Review

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RNA-mediated heterochromatin formation at repetitive elements in mammals

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Abstract

The failure to repress transcription of repetitive genomic elements can lead to catastrophic genome instability and is associated with various human diseases. As such, multiple parallel mechanisms cooperate to ensure repression and heterochromatinization of these elements, especially during germline development and early embryogenesis. A vital question in the field is how specificity in establishing heterochromatin at repetitive elements is achieved. Apart from *trans*-acting protein factors, recent evidence points to a role of different RNA species in targeting repressive histone marks and DNA methylation to these sites in mammals. Here, we review recent discoveries on this topic and predominantly focus on the role of RNA methylation, piRNAs, and other localized satellite RNAs.

Keywords chromatin; development; repeats; RNA; transposons
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Introduction

Genomes are complex ecosystems that have been shaped by the two major forces of natural selection and genetic drift. They are inhabited by diverse communities of selfish genetic elements as well as inert, opportunistic sequences. Opposite selective forces create a balance between parasitic expansion and host homeostasis, which eventually benefits both sides (Rebollo *et al*, 2012; Chuong *et al*, 2017; Platt *et al*, 2018). In line with this functional relevance, repetitive elements are not randomly distributed across the genome, but rather reside in specific chromatin regions, which allow their tight control (Bourque *et al*, 2018). The developing germline is considered the major battlefield in the war against these elements. Animals, such as mammals, in which the germ cells are specified from the soma, face an additional threat, as reprogramming of the epigenome provides windows of opportunity for their expansion (Ozata *et al*, 2019). Studies over the last decades have shown that most of these elements rely on transcription in one way or another to execute their function (Bourque *et al*, 2018; Smurova & De Wulf, 2018; Talbert & Henikoff, 2018). How cells harness this dependency to conspire their strategies against them is going to be the focus of this review.

Seminal studies in many model organisms such as fission yeast, nematodes, and flies, have established the eminent role of RNA molecules in this process (Holoch & Moazed, 2015; Martienssen & Moazed, 2015; Bhattacharjee *et al*, 2019; Weiser & Kim, 2019; Duempelmann *et al*, 2020; Padeken *et al*, 2022). Recent evidence from mammalian systems further supports the notion of context-specific RNA-mediated recruitment of chromatin modifying complexes as a general mechanism across species. Since the canonical mechanisms of silencing repetitive elements by heterochromatin have been extensively reviewed in the past, here we will only briefly discuss it and instead focus on the emerging role of RNA molecules in heterochromatic silencing in mammalian cells (Goodier & Kazazian, 2008; Rowe & Trono, 2011; Leung & Lorincz, 2012; Goodier, 2016; Groh & Schotta, 2017; Kwon & Chung, 2020; Padeken *et al*, 2022). In this review, we examine specifically how low-level transcription of repetitive elements and the resulting RNA transcript contribute to heterochromatin formation in mouse and human cells. First, in the following chapter and accompanying boxes, we will introduce the different types of repetitive elements present in mammalian genomes and briefly outline the general schemes and patterns used to silence repeats across different species.

Diversity of repetitive elements and general mechanisms for their silencing

The sequence complexity of the mammalian genomes is diverse, and a relatively large portion of it comprises repetitive sequences. These sequences can be categorized based on their degree of repetitiveness, their organization, or their functional role. They can be found in both coding and non-coding regions and can be arranged in tandem or dispersed across the genome (López-Flores & Garrido-Ramos, 2012; Garrido-Ramos, 2017). Those that are arranged in tandem are mainly satellite sequences (Box 1), whereas transposable elements (TE, Box 2) comprise the dispersed portion of the repetitive sequences. According to the Repeat Masker database (Smit *et al*, n.d.), together both repeat groups account for ~52 and ~45% of the human and the mouse genome respectively. Satellite sequences and TEs are traditionally linked to constitutive heterochromatin (Box 3); however, their location within the genome might

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Box 1. Satellite sequences

Named after the distinct characteristic "satellite" bands that they form when applied on density-gradient of cesium chloride, satellite sequences consist of a variable number of tandem repeats (VNTRs). Conventionally, depending on the length of the repeat unit, VNTRs can be subdivided into microsatellites (< 10 nucleotides - also known as Simple Sequence Repeats or Short Tandem Repeats), mini-satellites (≥ 10 nt) or satellites (≥ 150 nt; Gemayel *et al*, 2010). The former two groups, share many common characteristics, such as that they can be found in protein-coding, non-coding, and regulatory regions, they exhibit higher mutation rates than the average in the genome, and they are highly polymorphic, that is, exhibiting variations in the number of repeat units among individuals in a population (Fan & Chu, 2007). It has been estimated that around 10–20% of eukaryotic genes and promoters contain unstable repeat series. Specific gene families are significantly enriched in such elements, which suggests that they might be subjected to higher evolution rates (Gemayel *et al*, 2010; López-Flores & Garrido-Ramos, 2012). Additionally, microsatellite instability has been linked to many genetic diseases such as Huntington disease, spinobulbar muscular atrophy (SBMA-Kennedy disease), spinocerebellar ataxias, fragile X syndrome, Friedreich ataxia, myotonic dystrophy type 1 and 2 and others (Madireddy & Gerhardt, 2017; Orr & Zoghbi, 2007).

Satellite DNA is a major component of constitutive heterochromatin, mainly located in the centromeric, pericentric and subtelomeric regions of chromosomes. Typically, they consist of 150–180 or 300–360 bp, corresponding to mono- and di-nucleosomes, and account for 2.6 and 0.2% of the human and mouse genome respectively (Garrido-Ramos, 2017). Their monomers might include micro- and/or mini-satellites and they are usually organized in long arrays of hundreds, or thousands, of not strictly identical copies. The primate-specific α-satellite elements are a remarkable example of centromeric repeats with functional relevance. While their monomers consist of 171-bp, they are usually found as dimers. Most monomers contain a binding site for the Centromere Protein B (CENP-B), a protein that enhances recruitment of the centromere-specific H3 histone CENP-A and facilitates kinetochore formation and function (Fachinetti *et al*, 2015; Otake *et al*, 2020). Satellite DNA sequences are also predicted to form secondary structures such as non-beta form DNA, G-quadruplexes, and dimeric i-motif structures, which can potentially act as platforms of protein recruitment or exclusion. Such structures are thought to be involved in chromatid cohesion post replication and telomere maintenance (Thakur *et al*, 2021). Furthermore, different types of satellite sequences carry distinct heterochromatin components which are associated to specific epigenetic features such as replication timing, nuclear localization and chromatid cohesion (Guenatri *et al*, 2004; Otake *et al*, 2020). In mice for instance, centromeric minor satellite chromatin is organized with CENP-A/B/C molecules while the pericentric major satellite repeats are not. Such distinct chromatin features allow major satellite sequences from different chromosomes to cluster together during interphase, and also prolong resolution of sister chromatids at these sites until metaphase (Guenatri *et al*, 2004). Thus, satellite sequences can play an important role in maintaining genome stability throughout the cell cycle.

Box 2. Transposable elements

Transposable elements (TEs), or transposons, are interspersed repeat sequences which, unlike satellites, either have or had the ability to change their location around the genome. In principle, TEs carry the regulatory and the coding sequences to catalyze their self-transposition. However, a large number of them rely on the functional machinery encoded by other copies of TEs, distinguishing them into autonomous and non-autonomous transposons. Their ability to move and replicate affects the genome by generating plasticity and heterogeneity (Goodier & Kazazian, 2008; Bourque *et al*, 2018). They comprise about 48.49 and 41.73% of the human and mouse genome respectively, and can be classified into two major classes (retrotransposons and DNA transposons), based on the presence or absence of an RNA intermediate involved in their mechanism of transposition. According to the classification system proposed by Wicker *et al* (2007, 2009a, 2009b), TEs can be further subdivided into orders, based on the insertion mechanism they deploy, a distinction which best reflects major differences in their structural organization.

Class I elements, best known as retrotransposons, are the major contributors of the repetitive fraction in mammals and they can be categorized into five orders, that is, long terminal repeat (LTR) retrotransposons, the long and short interspersed nuclear elements (LINEs & SINEs), the Penelope-like and the *Dictyostelium* intermediate repeat sequence-like (DIR-like) elements. Their transposition relies on an RNA intermediate which is reversed transcribed by a TE-encoded reverse transcriptase. This mechanism is usually described as a "copy & paste" due to the fact that each transposition event produces a new copy, integrated into a new location. On the other hand, Class II elements, also known as DNA transposons, are found in all eukaryotes. Such TEs account for 3.7 and 1.4% of the human and the mouse genome respectively, and their mechanism of transposition can be categorized as "cut & paste" (Kapitonov & Jurka, 2008). In humans, Class II elements, that is, DNA transposons have been inert for the past 50 million years with the last evidence of activity before the divergence between humans and new world monkeys (Lander *et al*, 2001).

Despite their large numbers, the vast majority of TEs progressively erode until they become no longer capable of transposition. For instance, it has been estimated that more than 99.9% of the ~500,000 copies of human LINE1 elements are fixed and immobile, with about 100 of them, known as "hot LINE1s", remaining active (Sassaman *et al*, 1997; Brouha *et al*, 2003). Still, transposable elements are an important source of mutations and polymorphisms in mammals. In laboratory mice, TE insertions are responsible for ~10% of all *de novo* mutations (Maksakova *et al*, 2006), while for humans it is estimated that this rate is ~1/21 births for 7SL-Alu (Xing *et al*, 2009) and 1/95–1/270 births for LINE1 elements (Ewing & Kazazian, 2010). More than 120 of such insertions have been associated with human diseases such as hemophilia A & B, cystic fibrosis, Duchene muscular dystrophy, beta-thalassemia, hypercholesterolemia, Apert syndrome, neurofibromatosis, leukemias and cancers (Cordaux & Batzer, 2009; Hancks & Kazazian, 2016). Thus, controlling TE expression is of great importance for genome stability and health.

deviate, with regards to the evolutionary history of each repeat type (López-Flores & Garrido-Ramos, 2012; Sultana *et al*, 2017).

