at different stages during development and their implications for disease and medical applications.

5.2.3. TET1 and retroviral silencing

DNA methylation evolved as a defense mechanism against viruses in bacteria (Blow et al., 2016). During embryonic development, DNMT1 KO mouse embryos are lethal after 8.5 days and the severe phenotype is coupled to the loss of DNA methylation and the activation of ERVK elements (Grosswendt et al., 2020; Li et al., 1992; Walsh et al., 1998). However, mESCs without DNMT1 are viable and the loss of DNA methylation only leads to a modest activation of retroviruses (Lei et al., 1996; Matsui et al., 2010). In addition, loss of DNMT1 in primordial germ cells does not lead to an upregulation of ERVs, suggesting alternative silencing mechanisms at certain time points of development (Liu et al., 2014b; Walsh et al., 1998). In mammalian germ cells and early embryonic development silencing of ERVs is mediated by mechanisms entailing the deposition of H3K9me3 by TRIM28-SETDB1 (Liu et al., 2014b; Matsui et

al., 2010; Rowe et al., 2010). The deletion of DNMT1 or DNMT3A/B has no effect on H3K9me3 in mESCs, showing that TRIM28-SETDB1 are the predominant ERV silencing mechanism during pluripotency (Lehnertz et al., 2003). We found that TET1, independent of DNA demethylation activity, but together with SIN3A regulates ERV expression in mESCs (Figure 11a, Publication I). Altogether, this raises several questions: What is the precise role and mechanism of TET1 in ERV silencing? Why is TRIM28-SETDB1 the predominant silencing mechanism in mESCs and PGCs? Why is DNA methylation the main ERV silencing mechanism in differentiated cells?

The expression of specific retroviruses, like ERVL elements, is critical for embryonic development and regulates networks of embryonic marker genes (Macfarlan et al., 2012). TRIM28-SETDB1 silencing might allow greater genome plasticity to regulate ERV expression, whereas DNA methylation serves as a stable silencing mechanism in differentiated cells. This would require histone modifications to have a higher turnover rate than DNA methylation. Historically, histone acetylation is received as highly dynamic and today we know that all histone modifications are reversible to some extent (Millán-Zambrano et al., 2022). The turnover rate of histone acetylation ranges from 2 to 40 min (Waterborg, 2002), while H3K9me3 is completely demethylated in 2h (Liu et al., 2017). Global DNA demethylation with a certain significance in human cells occurs after 2h (Yamagata et al., 2012) and demethylation at single genes was observed even after 20 min (Lucarelli et al., 2001). Altogether, the reported turnover rates of histone modifications and DNA methylation are rather similar and can not explain why H3K9me3 is the dominant epigenetic silencing mechanism for ERVs in mESCs and PGCs. However, a systematic quantification of histone modification and DNA methylation turnover rates in a developmental context is missing.

While we cannot conclude that the histone modification turnover rates are important for genome plasticity, we know that histone methylation and acetylation are crucial marks of epigenetic regulation (Rice and Allis, 2001). This doctoral work proposes that TET1-SIN3A/HDAC deacetylation at ERV regions is necessary for TRIM28-SETDB1 to place H3K9me3 (Figure 11a, Publication I). The hypothesis is that the H3K9 deacetylation is crucial for H3K9me3 installation and subsequent silencing. To test the hypothesis, dCas9 fused to HDAC1/2 could be targeted to specific upregulated ERVs in Tet1 KO mESCs. The removal of H3K9ac at targeted ERVs should allow the installation of H3K9me3 and the subsequent ERV repression. While this doctoral work is lacking direct mechanistic proof, the loss of H3K9me3 might explain the dissociation

of HP1 proteins and the reduction of H4K20me3 in Tet1 KO mESCs (Publication I). However, the strong global effects on HP1 localization and H4K20me3 levels suggests that also other epigenetic mechanisms are involved. The chromatin IP of TET1 identified the kinase Aurora B, which phosphorylates H3S10 subsequently evicting HP1 proteins from chromatin (Fischle et al., 2005; Hirota et al., 2005). Interestingly, preliminary analysis suggests that H3S10P is enriched in Tet1 KO mESCs in our histone modifications LC-MS/MS dataset from Publication I (Figure 11b). Further, H3S10P is well established as a mitotic mark and TET1 is described to regulate mitotic gene expression (Yamaguchi et al., 2012). These findings suggest that TET1 could directly regulate the kinase activity of Aurora B (Figure 11b) or indirectly via controlling mitotic gene networks. Future studies need to verify a direct interaction of TET1 and Aurora B, for example by performing co-immunoprecipitation and a kinase activity assay. Although still elusive at the moment, a functional interplay of TET1 and Aurora B could be an interesting study subject and an explanation for the global HP1 delocalization and H4K20me3 loss in Tet1 KO mESCs. As discussed, the mechanism by which TET1 is involved in heterochromatin formation and specific ERV regulation remains to be further characterized and will be a fascinating topic for future research.

