Review

Rodrigo Maldonado and Gernot Längst*

The chromatin – triple helix connection

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Abstract: Mammalian genomes are extensively transcribed, producing a large number of coding and non-coding transcripts. A large fraction of the nuclear RNAs is physically associated with chromatin, functioning in gene activation and silencing, shaping higher-order genome organisation, such as involvement in long-range enhancer–promoter interactions, transcription hubs, heterochromatin, nuclear bodies and phase transitions. Different mechanisms allow the tethering of these chromatin-associated RNAs (caRNA) to chromosomes, including RNA binding proteins, the RNA polymerases and R-loops. In this review, we focus on the sequence-specific targeting of RNA to DNA by forming triple helical structures and describe its interplay with chromatin. It turns out that nucleosome positioning at triple helix target sites and the nucleosome itself are essential factors in determining the formation and stability of triple helices. The histone H3-tail plays a critical role in triple helix stabilisation, and the role of its epigenetic modifications in this process is discussed.

Keywords: chromatin; chromatin-associated RNA; lncRNA; nucleosome; RNA-DNA triple helix

1 Introduction

About 2 m of DNA must be functionally organised in a eukaryotic nucleus with a typical diameter of 5–10 µm. DNA is associated with histone and non-histone proteins and RNA, forming a complex and highly variable structure called chromatin. Chromatin is not evenly distributed in the nucleus but is structured into subcompartments (referred to as membrane-less organelles or nuclear bodies), dynamically partitioning the genome in a self-organising manner (Cook and Marenduzzo 2018; Frank and Rippe 2020; Misteli 2007).

The fundamental packaging unit of chromatin is the nucleosome, composed of a histone octamer wrapped by 147 base pairs of DNA (Finch et al. 1977; Luger 2003; Luger et al. 1997). The unstructured histone tails are modified by phosphorylation, methylation, acetylation, and other post-translational modifications forming a histone code that helps to partition the genome into distinct domains such as euchromatin and heterochromatin (Ruthenburg et al. 2007).

The placement and removal of histone post-translational modifications are precisely controlled by proteins that set ("writers") and erase ("erasers") these modifications (Zhang et al. 2015). Histone modifications are dynamically regulated and established in response to various developmental and environmental cues. The functional impact on modified chromatin domains is exerted by "reader" proteins, resulting in chromatin structure and gene expression alterations.

Euchromatin is an open chromatin state and is associated with active transcription, being enriched in specific histone tail modifications. These modifications include the di-and tri-methylation of histone H3 at lysine postion 4 (H3K4me2/3), tri-methylation at position 36 (H3K36me3) and a general hyperacetylation of histone tails (Bannister and Kouzarides 2011; Talbert and Henikoff 2021). In contrast, heterochromatin corresponds to inactive genomic domains, carrying repressing modifications of the histone tails and being less accessible to the transcription machinery. Heterochromatin can be further subdivided into facultative heterochromatin, formed at genomic regions containing developmentally regulated genes, and constitutive heterochromatin, maintaining the inactive form of the highly repetitive regions of the genome (Trojer and Reinberg 2007). Constitutive heterochromatin is characterised by repressive histone marks such as H3K9me3, recruiting Heterochromatin Protein 1 (HP1) (Janssen et al. 2018).

The chromatin landscape is not only determined by histone modifications but also RNA is specifically associated with chromatin, playing central roles in nuclear architecture and gene regulation (Thakur and Henikoff 2020). In facultative heterochromatin, for example, mammalian X-chromosome inactivation is regulated by the long noncoding RNA (lncRNA) Xist. The well-studied *Xist* lncRNA is physically associated with the entire inactive X chromosome and recruits multiple protein