Molecular mechanisms controlling the expression of repeats always require sophisticated pathways that can discern these elements from their own genes. One such pathway relates to intronless transcription, a particular feature of repeat RNAs which rely on reverse transcription. Importantly, this feature is sufficient to initiate a specific innate immunity response, a cellular mechanism also deployed against retroviruses (Seczynska *et al*, 2022). Other mechanisms for repeat recognition have also evolved and relate to the DNA sequence of repeats themselves. Both processes allow for the deployment of distinct strategies to old better adapted elements, and young, less mutated, and likely more deleterious ones. The suppression of elements via DNA-binding proteins, such as transcription factors (TF), is frequently used to target evolutionary old families

Box 3. Heterochromatin in mammals

Chromatin organization differs in space and time, both in terms of its epigenetic marks and the level of compaction. These differences influence accessibility to genetic information, thereby allowing for timely and selective expression of genes. Nevertheless, chromatin organization serves additional roles in the nucleus, such as facilitating attachment to nuclear lamina, chromosome segregation, prevention of telomere erosion, and silencing of repetitive elements (Allshire & Madhani, 2018; Janssen *et al*, 2018). These rather "housekeeping" functions are extremely important in maintaining genome stability and are particularly linked to condensed chromatin, termed as heterochromatin.

Differentiating it from euchromatin based on cytological examination, heterochromatin was initially described as the portion of chromatin that consistently fails to decondense following mitosis. Research over the past decades has shown that this definition better reflects a constitutive, rather than the cell-type specific and developmentally regulated heterochromatin, now referred to as facultative heterochromatin. Different "flavors" of heterochromatin, as a concept, have been proposed and discussed in the past (Allshire & Madhani, 2018; Janssen *et al*, 2018; Thakur *et al*, 2021; Żylicz & Heard, 2020). Typically, heterochromatin forms at the centromeres, the telomeres, as well as their adjacent regions (pericentric/pericentromeric and subtelomeric respectively). The main features of constitutive heterochromatin in mammalian genomes are the enrichment for certain marks, for example, 5-methyl-cytocine (5mC) on DNA, histone H3 di- and tri- methylation on Lysine 9 (H3K9me2/3) as well as histone H4 Lysine 20 trimethylation (H4K20me3); the absence of histone acetylation; the presence of the heterochromatin protein 1 homologs (HP1α/β/γ); the distinct subnuclear localization in the periphery or in chromocenters; and the presence of repetitive sequences (Janssen *et al*, 2018). Most of these characteristics share a high degree of interdependence and constitute multiple lines of protection that secure the functionality of heterochromatin.

Efficient heterochromatinization depends primarily on histone modifications. For instance, H3K9me2/3 marks are essential for both establishment and maintenance of heterochromatin. Numerous enzymes involved in this process, including the G9A:GLP complex, which is responsible for deposition of H3K9 mono- and di-methylation, and the SETDB1/2 and SUV39H1/2 complexes, which catalyze di- and tri- methylation of H3K9 residues (Janssen *et al*, 2018; Padeken *et al*, 2022). These Lysine methylation marks, in turn, can be recognized by proteins containing specific domains such as the chromodomain, the Tudor domain and others (Yap & Zhou, 2010, 2011). Such proteins can either be enzymes themselves or act as platforms for recruitment of additional factors. A remarkable example is the HP1 protein which can recognize H3K9me3 via its chromodomain, bind RNA molecules, and recruit additional HP1 units, as well as the SETDB1, SUV39H1/2 and SUV420H1/2 enzymes (Muchardt *et al*, 2002; Schultz *et al*, 2002; Yamamoto & Sonoda, 2003; Schotta *et al*, 2004; Bosch-Presegué *et al*, 2017). These unique characteristics of HP1 place it at the center of heterochromatin formation, maintenance, and spreading.

In addition to histone modification, DNA methylation is also the major type of chromatin modification and it has been reported that the enzymes required for H3K9 methylation, such as G9a, SETDB1 & SUV39H1/2, can influence *de novo* DNA methylation, which is highly correlated with gene silencing (Lehnertz *et al*, 2003; Dong *et al*, 2008; Leung *et al*, 2011; Liu *et al*, 2014; Du *et al*, 2015). In almost all somatic cells of mice and human, ~70% of every cytosine in the context of CpG dinucleotides is found to be methylated (Li & Zhang, 2014). During germline development this DNA methylation is erased nearly completely, only to be re-established in the gametes in a sex-specific manner. Following fertilization, these levels will once again become severely reduced before establishing somatic patterns during implantation (Smith & Meissner, 2013; Greenberg & Bourc'his, 2019; Zeng & Chen, 2019). The enzymes responsible for deposition of the mark and maintenance through multiple cell cycles are DNMT3A/B:DNMT3L complexes and DNMT1 respectively. Genetic ablation of DNMT1 in somatic cells or the mouse embryo is lethal due to increased genomic instability highlighting its role in TE repression (Li *et al*, 1992; Jackson-Grusby *et al*, 2001; Chen *et al*, 2007). Additionally, both the mark itself and the DNMT proteins can directly or indirectly recruit chromatin modifying complexes, such as histone deacetylases (HDACs) and the heterodimeric histone methyltransferase complex G9a:GLP (Jones *et al*, 1998; Nan *et al*, 1998; Fuks *et al*, 2001; Deplus *et al*, 2002; Estève *et al*, 2006). Together, this chromatin machinery allows for stable repression of heterochromatic regions particularly keeping repetitive loci in check.

with higher mutational burden, even if they are not actively transcribed. However, a recent study argues for the importance of TF-based suppression for young families as well (Wolf *et al*, 2020). While mechanisms resulting in repeat silencing have diverged among species these basic RNA- and DNA-dependent strategies of repeat targeting are used throughout the animal kingdom.

Three major RNA-based strategies are deployed against repetitive elements across species, generally referred to as transcriptional, co-transcriptional, and post-transcriptional gene silencing (TGS, CTGS, and PTGS). This review will focus on the TGS, where the major mode of action involves retention of the nascent unspliced RNA repeat transcripts on chromatin. These transcripts in turn act as platforms for co-operative assembly of complexes that facilitate heterochromatin establishment and spreading over repetitive elements. Once the heterochromatin environment is established, chromatin-binding proteins further stabilize the retention of repeat RNAs on chromatin, hence sustaining the cycle of transcription-dependent chromatin silencing. As these nascent transcripts can in principle be deleterious for cells, it is of no surprise that this type of TGS is often found tightly interconnected with CTGS and PTGS, which are in essence sophisticated nuclear or cytoplasmic RNA decay systems respectively. Exciting findings revealed additional layers of regulation at the level of transcription elongation, splicing control, and translation; however, such mechanisms fall out of the scope of this review (Guang *et al*, 2010; Wilson & Doudna, 2013; Wang *et al*, 2015; Attig *et al*, 2018). All in all, the concerted activity of RNA- and DNA-dependent strategies offers leakproof repression of repeat expression at every level.

As mentioned before, establishment of such stable silencing typically requires for the retention of repeat transcripts on chromatin. Indeed, studies in budding yeast, and in other model organisms, suggest that slowly processed transcripts can be actively retained on chromatin and then targeted for degradation in an exosome-dependent manner (Custodio *et al*, 1999; Zenklusen *et al*, 2002; de Almeida *et al*, 2010; Eberle *et al*, 2010; Kallehauge *et al*, 2012; Muniz *et al*, 2021). Many interesting hypotheses were proposed on the role, the control, as well as the mechanisms of action of chromatin-associated RNAs (Dinger *et al*, 2009; Deveson *et al*, 2017; Smurova & De Wulf, 2018; Talbert & Henikoff, 2018; Li & Fu, 2019; Muniz *et al*, 2021; Trigiante *et al*, 2021) Inspiring work across species has provided insight into how specificity could be achieved in different contexts (Wilson & Doudna, 2013; Holoch & Moazed, 2015; Martienssen & Moazed, 2015; Duempelmann *et al*, 2020). In many cases, there is involvement of protein complexes containing members of the Argonaute protein family in association with small RNA molecules that act as specificity factors via base pairing. These

processes were best described in *Schizosaccharomyces pombe* (Holoch & Moazed, 2015; Martienssen & Moazed, 2015; Duempelmann *et al*, 2020), where double-stranded RNA precursors, generated from pericentric DNA repeats are processed into small interfering RNAs (siRNAs) primarily by the collaborative action of the RNA-dependent RNA polymerase complex (RDRC) and the ribonuclease Dicer 1 (Dcr1p; Volpe *et al*, 2002; Motamedi *et al*, 2004; Verdel *et al*, 2004; Martienssen & Moazed, 2015). Thanks to RDRC activity the RNAi response is amplified, and siRNAs are then loaded to an Argonaute-containing complex, referred to as RNA-induced transcriptional silencing (RITS) complex. The loaded RITS is responsible for directing and tethering of the H3K9 methylation machinery to proximal nucleosomes (Irvine *et al*, 2006; Schalch *et al*, 2009). H3K9 methylation in turn recruits homologs of the vertebrate heterochromatin protein 1 (HP1) and co-repressor complexes (see Box 3). This allows for histone deacetylation and heterochromatin spreading. Likewise, RDRC and RITS complexes are also capable of recognizing H3K9 methylation marks, hence further sustaining siRNA biogenesis, heterochromatin maintenance and spread (Volpe *et al*, 2003; Bühler *et al*, 2006). Similar RNA interference-dependent (RNAi) pathways have been identified in other model organisms such as *Caenorhabditis elegans* (Gu *et al*, 2012; Weiser & Kim, 2019; Duempelmann *et al*, 2020) and *Droshophila melanogaster* (Pal-Bhadra *et al*, 2004; Bhattacharjee *et al*, 2019). These systems provide elegant models for studying how RNAs mediate specific targeting of heterochromatin formation to repeat DNA.