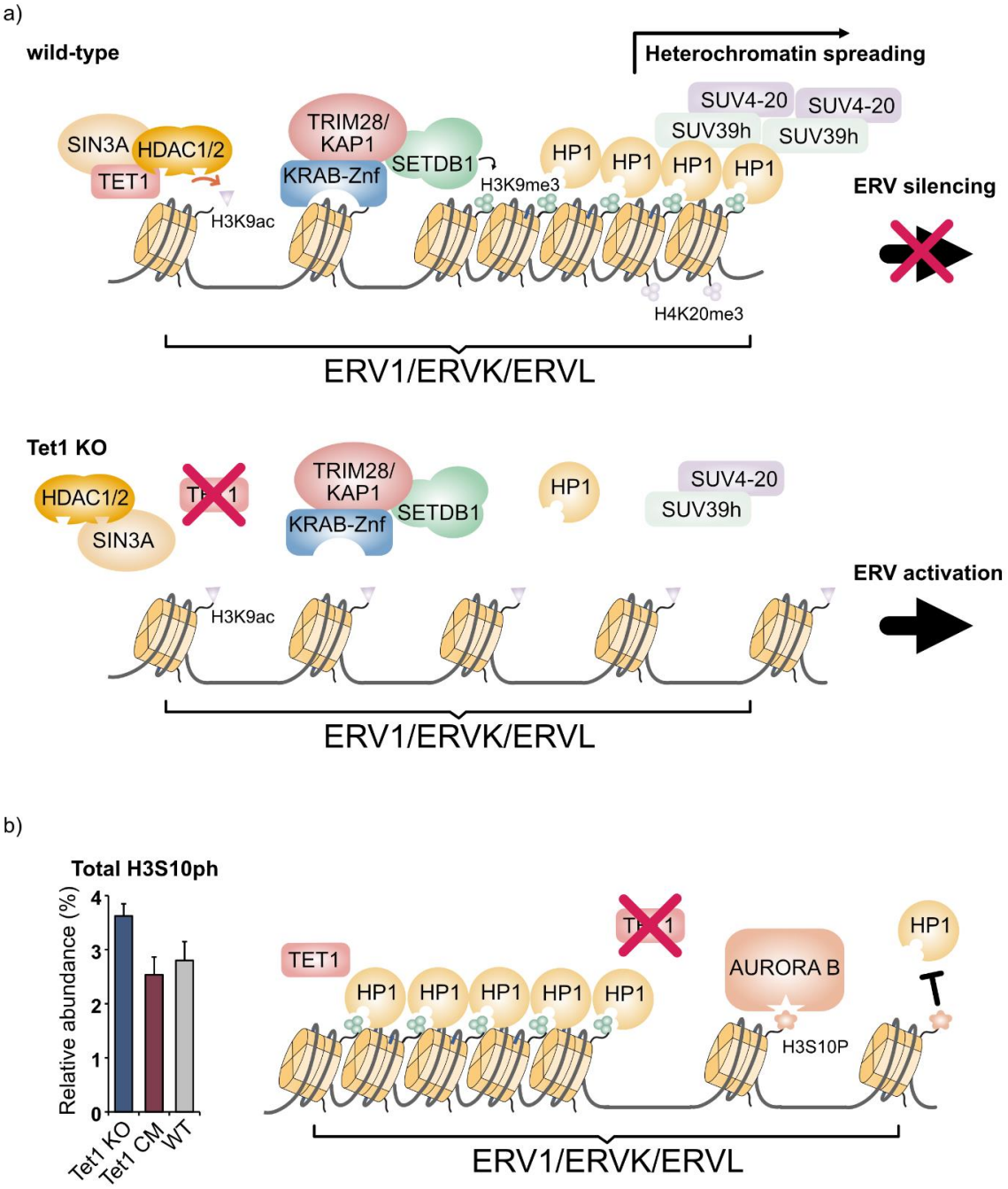


Figure 11. Potential non-catalytic mechanisms of TET1 in ERV regulation. a) Proposed model of TET1 regulating heterochromatin formation and ERV silencing independent of DNA demethylation. In wild-type mESCs, TET1 facilitates the deacetylation of H3K9ac by SIN3A-HDAC1/2. The deacetylated H3K9 is recognized by KRAB-Znf, which recruits TRIM28/KAP1 and the H3K9 methyltransferase SETDB1. HP1 proteins bind H3K9me3, recruit SUV39h and SUV4-20 for the installation of H3K9me3 and H4K20me3, leading to subsequent heterochromatin spreading and ERV silencing. In Tet1 KO mESCs SIN3A-HDAC1/2 cannot deacetylate H3K9ac at ERV elements, inhibiting heterochromatin formation and causing ERV activation. b) Relative levels of H3S10P measured by LC-MS/MS in WT, Tet1 KO and Tet1 CM

mESCs (data from Publication I, plot created from Andrey Tvardovskiy) and proposed model of TET1 and AURORA B regulating HP1 recruitment.

5.3. RNA methylation

5.3.1. METTL enzymes in development and disease

m^6A contributes to multiple cellular processes and is present on mRNA, spliceosomal RNA, non-coding RNA and 18S and 28S rRNA (He and He, 2021). In human rRNA only two m^6A sites exist, in 18S rRNA at position A1832 and in 28S rRNA at position A4220. We and others identified METTL5 as the RNA methyltransferase required for m^6A formation at position A1832 (Publication V) (van Tran et al., 2019; Xing et al., 2020). In a collaborative project I contributed to describe the importance of METTL5 for animal development, translation, pluripotency and differentiation (Publication V). However, the study lacks direct evidence that the observed phenotypes are the cause of impaired m^6A formation in 18S rRNA. The multiple drastic phenotypes upon METTL5 loss rather suggest additional functions besides 18S rRNA methylation. In the fractionation assays, 18S rRNA was the predominant RNA species losing m^6A in METTL5 KO cells, yet the experiment was performed on total RNA and it is possible that METTL5 also methylates other RNA species. To note, m^6A is the most abundant modification on mRNA (Roundtree