Even though RDRC/RITS complexes do not exist in mammals, Dicer can still downregulate the expression of centromeric repeats and transposable elements in mouse embryonic stem cells (mESCs; Kanellopoulou *et al*, 2005). An alternative class of small RNAs has also evolved to repress TEs in the germline of many organisms including mammals. These so-called Piwi-interacting RNAs (piRNAs) are defined by their interaction with the Piwi clade of Argonaute proteins. They are primarily derived from single-stranded long non-coding RNAs, from loci which can be described as graveyards of TEs. piRNAs also profit from an RDRC-independent amplification mechanism that is often referred to as the "ping-pong" cycle. In mice, it relies on the RNA slicing capacity of the protein called Piwi-like 2 (PIWIL2 or MILI). Their biogenesis and function in restricting retrotransposons has been extensively reviewed elsewhere (Ernst *et al*, 2017; Czech *et al*, 2018; Ozata *et al*, 2019). Importantly for this review, piRNAs in mammals not only act at the level of PTGS, but are also involved in regulating heterochromatin and TGS of repetitive elements. However, before describing these new discoveries, it is pertinent to briefly introduce why repetitive elements need to be controlled, as well as the canonical RNA-independent pathways involved in their silencing.

Canonical silencing of repetitive elements in mammals

Both satellite repeats and transposable elements act at the verge of evolution and numerous detailed previous reviews have discussed their role in reshuffling the genome, especially at regulatory elements, and in acting as building blocks for new genes (Goodier & Kazazian, 2008; Cordaux & Batzer, 2009; Rebollo *et al*, 2012; Elbarbary *et al*, 2016; Bourque *et al*, 2018; Platt *et al*, 2018; Rodriguez-Terrones & Torres-Padilla, 2018; Thakur *et al*, 2021; Fueyo *et al*, 2022). There are multiple examples of "domestication", or so-called exaptation of transposable elements as they acquire new and important regulatory functions (Gerdes *et al*, 2016). For instance, mouse oocytes express an alternative isoform of Dicer, driven by an intronic LTR retrotransposon, and this specific TE insertion is particularly important for meiosis and female fertility (Flemr *et al*, 2013). However, many changes to a repetitive element are not well tolerated by the cells and can lead to catastrophic consequences.

Mobilization and aberrant transcription of transposable elements, poses a potentially lethal threat to cells, in particular their genomic integrity and transcriptional regulation (Orr & Zoghbi, 2007; Hancks & Kazazian, 2016). In case of LINE1 elements for example, the very mechanism of transposition itself results in novel insertions and occasionally also small deletions (Moran *et al*, 1996; Gilbert *et al*, 2002; Beck *et al*, 2011; Richardson *et al*, 2014). Another remarkable example of TE interference with the gene activity is that of Intra-cisternal A Particles (IAPs). Such elements can act as sites of heterochromatin nucleation and spread into flanking regions, as assessed by chromatin profiling of H3K9me3 and H4K20me3 marks (Rebollo *et al*, 2011). Though in rare cases, this results in transcriptional silencing of nearby genes, hence potentially jeopardizing cell fitness. TEs can also form new gene regulatory elements. For example, an IAP element inserted up to 100 kb upstream the Agouti gene acts as a cryptic promoter and drives ectopic expression of the latter. As a result, incomplete epigenetic silencing of the element leads to mosaic Agouti expression, and the heritable pleiotropic phenotype of yellow fur, obesity, diabetes, and increased tumor-susceptibility (Duhl *et al*, 1994; Morgan *et al*, 1999). It is now well-documented that transposable elements are primary sources of mutations (see Box 2) and can interfere with regulation of gene expression at multiple levels. Both features are tightly linked to disease (Hancks & Kazazian, 2016). Therefore, heterochromatin formation and silencing of repetitive elements should in most cases be stable, even though at some developmental stages their de-repression can provide a source of genetic variability.

Stable repression is particularly important for TEs, which account for the more abundant and active portion of repetitive elements. In almost every somatic tissue, TEs are kept silenced within the heterochromatin compartment, which is established by the concerted action of multiple factors. Apart from the RNA-mediated mechanisms the canonical RNA-independent repression plays a vital role. The latter process typically depends on Kruppel-associated box (KRAB)-containing zinc finger proteins. This class of TFs can recognize and bind to retroviral DNA sequences resembling the structure of LTRs (see Box 2) and recruit the KRAB domain binding corepressor KAP1/TRIM28. These corepressors in turn can induce heterochromatin formation by recruiting histone deacetylases, the DNA methylation machinery, heterochromatin protein 1 (HP1), and the histone methyltransferase SETDB1/ESET (Friedman *et al*, 1996; Schultz *et al*, 2001; Schultz *et al*, 2002; Sripathy *et al*, 2006; Wolf & Goff, 2009; Rowe *et al*, 2010; Quenneville *et al*, 2012; Turelli *et al*, 2014; Wolf *et al*, 2015). Unlike the RNA-dependent repression, KRAB factors can also target mutated TEs, which are no longer transcribed but retain the necessary sequence motif for zing finger domain binding. Moreover, this canonical pathway can also maintain repression in cellular contexts where heterochromatin would not allow for even low-level TE expression.

Although various mechanisms contribute to TE silencing at the chromatin level across tissues, their relative importance is context specific. For instance, while the role of H3K9 histone methyltransferases in TE silencing of mouse ESCs is well documented (Hutnick *et al*, 2010; Matsui *et al*, 2010; Rowe *et al*, 2010; Karimi *et al*, 2011; Bulut-Karslioglu *et al*, 2014; He *et al*, 2015; He *et al*, 2019), DNA methylation seems to be particularly crucial in maintaining TE silencing in somatic tissues (Walsh *et al*, 1998; Hutnick *et al*, 2010; Roulois *et al*, 2015). Conversely, extensive DNA demethylation in mouse ESCs is well tolerated leading to only a transient TE upregulation, which is subsequently reversed thanks to *de novo* deposition of H3K27me3 and H3K9me2/3 repressive marks (Walter *et al*, 2016). In line with these findings, TEs' DNA methylation is partially erased at two developmental stages, that is, pre-implantation stages and germline development (Greenberg & Bourc'his, 2019), two periods when cells reprogram their epigenome to acquire either parent-independent or sex-specific chromatin patterns, respectively. These developmental windows are thus particularly sensitive to derepression of transposons. Moreover, how silencing complexes are specifically targeted to TEs in the absence of DNA methylation is an active field of research. One intriguing finding comes from germline development in mice, where Alexei Aravin and others have identified a male-specific RNA pathway that mediates TE silencing, both in an epigenetic and in a post-transcriptional manner (Aravin *et al*, 2006, 2008; Kuramochi-Miyagawa *et al*, 2008, 2010; Ernst *et al*, 2017; Greenberg & Bourc'his, 2019; Ozata *et al*, 2019). While RNA-mediated regulation of TE silencing in other organisms had been extensively studied (Kwon & Chung, 2020), its role in mammals and its potential interaction with chromatin repressive mechanisms remained unclear until recently. In the following section, we will discuss recent advances in this field.

RNA-mediated silencing of repetitive elements in mammals

Chromatin-associated RNAs

While much is known about how heterochromatin maintains silencing of repetitive elements, the question of how it is targeted in the first place remains more elusive. The KRAB/TRIM28 pathway is an elegant example of how this can be achieved through trans-acting DNA binding proteins with silencing activity. However, a large proportion of repetitive elements are heterochromatinized independently of TRIM28 (Rowe *et al*, 2010; Maksakova *et al*, 2013). Interestingly, nuclear RNAs have recently emerged as alternative mediators of specificity for silencing both *in cis* and *in trans*.

In the nucleus, RNAs can be found either in a soluble state in the nucleoplasm, associated with the nuclear matrix or the chromatin itself. Chromatin-associated RNAs (caRNAs) can be divided into either nascent transcripts that remain at their site of synthesis, acting *in cis*, or transcripts that are released from the transcription site in order to act at different loci, known as *trans*-acting RNAs. Even though caRNAs can be found almost everywhere in the genome, in most cases their function remains elusive. They are generated mainly from genic sequences (~80%, including long ncRNA), primarily intronic, while their targets can be both protein coding and intergenic regions (Li *et al*, 2017; Zhou *et al*, 2019; Bonetti *et al*, 2020). Based on chromatin-fractionation protocols (see Box 4), a large portion of the caRNAs, are mapped to promoters, enhancers and importantly for this review also repeat elements. For example in a recent study, Xu *et al* found that more than 50% of the IAPez elements (Intra-cisternal A Particles), which belong to the family of LTR-ERV retrotransposons, associate with chromatin at their sites of origin thus highlighting their potential for regulating chromatin state.

Despite the highly context-specific mode of action of caRNAs, certain principles can be described. In naïve mESCs, Li *et al* (2017) reported that around 63% of caRNAs act in trans and the vast majority of them (~90%) exhibit non sequence-specific interactions. In fact, most of the regulatory caRNAs are likely to be found in complexes with RNA-binding proteins (Lunde *et al*, 2007; Hentze *et al*, 2018), which can recognize an RNA sequence motif, the structure of the RNA molecule itself (single stranded, double stranded etc), a higher-order structure, such as a stem-loops and pseudoknots, a specific RNA-modification or a combination of the above. With regards to their function, traditionally caRNAs include the fraction of the nascent pre-mRNAs, the small nuclear RNAs (snRNAs) of the splicing machinery, some small nucleolar RNAs (snoRNAs), long non-coding (lncRNAs), promoter upstream transcripts (PROMTs), enhancer RNAs (eRNAs), stable intronic sequence RNAs (sisRNAs), and the repeat-derived RNAs which we will focus on in the remainder of this review (Chan & Pek, 2019; Li & Fu, 2019; Khelifi & Hussein, 2020; Agostini *et al*, 2021).

The inherent characteristics of RNA molecules render them highly potent and versatile candidates for chromatin regulation. First, their ability of base pairing provides an opportunity for locus-specific interactions. One such well-studied case is the formation of RNA:DNA hybrids, also known as R loops (Santos-Pereira & Aguilera, 2015). Typically, R loops are formed when nascent RNAs anneal back to their template, displacing the complementary DNA strand. This results in the formation of a transient three-strand structure, which predominantly occurs in GC-skewed regions, often found in regulatory sequences, such as promoters and enhancers,

Box 4. Studying RNA-chromatin interactions

Establishing RNA-chromatin interactions is not a trivial process as there are limitations in the methods used for their identification. Chromatin fractionation protocols, which are commonly used for detection of ca-RNAs, retain RNA molecules that are associated not only with chromatin but also the general "nuclear matrix" (Creamer *et al*, 2021). Moreover, this approach, though unbiased, does not provide information on the mechanism how they are generated nor their mode of action. Other more sophisticated methods, which are based on proximity ligation, can provide further insights on RNA localization on chromatin (Li & Fu, 2019; Mishra & Kanduri, 2019; Kato & Carninci, 2020). The choice of the most suitable technique however requires taking into consideration parameters such as the amount of input material, the required coverage and the mapability of reads. MARGI-seq (Sridhar *et al*, 2017), CHAR-seq (Bell *et al*, 2018), GRID-seq (Li *et al*, 2017; Zhou *et al*, 2019), RD-SPRITE (Goronzy *et al*, 2022) and RADICL-seq (Bonetti *et al*, 2020) are the most recent additions to the toolbox for identification of RNA–DNA/chromatin interactions. Out of all, the latter is the one provides a considerable advantage, as it allows for removal of nascent transcripts from the sequenced pool through addition of the RNA Pol II inhibitor actinomycin D in combination with RNAse H treatment.

but also repetitive elements, centromeres and highly transcribed genes (Santos-Pereira & Aguilera, 2015; Niehrs & Luke, 2020). Such structures have recently been identified as important regulators of gene expression, by impeding transcription factor binding at promoters, hence conferring transcriptional silencing, or by excluding repressors and thus promoting transcriptional activation (García-Muse & Aguilera, 2019). Furthermore, RNAs can be found in association with the DNA in a structure called triplex. This structure is formed when an RNA molecule invades the major groove of polypurine double-stranded DNA, and forms interactions via Hoogsteen base-pairing (Li & Fu, 2019; Mishra & Kanduri, 2019). However, most commonly RNAs exert their function through protein-mediated interactions. In such cases, the RNA plays the role of the scaffold for one or more RNA-binding protein, which is in direct contact with chromatin. One of the best well-studied example of a caRNA is *Xist*, a lncRNA recruiting a plethora of transcriptional repressors and chromatin modifiers to inactivate one of the X chromosomes in female placental mammals (Żylicz & Heard, 2020). All in all, caRNAs are a varied class of RNAs that encompass many repeat elements. Such RNAs are closely associated with chromatin and thus have a potential to function as specificity factors initiating heterochromatin formation. Another feature of RNAs molecules, highly prevalent in caRNAs, is their covalent modifications, also known as the epi-transcriptome. Hitherto, more than 170 modifications have been identified in the MODOMICS database (Boccaletto *et al*, 2018), with the N-6 methyl-adenosine (m6A) being one of the major and one of the most abundant (Zhang *et al*, 2020). In the sections below, we will discuss the importance of this RNA mark for RNA-mediated heterochromatic silencing of TEs.

Role of m6A in epigenetic silencing of TEs

Chemical modifications of RNAs have emerged as important mediators of TE silencing at the RNA as well as the chromatin level. Recent technical and methodological advances allowed for the accurate detection of RNA modifications, especially of the most abundant m6A (Wang & Jia, 2020). Similarly to histone modifications, m6A dynamics rely on the concerted action of "writer" and "eraser" complexes. The major m6A methyltransferase complex (MTC) in polyA RNAs consists of the METTL3, which exerts the catalytic activity, the METTL14, which is an allosteric activator of METTL3, and other proteins such as WTAP and ZC3H13, which direct and facilitate interactions of the complex with other molecules (Huang *et al*, 2020; Kan *et al*, 2022). The m6A mark deposition occurs co-transcriptionally (Ke *et al*, 2017), primarily within the context of the DRm6ACH consensus sequence (D = G, A, or U, R = G or A, H = A, C, or U). In mammals METTL3 is perhaps the sole enzyme responsible for this activity in DRm6ACH motif (Geula *et al*, 2015; Liu *et al*, 2020a; preprint: Poh *et al*, 2021) on the other hand, there are two main demethylases: ALKBH5 and the FTO (Huang *et al*, 2020).

Various "reader" proteins are also involved in regulating the multifaceted effects of m6A modification. Among the best studied are the YT521-B homology (YTH) domain-containing proteins, which include YTHDF1/2/3 and YTHDC1/2 (Shi *et al*, 2019). So far, m6A modification has been linked to the regulation of transcript splicing, nuclear export, translation, and mRNA decay (Wang *et al*, 2015; Xiao *et al*, 2016; Roundtree *et al*, 2017; Shi *et al*, 2017). As such, m6A is involved in virtually every physiological process (Huang *et al*, 2020; Kan *et al*, 2022), with pluripotency being one of the most pronounced (Batista *et al*, 2014; Wang *et al*, 2014; Geula *et al*, 2015; Wen *et al*, 2018; Chen *et al*, 2021; Liu *et al*, 2021, 2020a). *Mettl3-ko* mouse embryos exhibit early embryonic lethality by E8.5 (Geula *et al*, 2015). Consistently, conversion of mESCs failed to efficiently exit naïve pluripotency and upregulate gastrulation markers. Hence, m6A could play an important role in the execution of specific and highly controlled transcriptional programs. In line with this, m6A has recently attracted much attention with regards to various diseases including cancer, liver steatosis, and heart disease (Huang *et al*, 2020; Yang *et al*, 2020; Jiang *et al*, 2021). In the following paragraphs, we are discussing the mechanistic aspects of how m6A functions in the context of TE repression.

Almost every type of RNA can be modified with m6A and this also includes caRNAs (Huang *et al*, 2020). Approximately 15–30% of caRNAs contain m6A in mouse ESCs (Liu *et al*, 2020a). It is noteworthy that m6A methylation on caRNAs is not uniformly distributed among different classes of RNAs, with around 35% of repeat-derived caRNAs being enriched with m6A (Liu *et al*, 2020a). According to recent studies, LTR-ERVK transcripts and especially from IAPez elements, exhibit high enrichment for m6A modification and METTL3 protein occupancy at their 5'UTRs (Chelmicki *et al*, 2021; Liu *et al*, 2021; Xu *et al*, 2021). This is in contrast to most polyA and non-caRNAs which carry m6A methylation primarily at their stop-codon regions and 3'UTRs (Dominissini *et al*, 2012; Meyer *et al*, 2012; Xu *et al*, 2021). METTL3 not only binds and modifies RNAs, it can also be detected in proximity to specific genomic regions that are often associated with retrotransposons. In mESCs about 90% of METTL3 peaks on chromatin co-localize with H3K9me3 and H4K20me3 (Xu *et al*, 2021), indicating a potential function of m6A in targeting heterochromatin formation. In naïve pluripotent cells, these repressive marks mainly decorate telomeric, satellite, and long terminal repeats (LTRs), such as IAPs and early transposon elements (ETns; Martens *et al*, 2005; Mikkelsen *et al*, 2007; Efroni *et al*, 2008; Wen *et al*, 2009; Zylicz *et al*, 2015; Schlesinger & Meshorer, 2019). Indeed METTL3 chromatin-binding is detected at repeat regions in ESCs but this pattern changes drastically upon differentiation, with METTL3 becoming predominantly bound to promoters of specific active genes (Xu *et al*, 2021). This is likely related to previous findings that m6A modification regulates the pluripotency network during differentiation (Batista *et al*, 2014; Wang *et al*, 2014; Geula *et al*, 2015; Zhang *et al*, 2020; Chen *et al*, 2021; Liu *et al*, 2021), but also the downregulation of LTR-ERV retrotransposons upon exit from pluripotency (Martens *et al*, 2005; Macfarlan *et al*, 2012; Göke *et al*, 2015; Grow *et al*, 2015; Chuong *et al*, 2017; Chen *et al*, 2021). Therefore, METTL3 specifically in pluripotent cells, localizes to lowly transcribed repeats/retrotransposons where it might regulate their expression and heterochromatin environment.