et al., 2017) and other METTL enzymes are involved in m^6A formation on mRNA (Wong and Eirin-Lopez, 2021). A complex of METTL3/METTL14 for example regulates m^6A formation on mRNA of the pluripotency genes *Nanog*, *Klf2*, *Sox2* and *Zfp42* (Batista et al., 2014; Geula et al., 2015). Generally, METTL enzymes have been shown to be involved in pluripotency and differentiation. METTL8 inhibits c-Jun N-terminal kinase (JNK) pathway (Gu et al., 2018a) and METTL16 is involved in splicing (Mendel et al., 2018; Pendleton et al., 2017), both are important for differentiation. The loss of m^6A in METTL3 KO mESCs increases mRNA stability and translation leading to “hyperpluripotency” of mESCs (Batista et al., 2014; Geula et al., 2015). Interestingly, upon loss of METTL5 we observed the opposing phenotype to METTL3 KO mESCs. The loss of METTL5 causes a downregulation of core pluripotency markers and a differentiated morphology of mESCs (Publication V). In a next step, it will be intriguing to study whether METTL5 also methylates transcripts of core pluripotency genes and whether methylation of rRNA affects pluripotency. To test this WT and METTL5 KO mESCs can be compared

using m^{6}A sequencing (m^{6}A-seq) (Dominissini et al., 2012). Moreover, depending on different reader proteins, m6A methylation can increase or decrease the transcript stability (Roundtree et al., 2017; Wang et al., 2014b). To study the effects of METTL5 on mRNA stability, mESCs can be treated with Actinomycin D to stop RNA synthesis and the amount of RNA can be measured by RT-qPCR. In METTL5 KO mESCs pluripotency genes are downregulated (Publication V), suggesting that m^{6}A sites targeted by METTL5 promotes transcript stability. Interestingly, m^{6}A formation by METTL3 has the opposite effect and causes mRNA instability (Batista et al., 2014; Geula et al., 2015). METTL3 and METTL5 might target different m^{6}A sites and/or associate with different reader proteins promoting or decreasing RNA stability. It is tempting to speculate that METTL3 and METTL5 thereby coordinate the balance between pluripotency and differentiation.