The fate of m6A-modified transcripts associated with chromatin remains elusive. Overall, METTL3 and YTHDC1 seem to bind chromatin-associated retrotransposon transcripts, particularly the *cis*-acting LINE1 (L1) and ERV superfamilies, such as the ERVL and ERVK/IAP. Once bound, METTL3/YTHDC1 down-regulate TE expression on many levels and in an m6A-dependent manner (Chelmicki *et al*, 2021; Liu *et al*, 2021; Xu *et al*, 2021). Indeed, the acute auxin-inducible degradation of both METTL3 and METTL14 in mESC resulted in a strong increase in the stability of IAP transcripts. This was measured by tracking RNA levels upon inhibiting

RNAPI and RNAPII-transcription with actinomycin D (Chelmicki *et al*, 2021). These elegant experiments indicate an important role of m6A in PTGS at IAP elements. Indeed, further analysis showed that YTHDF2 can bind and downregulate IAP and other ERVK transcripts, in an m6A-dependent manner (Chelmicki *et al*, 2021). Similar to these results, YTHDC1 can also recognize m6A-modified repeat RNAs (Fig 1) and targets them for degradation (Liu *et al*, 2020a). This function is presumably facilitated by an interaction of m6A readers with core components of the nuclear exosome targeting (NEXT) complex such as RBM7 and ZCCHC8 (Liu *et al*, 2020a). In contrast, chronic loss of METTL3 in mESC resulted in derepression of IAPez but had no effect on the stability of these transcripts, indicating transcriptional regulation mechanisms to be responsible for their upregulation (Xu *et al*, 2021).

Thus, METTL3 plays a dual function in regulating the abundance of m6A-marked transcripts, both through transcriptional and post-transcriptional repression (Chelmicki *et al*, 2021; Liu *et al*, 2021; Xu *et al*, 2021). In mESC chromatin accessibility and nascent transcription are dramatically hampered by the catalytic activity of METTL3 and the presence of the m6A reader, YTHDC1 (Liu *et al*, 2020a). Studies in METTL3 knock-out cells revealed that this increase in chromatin accessibility is accompanied by a global increase in epigenetic modifications associated with euchromatin, such as H3K4me3 and H3K27ac (Liu *et al*, 2020a), and depletion of heterochromatin- and repeat-associated marks such as H3K9me2 (Li *et al*, 2020), H3K9me3 and H4K20me3 (Xu *et al*, 2021). Interestingly, the expression of the respective epigenetic writers or erasers remained unchanged, which indicated a role of m6A in recruitment of histone modifiers on chromatin. Furthermore, loss of METTL3 resulted in decreased levels of H3K9me3 and H4K20me3 specifically on IAPEz loci, which is furthermore accompanied by a reduction in the histone variant H3.3 and of DNA methylation. Notably, the effect of METTL3-KO on DNA methylation was not restricted to IAP elements. According to Chelmicki *et al* (2021), however, the changes of H3K9me3, H3K4me3, and H3K27ac that have been reported in knock-out cell lines, could not be reproduced when acute depletion of both METTL3:METTL14 proteins was deployed. Nonetheless, transcription upregulation of ERVs was immediate and more pronounced than in chronic loss of function models (Chelmicki *et al*, 2021). These results highlight the predominant function of m6A on post-transcriptional regulation of TEs and its secondary and possibly more protracted role in epigenetic regulation of TE loci (Fig 1).

On a mechanistic level, the broader MTC complex consists of multiple subunits which regulate and direct its function. METTL3 localization on chromatin depends on its catalytic activity, as well as on METTL14 and other partners of the MTC complex, such as WTAP and ZC3H13 (Xu *et al*, 2021). Once localized on chromatin however, its catalytic activity is no longer required for deposition of H3K9me3 (Xu *et al*, 2021), indicating that MTC can function as an m6A-independent secondary scaffold. Indeed, METTL3 and YTHDC1 physically bind to the TRIM28/SETDB1 histone methyltransferase complex in ESC and potentiate its recruitment specifically to IAPez elements (Liu *et al*, 2021; Xu *et al*, 2021). Consistently, SETDB1 loss increases the abundance of IAPez transcripts, which are now able to acquire more m6A methylation. Moreover, YTHDC1 is also found to be enriched at LINE1 and IAPez elements on chromatin level. Its localization relies on METTL3 catalytic activity, as well as its own ability to recognize m6A modification (Xu *et al*, 2021). Both YTHDC1 and SETDB1 potentiate the localization of METTL3 on chromatin and promote heterochromatin formation, suggesting a positive feedback loop (Xu *et al*, 2021). All in all, recent findings have uncovered an important role for the m6A machinery in RNA stability and targeting heterochromatin formation to specific retrotransposons. Next, we will discuss another group of RNA-binding proteins-SPOC-domain containing factors, which can also link transcripts, the m6A machinery and chromatin repressors.

SPOC-domain containing proteins

Studies related to X-chromosome inactivation revealed Spen paralogue and orthologue C-terminal (SPOC) domain proteins as potential factors bridging chromatin associated RNAs with the m6A machinery and chromatin repressors (Shi *et al*, 2001; McHugh *et al*, 2015; Moindrot *et al*, 2015; Dossin *et al*, 2020). SPEN and SPOCD1 are two such proteins, that both exhibit RNA binding activity and can interact with chromatin remodeling and/or repressor complexes. Depletion of either SPOC-protein was found to result in upregulation of ERVK, ETn, and LINE1 transposable elements in specific cellular contexts (Carter *et al*, 2020; Zoch *et al*, 2020). With regards to SPEN, it is reported to specifically repress the expression of ERVKs and this is accompanied by a loss in H3K9me3 and a substantial gain in chromatin accessibility (Carter *et al*, 2020; Zoch *et al*, 2020). As mentioned in the previous chapter similar chromatin effects have been observed upon METTL3 and YTHDC1 knock-out, which also results in a more active chromatin profile with increased H3K27ac and H3K4me3 levels at repeat elements (Carter *et al*, 2020; Liu *et al*, 2020a; Xu *et al*, 2021). However, the exact mechanism of SPEN-regulated ERVK transcription remains unknown. Interestingly, SPEN can interact with the m6A RNA methylation machinery, as well as other co-repressors such as NuRD and NCoR/SMRT (Dossin *et al*, 2020). Moreover, SPEN's three RNA-binding domains were reported to directly bind to ERVK RNAs (Carter *et al*, 2020). Thus, it is tempting to speculate that SPEN localizes to ERVK transcripts retained on chromatin where it can bridge m6A machinery with chromatin repressors. Intriguingly, mESCs lacking SPEN cannot efficiently differentiate, a phenotype consistent with the effects observed in absence of m6A-related proteins (Carter *et al*, 2020; Chen *et al*, 2021; Geula *et al*, 2015). Nevertheless, *Spen-ko* mice can survive up to embryonic day ~E13.5 in contrast to *Mettl3-ko* mice, which are aborted earlier (Geula *et al*, 2015; Kuroda *et al*, 2003), indicating that additional players must be involved.

Similarly to SPEN, SPOCD1 is also involved in silencing of repeat elements but it does so by linking the piRNA pathway to chromatin regulation. In mouse male germ cells SPOCD1 interacts with MIWI2, a major regulator of the piRNA pathway, which plays distinct functions in the cytoplasm and the nucleus. While extensive work has established a post-transcriptional regulatory role of cytoplasmic piRNAs, the nuclear MIWI2 is proposed to target DNA methylation to transposable elements (Aravin *et al*, 2006; Carmell *et al*, 2007; Aravin *et al*, 2008; Kuramochi-Miyagawa et al, 2008; Kuramochi-Miyagawa et al, 2010; Watanabe *et al*, 2018). Indeed, *Miwi2-ko* mice not only show upregulation of LINE1 elements during spermatogenesis but also their DNA demethylation (Kuramochi-Miyagawa *et al*, 2008). A model whereby piRNA-loaded MIWI2 targets SPOCD1 to specific repetitive element loci has been proposed (Fig 2). At these loci, SPOCD1 can recruit *de novo* DNA methylation machinery