In another collaborative study I contributed to identify METTL6 as a tRNA^{Ser}-specific 3-methylcytidine (m3C) methyltransferase (Publication VI). The loss of METTL6 in mESCs causes impaired pluripotency and promotes the differentiation towards endodermal lineages (Publication VI). METTL6 KO mice have metabolic defects but develop normally (Publication VI), which suggests that other enzymes like METTL2 (Xu et al., 2017) compensate for the loss of METTL6 during embryonic development. However, the question remains how m3C methylation of tRNA^{Ser} isoacceptors promotes specific lineages and expression of pluripotency factors. Interestingly, the loss of METTL6 leads to an increase in mRNA abundance and ribosome occupancy of lineage marker genes like *Nodal*, *Fgf4* and *Pdgfa,* but also of epigenetic enzymes like *Dnmt3b*. The upregulation of the *de novo* methyltransferase DNMT3B is in fact a hallmark of lineage priming (Publication IV). It will be interesting to study whether METTL6 directly regulates DNMT3B in mESCs or whether this is a secondary effect upon METTL6 loss. Of note, increased ribosome occupancy correlates with higher mRNA abundance and is not necessarily directly caused by the loss of tRNAs (Publication VI) (Chou et al., 2017; Thompson et al., 2016). Therefore, future studies will have to show if biological secondary effects cause the differentiation phenotype in METTL6 KO mESCs and whether m3C methylation of tRNA^{Ser} isoacceptors is directly involved in regulating the balance between pluripotency and differentiation.

While the catalytic activity of METTL enzymes is heavily studied, they also harbor the potential of catalytically independent functions. This accounts also for the group of METTL enzymes. METTL3 for instance was shown to also have non-catalytic

functions and can act as a scaffold for other proteins (Lin et al., 2016). Additionally, METTL3 can promote translation independent of its catalytic activity by recruiting translation initiation factors (Lin et al., 2016). This finding highlights the importance of including catalytic mutants into future studies to identify potential mechanisms beyond the enzymatic function of RNA modifiers. It is intriguing to speculate that METTL5 and METTL6 might also harbor functions independent of their RNA methyltransferase activity. In general, the detailed mechanisms of METTL5 and METTL6 in a developing embryo remain widely unclear. The impact of RNA modifications on pluripotency and differentiation is a fascinating study subject and future studies will shed light on the impact of METTL proteins and RNA modifications on development and disease.

5.4. Crosstalk - Histones, DNA methylation and RNA modifications

Until today, the majority of studies have described and reported about the catalytic functions of TET1 during development and disease. However, the main focus of this doctoral work was to dissect the non-catalytic functions of TET1 in mESCs. I have found that TET1 regulates chromatin, gene and ERV expression independent of DNA demethylation (Publication I). Further, I have identified TET1 as an epigenetic hub and regulator of the histone modification landscape, which correlates with the gene and ERV expression (Publication I). Despite the important non-catalytic functions of TET1 described in this doctoral work, TET1 also regulates the expression of important developmental genes, like *Dppa3* and *Lefty* via active DNA demethylation (Publication III) (Dai et al., 2016). Astonishingly, the catalytic activity of TET enzymes is not limited to DNA, but TET-mediated 5hmC also occurs on mRNA, regulating RNA stability of developmental genes (Fu et al., 2014; Lan et al., 2020). Altogether, TET1 is an incredibly versatile epigenetic player which can regulate DNA modifications, RNA modifications, histone modifications and can serve as a scaffold protein for different epigenetic modifiers. The example of TET1 perfectly demonstrates that epigenetic processes are tightly connected and that the crosstalk of epigenetic marks can be highly complex. Today, the rise of genome-wide studies more and more reveals the complex crosstalk among those epigenetic marks (Janssen and Lorincz, 2022). In the

scope of this doctoral work I studied epigenetic processes at the level of DNA, RNA and histone modifications.