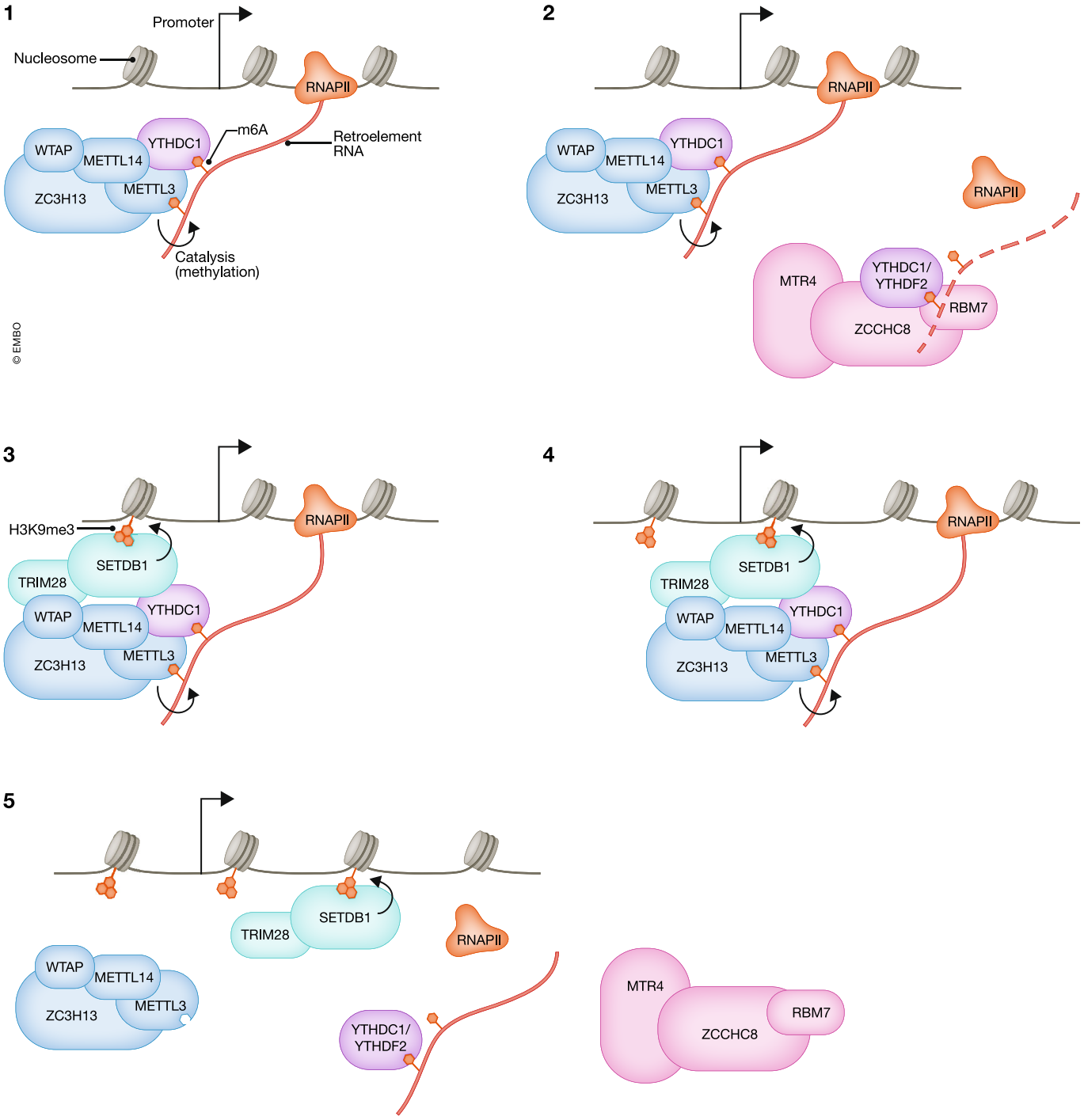

Figure 1. RNA m6A-directed formation of heterochromatin in ES cells.

Genomic loci of retrotransposons such as LINE1 and IAP elements are usually decorated by the H3K9me3 and H4K20me3 chromatin marks (Mikkelsen *et al*, 2007). The former mark is often required for the deposition of the latter, as well as for efficient chromatin silencing (Schotta *et al*, 2004). N6-adenine methylation (m6A) on chromatin-associated retrotransposon RNAs can alter heterochromatic landscape and lead to transcriptional silencing. (1) At first, m6A is deposited on nascent retrotransposon RNAs by the METTL3-METTL14 complex toward the 5′ end of chromatin-associated RNAs (Xu *et al*, 2021). Different m6A readers recognize this modification and mediate various functions, however, the full complexity of their network remains elusive. One such m6A reader YTHDC1, seems to positively regulate METTL3-METTL14 complex formation. (2) YTHDC1 as well as YTHDF2 are also involved in targeted degradation of the m6A labeled RNAs via the Nuclear Exosome Targeting Complex (NEXT; Liu *et al*, 2020a; Chelmicki *et al*, 2021; Chen *et al*, 2021; Garland *et al*, 2022). (3) m6A on such RNAs can also lead to METTL3-dependent recruitment of the histone methyltransferase complex SETDB1-TRIM28. (4, 5) Subsequent spreading of H3K9me3 mark on proximal nucleosomes results in decreased chromatin accessibility and transcriptional silencing (Liu *et al*, 2020a; Xu *et al*, 2021).

(Fig 2) and subsequent H3K9me2/3-deposition can take place through G9a, SETDB1, or SUV39H1/2, in a DNA methylation-dependent or -independent manner (Peters *et al*, 2001; Estève *et al*, 2006; Tachibana *et al*, 2007; Fritsch *et al*, 2010; Quenneville *et al*, 2012; Pezic *et al*, 2014; Ernst *et al*, 2017; Greenberg & Bourc'his, 2019). In line with this model, loss of SPOCD1 results not only in the upregulation of young subfamilies of retrotransposons but also their drastic DNA demethylation, which phenocopies MIWI2-KO (Zoch *et al*, 2020). Recent studies have identified DNMT3C as an important player in establishing this piRNA-dependent DNA methylation of transposable elements in the mouse germline (Barau *et al*, 2016; Jain *et al*, 2017). TEX15 is also proposed to indirectly recruit the H3K9me3 machinery (Schöpp *et al*, 2020). All in all, recent findings suggest that SPOCD1 provides the direct mechanistic link between the piRNA pathway and heterochromatin formation in the male mouse germline.

Another SPOC domain-containing protein that recently attracted much attention is the transgene activator suppressor (TASOR/FAM208A/RAP140/C3ORF63/KIAA1105). Together with the M-phase phosphoprotein 8 (MPHOSPH8/MPP8) and Periphilin 1 (PPHLN1), they constitute a complex known as human silencing hub (HUSH) which bridges TE transcripts to chromatin modifiers (Tchasovnikarova *et al*, 2015). Initially, TASOR and its partners were identified as targets in screens for suppressors of position effect variegation (PEV), human immunodeficiency retrovirus (HIV) as well as evolutionarily young retro-transposons in mammalian cells (Yeung *et al*, 2009; Liu *et al*, 2011, 2018; Tchasovnikarova *et al*, 2015; Fukuda *et al*, 2018; Robbez-Masson *et al*, 2018). Importantly for this review, the HUSH complex has also recently been linked to RNA. Indeed, Periphilin 1 can bind transcripts derived from evolutionary young LINE1 TEs (Seczynska *et al*, 2022). Moreover, the self-associating properties of the arginine/tyrosine-rich disordered N-terminal region of Periphilin 1 seems to be crucial for HUSH assembly, HUSH-dependent silencing and H3K9me3 deposition (Prigozhin *et al*, 2020). Based on protein homology to other arginine-rich disordered domains it has been proposed that the N-terminal domain of periphilin could self-assemble into large solid-like ribonucleoprotein particles on nascent transcripts (Prigozhin *et al*, 2020). Nevertheless, Periphilins' structure potentially precludes it from inducing the formation of fibers hydrogels or liquid–liquid phase separation typically associated with transcriptional silencing, RNA poll II and splicing machinery exclusion (Strom *et al*, 2017; Maharana *et al*, 2018; Gao *et al*, 2019; Ries *et al*, 2019; Fu & Zhuang, 2020; Liu *et al*, 2020b; Cheng *et al*, 2021).

Another component of the HUSH complex linked to RNA-mediated silencing is TASOR. Just like other SPOC domain-containing proteins, it functions as an assembly platform, linking repeat RNAs and RNA-machinery (RNA Polymerase II) to transcriptional co-repressors (Castello *et al*, 2012; Douse *et al*, 2020; Matkovic *et al*, 2022). Indeed, the HUSH complex directs H3K9me3 deposition in conjunction with the histone methyltransferase SETDB1, its methylated chaperone ATF7IP (Fig 3; Tchasovnikarova *et al*, 2015; Timms *et al*, 2016; Tsusaka *et al*, 2018), and furthermore interacts with the chromatin remodeler MORC2 (Tchasovnikarova *et al*, 2017; Liu *et al*, 2018). The HUSH complex is known to interact with chromatin also by recognizing H3K9me3 and recruitment to retroviral DNA through MPP8 (Fig 3; Zhu *et al*, 2018). Nevertheless, HUSH binding sites on chromatin comprise only ~1% of SETDB1 binding sites but the majority of those relate to potentially dangerous LINE1 transposable elements located in introns of

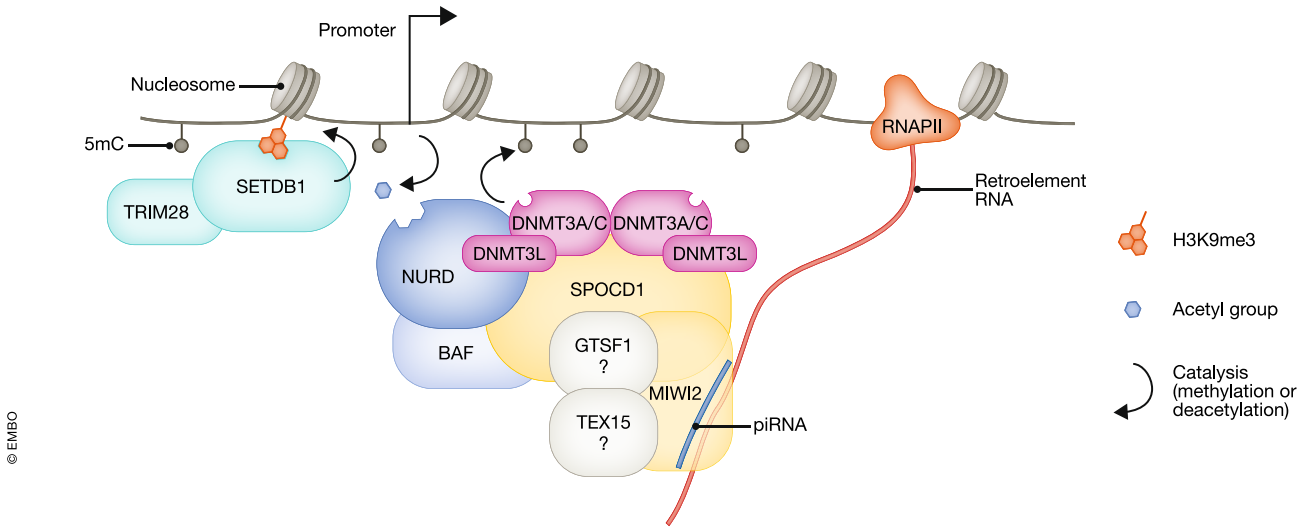

Figure 2. piRNA-directed DNA methylation in the developing germline.

The developing germline undergoes extensive changes on its epigenome. During this period, cells are particularly vulnerable to active retrotransposition (Ernst *et al*, 2017). To cope with this, the germ cells utilize a small RNA-mediated silencing pathway, which can act both on transcriptional and post-transcriptional level. It was recently proposed that these small RNAs, known as piRNAs, may direct the MIWI2-SPOCD1 complex to nascent retrotransposon transcripts, in a sequence-specific manner. This complex interacts with several other epigenetic modifiers such as the DNA methylation machinery as well as the NuRD, and the BAF chromatin remodeling complexes. Their recruitment results in the deposition of 5-methylcytosine (5mC) mark at promoters of specific retrotransposon subfamilies and it can potentially affect other important silencing marks such as H3K9me3, directed by SETDB1 (Greenberg & Bourc'his, 2019) or histone deacetylation, directed by the NuRD complex (Seto & Yoshida, 2014).

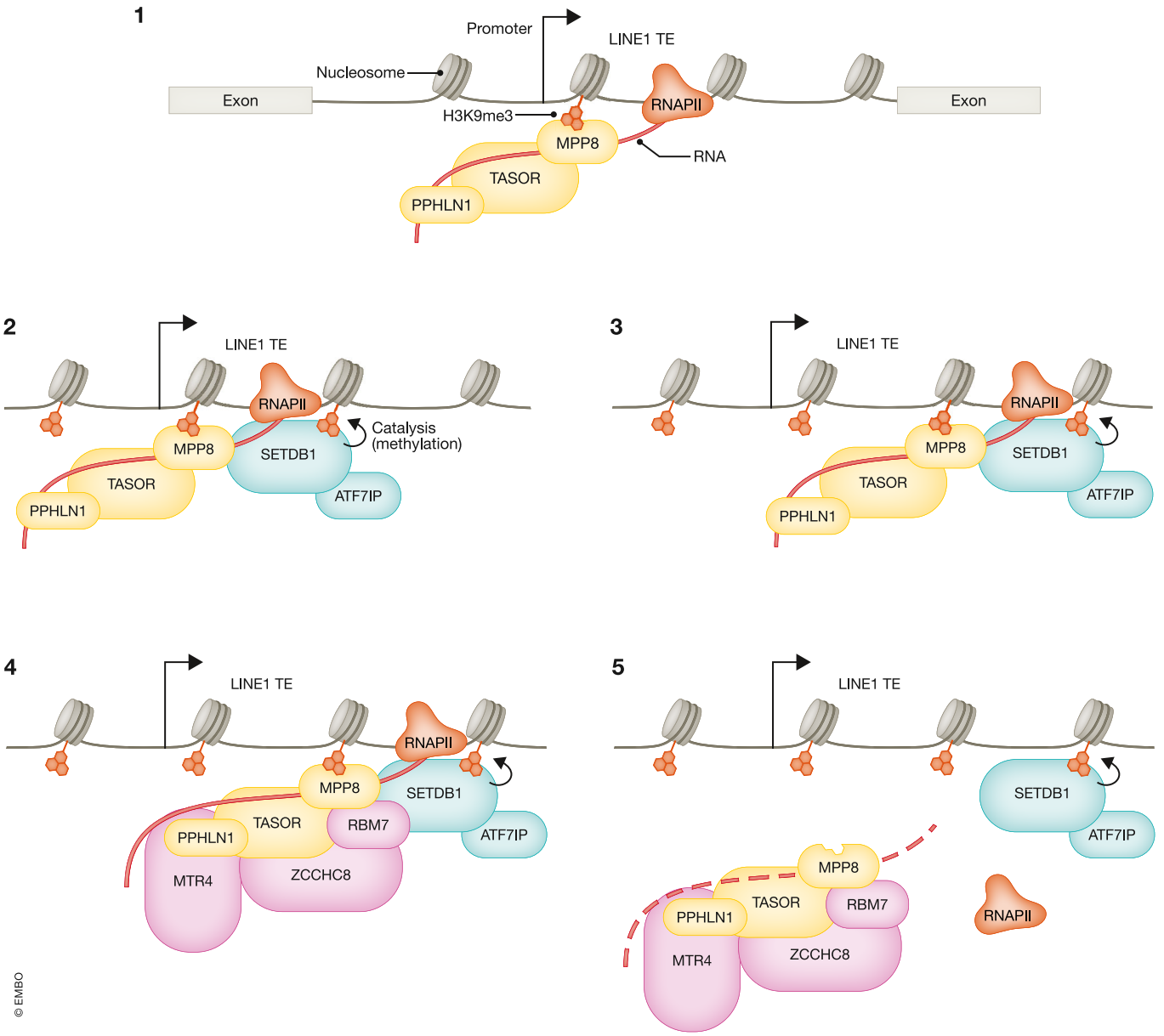

Figure 3. HUSH-mediated silencing.

The human silencing complex (HUSH) is known to regulate the expression of evolutionary young TEs, such as LINE1 elements, primarily residing within introns. (1) Core subunits of the complex, namely TASOR and PPHLN1, have intrinsic RNA binding capacity which allows them to bind nascent LINE1 transcripts (Castello *et al*, 2012; Prigozhin *et al*, 2020). (2) The complex is known to confer transcriptional silencing by recruiting SETDB1:ATF7IP on chromatin, and guiding H3K9me3 deposition at LINE1 loci in an MPP8-dependent manner (Tchasovnikarova *et al*, 2015; Timms *et al*, 2016; Douse *et al*, 2020). (3) The MPP8 subunit can also recognize H3K9me3 marks through its chromodomain, hence sustaining LINE1 heterochromatinization (Douse *et al*, 2020). (4) In parallel, both TASOR and MPP8 subunits directly interact with MTR4 and ZCCHC8 components of the Nuclear Exosome Targeting Complex (NEXT). (5) This allows for the recruitment of NEXT at TE loci allowing for targeted RNA decay (Garland *et al*, 2022).

transcriptionally active genes (Douse *et al*, 2020; Liu *et al*, 2018). Notably, such evolutionarily young transcribed LINE1 elements attract dozens of RNA binding proteins, some of which are known to interfere with TE expression (Attig *et al*, 2018). Seczynska *et al* (2022) recently reported that HUSH is targeted to newly invaded retroelements by recognizing long intronless transcripts, in a spliceosome-independent manner, thus highlighting the role of transcription in silencing of TEs. However, the exact molecular mechanisms of HUSH-dependent transcriptional silencing remain enigmatic. Lastly, the HUSH complex can potentially link epigenetic silencing of TEs and RNA decay in a way reminiscent of the m6A-dependent pathway and the RNA quality control in *S. pombe* (Garland *et al*, 2022; Matkovic *et al*, 2022). Specifically, it has been reported that MPP8 recruits the nuclear exosome targeting (NEXT) complex on chromatin via an interaction with its MTR4 and ZCCHC8 subunits, and drives RNA degradation of LINE1 and ERV

elements in naïve mESCs (Garland *et al*, 2022). The HUSH complex exhibits remarkable homology in domain organization and functional similarities with RITS, the complex responsible for silencing centromeric repeats in *S. pombe* (Douse *et al*, 2020) and it appears that the modes of action for silencing repetitive elements exhibit some conservation across evolutionary distant species.

Overall, according to the current model, SPOC domain-containing proteins act as repressor hubs that become functional only when recruited to chromatin through retrotransposon or non-coding RNAs. At these loci, they can associate with the m6A machinery, RNA Polymerase II and a multitude of chromatin co-repressors. Thus SPOC-domain proteins are important repressors of retrotransposons. However, alternative RNA-dependent strategies have evolved to regulate the expression of another class of repeat elements, the centromeric repeats. Regulation of these repetitive elements is crucial for overall genome stability in mammalian cells and will be discussed in further detail in the following section.

Satellite repeat silencing

Transcription of the centromeric and pericentric heterochromatin has an eminent role for the function of the centromere and thus genome stability. The first evidence for the presence of RNA molecules as a component of animal kinetochores dates back to electron micrography images generated in the 1970s (Rieder, 1979). Indeed, the notion that centromere-related heterochromatin and the repeat elements embedded within remain constantly silent has been disproven. Despite their low abundance, it is clear that centromeric RNAs are transcribed in a cell cycle-regulated manner by RNAPII, that they are capped and mostly polyadenylated (Lu & Gilbert, 2007; Talbert & Henikoff, 2018; Bury *et al*, 2020), and are required for centromere-nucleoprotein assembly and genome integrity (Bouzinba-Segard *et al*, 2006; Wong *et al*, 2007; Talbert & Henikoff, 2018). While multiple studies established that efficient silencing of pericentric satellite repeats is achieved by a positive feedback loop, involving H3K9me3-dependent SUV39H1 recruitment (Rea *et al*, 2000; Muchardt *et al*, 2002; Lehnertz *et al*, 2003; Peters *et al*, 2003; Yamamoto & Sonoda, 2003; Fritsch *et al*, 2010), until recently, the exact mechanism and link to RNA remained elusive.