TET1 regulates H3K9me3 levels at ERVs and global H4K20me3 levels (Publication I) and interestingly, DNMT1 activity at TEs was recently shown to be regulated by binding of H3K9me3 and H4K20me3 (Ren et al., 2020, 2021). Together these findings suggest a mechanism how TET1 indirectly regulates DNA methylation, namely via histone modifications and without direct DNA demethylation activity. While DNA methylation and H3K9me3 are important regulators of TEs (Janssen and Lorincz, 2022), RNA modifications and RNA-mediated mechanisms also play an important role in TE silencing. In mammals, small RNAs programme P-Element induced wimpy testis (Piwi) proteins to control TE expression (Aravin et al., 2007) and are considered to induce *de novo* DNA methylation (Aravin et al., 2008). In mESCs and in the male germline, TE-specific antisense transcripts can counteract the expression of TEs (Berrens et al., 2017) and tRNA-derived fragments (tRFs) can regulate specific ERVs (Schorn et al., 2017). Even more severe seems to be the effect of RNA modifications on TE transcripts. Recent studies show that m^6A levels control the stability and abundance of ERVs and LINE1 RNA in mESCs (Chelmicki et al., 2021; Liu et al., 2021a; Wei et al., 2022; Xu et al., 2021). The main function of m^6A is to control the RNA halflife of viral transcripts (Chelmicki et al., 2021). However, the RNA methyltransferase METTL3 can also directly interact with SETDB1 and TRIM28 and recruits them to IAP elements (Xu et al., 2021). The latest findings suggest that RNA modifications and RNA modifiers can control TE activity on a transcript level, but also by directly inducing H3K9me3-mediated heterochromatin formation. In total, the silencing of TE is mediated by DNA, RNA and histone modifications. In mESCs, DNA methylation and H3K9me3 seem to control distinct sets of TEs (Karimi et al., 2011), yet it is unclear which TEs are silenced by the different RNA mechanisms and how they are coordinated with DNA and histone modifications. The crosstalk of epitranscriptomics and epigenetics is not restricted to TEs, but is part of many biological processes (Kan et al., 2022). Importantly, epigenetics events like global DNA hypomethylation, GpG island hypermethylation and loss of specific histone marks are hallmarks of cancer (Fahrner et al., 2002; Fraga et al., 2005; Nguyen et al., 2002; Nishiyama and Nakanishi, 2021). Different RNA modifications and modifiers are also deregulated and hijacked by cancer cells (Jonkhout et al., 2017; Li et al., 2017; Liu et al., 2021c; Su et al., 2018). This raises the question how and whether the

interplay of epigenetic marks is linked to the development of various types of disease. Taking into account the close interplay among those epigenetic marks, it will be an important task to decipher the mechanistic interaction between enzymes, DNA, RNA and histone modifications to understand their implications for fundamental biological processes and future drug development.

6. Declaration of contributions as a co-author

Critical Role of the UBL Domain in Stimulating the E3 Ubiquitin Ligase Activity of UHRF1 toward Chromatin.

This study was designed and conceived by Ben Foster and Till Bartke. Paul Stolz created stable UHRF1 mutant cell lines using CRISPR/Cas, cultured embryonic stem cells, performed Immunofluorescence experiments, analyzed data and contributed to writing the manuscript.

Recent evolution of a TET-controlled and DPPA3/STELLA-driven pathway of passive DNA demethylation in mammals.

This study was designed and conceived by Christopher B. Mulholland, Sebastian Bultmann and Heinrich Leonhardt. Paul Stolz helped with cell line validation and performed fluorescence microscopy analysis.

Distinct and stage-specific contributions of TET1 and TET2 to stepwise cytosine oxidation in the transition from naive to primed pluripotency.

This study was designed and conceived by Christopher B. Mulholland, Sebastian Bultmann and Heinrich Leonhardt. Paul Stolz helped to culture embryonic stem cells and performed western blot experiments to validate the expression levels of the different TET proteins.

The rRNA m6A methyltransferase METTL5 is involved in pluripotency and developmental programs.

This study was designed and conceived by Valentina Ignatova and Robert Schneider. Paul Stolz generated stable METTL5 knock-out embryonic stem cells using CRISPR/Cas, performed differentiation experiments, studied the pluripotency phenotype and analyzed data.

METTL6 is a tRNA m3C methyltransferase that regulates pluripotency and tumor cell growth.

This study was designed and conceived by Valentina Ignatova and Robert Schneider. Paul Stolz generated stable METTL6 knock-out embryonic stem cells using CRISPR/Cas, performed embryoid body differentiation and studied the pluripotency phenotype.

_____	_____
Paul Stolz	Heinrich Leonhardt

7. Abbreviations

2OG	2-oxoglutarate
5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxmethylcytosine
5hmU	5-hydroxymethyluracil
5mC	5-methylcytosine
6mA	6-methyladenine
aa	Amino acid
AID	Auxin-inducible degron
BER	Base excision repair
bp	Base pair
BPTF	Bromodomain PHD-finger Transcription Factor
Cas	CRISPR associated
CENP-A	Centromere Protein A
ChIP	Chromatin Immunoprecipitation
CM	Catalytic mutant
CNS	Central nervous system
CpG	Cytosine-phosphate bond-guanine
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR-RNA
dCas9	Dead Cas9
dTag	Degron tag
DNA	Deoxyribonucleic acid
DMD	Duchenne muscular dystrophy
DME	5mC DNA glycosylase
DNMT	DNA methyltransferase

dNTPs	Desoxy nucleosidtriphosphat
DPPA3	Developmental pluripotency-associated protein 3
DSB	Double-strand break
E	Embryonic day
ERV	Endogenous retrovirus
Fe(II)	Iron(II)
H1	Histone 1
H2A	Histone 2A
H2B	Histone 2B
H3	Histone 3
H4	Histone 4
HC	Heterochromatin
HDAC	Histone deacetylase
HDR	Homology directed repair
HMCES	5-Hydroxymethylcytosine Binding, ES Cell Specific
HP1	Heterochromatin Protein 1
hTRM9L	tRNA methyltransferase 9-like
IAP	Intracisternal A-particle
ICM	Inner cell mass
kb	kilobase
KRAB-Znf	Krüppel-associated box domain zinc finger
LC-MS	Liquid chromatography - mass spectrometry
LIG1	DNA Ligase 1
LINE	Long interspersed nuclear elements
m^6A	*N*6-methyladenosine
m^7G	N^7-methylguanosine
MCM3	Minichromosome maintenance complex component 3
mEpiLC	Mouse epiblast-like cells
mESC	Mouse embryonic stem cell

METTL	Methyltransferase-like
MLL	Mixed lineage leukemia
MOF	Males absent on the first
mRNA	Messenger RNA
NGS	Next generation sequencing
NHEJ	Non-homologous end joining
NONO	Non-POU Domain Containing Octamer Binding
NPM1	Nucleophosmin 1
NSC	Neural stem cell
NSUN2	NOP2/Sun RNA methyltransferase 2
N-terminus	Amino-terminus
OGT	O-linked N-acetylglucosaminyltransferase
ORC2	Origin recognition complex subunit 2
PAM	Protospacer adjacent motifs
PAF15	PCNA-associated factor of 15 kDa
PCNA	Proliferating cell nuclear antigen
PRC2	Polycomb Repressive Complex 2
PGC	Primordial germ cell
PHD	Plant homeodomain
piRNA	PIWI-interacting RNA
PTM	Post-translational modification
QSER1	Glutamine and Serin Rich 1
RBBP	Retinoblastoma-Binding Protein
RING	Really interesting new gene
RNA	Ribonucleic acid
ROS1	Repressor of silencing 1
RP	Retinitis pigmentosa
rRNA	Ribosomal RNA
SALL4	Spalt Like Transcription Factor 4

SHERLOCK	Specific High Sensitivity Enzymatic Reporter Unlocking
SIN3A	SIN3 Transcription Regulator Family Member A
snRNA	Small nuclear RNA
SRA	SET- and RING-associated
SUV39h	Suppressor of variegation 3-9 homolog
SUV4-20	Suppressor of variegation 4-20
TALENS	Transcription activator-like effectors
TDG	Thymine DNA glycosylase
TE	Transposable element
TET	Ten-eleven-translocation
TET3o	TET3 oocyte
TET3s	Short TET3
TF	Transcription factor
TKO	Triple knockout
TRIM28/KAP1	Tripartite Motif Containing 28/KRAB-associated protein-1
tRNA	Transfer RNA
TTD	Tandem tudor
TTR	Transthyretin
UBL	Ubiquitin-like
UHRF1	Ubiquitin-like containing PHD and RING finger domains 1
VP64	VP16 activation domain
WGBS	Whole genome bisulfite sequencing
WT	Wild-type
ZFNs	Zinc-finger nucleases

8. References

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