One hypothesis is that repeat transcripts may be involved in appropriate localization of SUV39H enzymes. Inspired by the findings in fission yeast (Ishida *et al*, 2012), where small RNAs recruit chromatin repressors, recent studies in mouse and human cells identified the SUV39H1 chromodomain as a broad nucleic acid binding domain. Both *in vitro* and *in vivo*, this domain can interact with all types of oligonucleotides (Porro *et al*, 2014; Scarola *et al*, 2015; Johnson *et al*, 2017; Shirai *et al*, 2017) and its affinity for nucleic acids allows direct binding of RNAs from major satellite and α-satellite repeats, in mouse and human respectively. Both types of repeat RNAs remain associated with chromatin, likely due to RNA: DNA hybrids formation (Johnson *et al*, 2017; Shirai *et al*, 2017; Velazquez Camacho *et al*, 2017). Notably, SUV39H1's affinity for nucleic acids is independent of its ability to recognize H3K9me3 or to bind HP1 (Fig 4). Moreover, *in vitro* studies from Velazquez Camacho *et al* (2017) have showed that association of SUV39H enzymes with native nucleosomes can occur in an RNA-dependent manner. Indeed both RNAs and H3K9me3/HP1 contribute to pericentric chromatin retention of the enzyme, thereby preventing the spread of heterochromatin and potential position-effect variegation (Johnson *et al*, 2017; Shirai *et al*, 2017). Consistently, knocking-down major satellite repeats resulted in diffusion of SUV39H1 protein on chromatin, while SUV39H1 loss, leads to upregulation of major satellite repeats (Bulut-Karslioglu *et al*, 2014; Shirai *et al*, 2017; Velazquez Camacho *et al*, 2017).

However, not all types of repeats are linked to heterochromatin formation, and those which are, appear to function locally. In human cells, RNA-FISH combined with RNAse treatment revealed that ssRNAs generated from alpha-satellite repeats, but not beta- or Satellite III, colocalize with H3K9me3 enrichment surrounding the centromere/kinetochore center marker HEC1, suggesting specificity for pericentric chromatin (Johnson *et al*, 2017). These ssRNAs are transcribed primarily by RNAPI and remain associated at their sites of origin. In addition, double knock-out for SUV39H1 in human

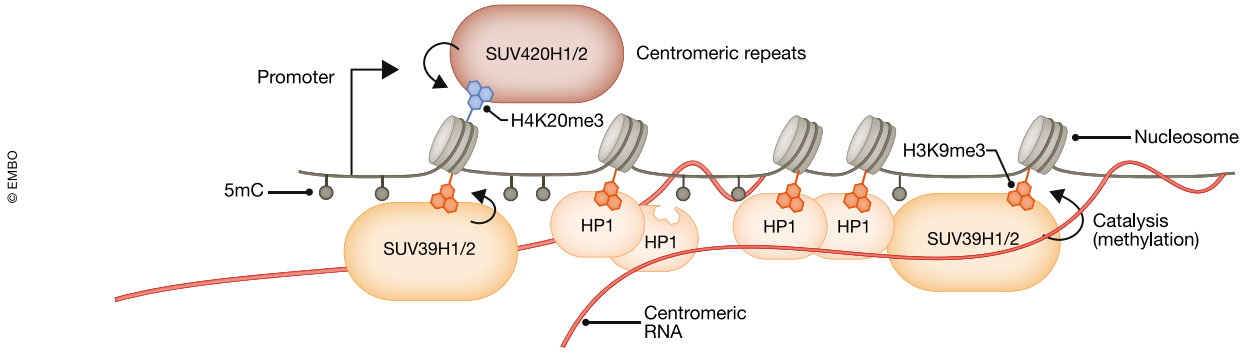

Figure 4. RNA–DNA hybrids mediate heterochromatin formation in centromeric repeats in HeLa and mouse ESC.

Deposition of H3K9me2/3 is involved in heterochromatin formation and transcriptional repression especially at repetitive elements (Bannister *et al*, 2001). Chromatin-associated RNAs produced by centromeric α-satellite repeats, remain *in cis* at the transcription site and act as platforms for stable association of SUV39 histone methyltransferase enzymes (Johnson *et al*, 2017; Velazquez Camacho *et al*, 2017). Particularly, the chromodomain of SUV39H1, which recognizes H3K9me2/3, has an intrinsic RNA and DNA binding affinity and promotes its centromeric localization (Shirai *et al*, 2017). Additional chromodomain-containing proteins such as HP1, which can also bind to RNA, H3K9me2/3 (Muchardt *et al*, 2002), and SUV39 histone methyltransferases (Yamamoto & Sonoda, 2003) are recruited at the site and mediate chromatin compaction.

DLD1 and HeLa cells, resulted in decreased levels of H3K9me3 as well as HP1 foci, accompanied by an increase in alpha-satellite repeat transcription. Notably, the transcription of alpha-satellite did not affect their localization. These results support a model in which repeat transcripts are part of a feedback loop which sustains their local chromatin silencing. Intriguingly, analysis of SUV39H1 mutants lacking either the ability to bind nucleic acids, H3K9me3 or both, showed an additive effect in the double mutant which might indicate an H3K9me3-independent, ssRNA-dependent contribution to heterochromatin formation (Johnson *et al*, 2017). Fluorescence recovery after photobleaching (FRAP) revealed that H3K9me3 deposition is the major determinant of SUV39H1 localization in pericentric chromatin while inhibition of RNA polymerase I increases SUV39H1's mobility (Johnson *et al*, 2017).

In summary, SUV39H enzymes exhibit both histone- and RNA-binding activity (Fig 4), mediating proper localization to centromeric and pericentric heterochromatin, primarily in an H3K9me3-dependent manner (Fig 4). Subsequent interaction with specific repeat-derived transcripts, retained at their sites of origins, restricts the mobility of the histone modifying enzymes and prevents heterochromatin invasion to euchromatic regions (Fig 4). Intriguingly, a very recent study points out that m6A RNA methylation might also be involved in retention of major satellite repeat RNAs on chromatin, in the form of RNA: DNA hybrids, though the exact mechanism requires further investigation (Duda *et al*, 2021).

Conclusion

Silencing of repetitive elements is crucial for genome stability and survival of organisms. Cells have developed multiple lines of defense which ensure tight regulation of their expression. In this review, we highlighted the importance of RNA-dependent mechanisms in mediating the heterochromatinization of repetitive elements primarily in embryonic stem cells and the germline. In these two developmental contexts the repetitive fraction of the genome is partially de-repressed. There, activation of TEs is important for regulating the expression of germline and pluripotency genes, but also is a direct consequence of global epigenome resetting. At these crucial developmental junctures, RNA-mediated processes help in *de novo* heterochromatin establishment and transcriptional repression. Recent evidence supports the notion that transcription of TE is one of their defining functional features which allows for specific targeting and efficient silencing. Thereby even newly transposed, potentially harmful TEs can be repressed just prior to, and shortly after, fertilization. Future studies characterizing the plethora of RNA-binding proteins should shed light on how stable TE repression is induced and the interplay between RNA-dependent and independent targeting mechanisms. Finally, in some contexts, such as satellite repeats in non-transposable repeats remain expressed at low levels. Counterintuitively, these transcripts promote the establishment of well-defined heterochromatin states, which might play a role in maintaining genome integrity. How wide-spread this phenomenon is and how it relates to the formation of phase-separated heterochromatin are questions that should be addressed in future work.

In this review, we have also highlighted some common features of RNA-mediated silencing pathways that contribute to specificity in *de novo* heterochromatin formation. Firstly, RNA-binding domains within chromatin repressor complexes often mediate their recruitment to repeat loci by *cis*-acting transcripts. Secondly, there is frequent involvement of the m6A machinery in repressing repeats. Finally, we highlight the use of both transcriptional repression at the chromatin level as well as post-transcriptional repression of the RNA transcripts themselves. The fact that mammals deploy such intricate and multi-layered regulatory strategies again stresses the importance of tightly regulating repeat expression, especially in preparation for the successful development.

The last two decades have marked a turning point in unraveling the role that RNA molecules play in maintaining genome stability and controlling chromatin. Major technological advances have allowed us to delve deeper into the intricacies of these complex biological phenomena. The identification of new players in these sophisticated regulatory networks has added layers of complexity to our understanding of how cells and organisms protect their genome from sequence erosion and precisely execute their transcriptional programs. Despite these discoveries it is still unclear how cells initially identify RNAs transcribed from multicopy elements and what is the mechanism behind their retention on chromatin. Answering such questions should help in uncovering the molecular requirements for *de novo* heterochromatin assembly at crucial developmental junctures. Another question that remains to be answered is how widespread the role of low-level transcription might be in inducing and maintaining heterochromatin formation. Does this mechanism apply to some host genes as well? In line with this, is there a role of low-level transcription of transposable elements (TEs) utilized as gene regulatory elements? Recent findings also highlighted the importance of the cellular context in which specific repeat repression mechanisms are deployed. However, we still do not fully understand in which contexts RNA-dependent mechanisms are particularly important. Might they also operate in specific adult or embryonic somatic tissues? We are finally beginning to shed light on the sequence of events that occur during repeat heterochromatinization. All in all, uncovering the multilayered mechanisms mediating repeat repression promises to have a profound impact on our understanding of how genome integrity is maintained in varying cellular and disease contexts.

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