***Corresponding author: Gernot Längst**, Regensburg Center for Biochemistry (RCB), University of Regensburg, D-93053 Regensburg, Germany, E-mail: gernot.laengst@ur.de. https://orcid.org/0000-0002-8232-1179
Rodrigo Maldonado, Institute of Anatomy, Histology, and Pathology, Faculty of Medicine, Universidad Austral de Chile, 5090000 Valdivia, Chile

complexes, including epigenetic modifiers that establish gene silencing and formation of facultative heterochromatin (Cerase et al. 2015; Rocha and Heard 2017). *Xist* is an example of a chromatin-associated RNA, specifically tethered to chromatin to exert its regulatory function, resulting in the compaction of chromatin and formation of the Barr body in mammalian cells. Also, other lncRNAs play transcriptional regulatory roles by interacting with various types of proteins or directly with chromatin and DNA, and they more often regulate chromatin structure and chromatin remodelling by interacting with epigenetic regulators (Sasso et al. 2022).

RNA plays a crucial role as an architectural factor, not only at the inactive X-chromosome but also at ribosomal genes in the nucleolus, (peri)centromeres, telomeres, and more globally, RNA shapes the nuclear architecture (Caudron-Herger and Rippe 2012; Dundr 2012; Li and Fu 2019; Mao et al. 2011; Nozawa and Gilbert 2019). In the last few years, our view on gene expression has changed due to the many roles of RNAs in gene regulation.

2 An overview to chromatin-associated RNAs

Genome-wide RNA expression profile showed that about 80 % of our genome is transcribed, giving rise to various classes of both protein-coding and noncoding RNAs (Djebali et al. 2012). The number of different noncoding RNAs in the human genome is still unknown because of their cell type specificity and sometimes low abundance in cells (Cabili et al. 2011). A recent analysis of the FANTOM5 consortium identified 27.919 different lncRNAs (with a length above 200 nt) in various human cell types (Hon et al. 2017). Many of these ncRNAs and nascent transcripts are integral components of chromatin, changing the organisation of chromatin, facilitating the recruitment of regulatory factors and serving as nuclear organisation factors (Caudron-Herger et al. 2011; Rodríguez-Campos and Azorín 2007; Tsai et al. 2010).

The stable association of RNA molecules with chromatin was shown more than four decades ago. Chromatin isolated from different organisms, such as peas, calf, chicken and fruit flies, exhibited 2–10 % of the total nucleic acids found in chromatin being stably associated RNA molecules (Bonner and Widholm 1967; Bynum and Volkin 1980; Holoubek et al. 1983; Huang and Bonner 1965; Huang and Huang 1969). Initially, it was suggested that these chromatin-associated RNAs were nascent transcripts still being tethered to chromatin via RNA polymerase or contaminants from the isolation procedure (Artman and Roth 1971; Bonner 1971). However, studies from the late 1970s described a possible role of these caRNAs in chromatin organisation. It was hypothesised that caRNAs might play an activating role in the regulation of transcription (Britten and Davidson 1969). In this hypothesis, caRNA functions as an "activator" for the transcription of an "acceptor gene" by sequence-specific interactions between RNA and DNA, suggesting that "chromosomal RNAs may function as a sequence detector for chromosomal proteins" (Bonner et al. 1968).

An initial protocol to purify caRNA was established by the Schibler group in 1994 (Wuarin and Schibler 1994), extracting chromatin and its associated RNA in the presence of a high concentration of urea and detergent. Since then, adaptations of this protocol and the development of high throughput techniques identified a heterogenous pool of caRNA species, being differentially associated with active and inactive fractions of chromatin (Fang et al. 2019; Kurup and Kidder 2018; Schubert et al. 2012; Soboleva and Tremethick 2018; Werner and Ruthenburg 2015). Furthermore, crosslinking and ligation of caRNA-DNA complexes allowed to detect the genomic binding sites of the caRNAs. Numerous variations of the techniques, like Red-C, MARGI-Seq, Char-seq, GRID-seq, RADICL-Seq and others, have been published (Bell et al. 2018; Bonetti et al. 2020; Gavrilov et al. 2020; Sridhar et al. 2017; Zhou et al. 2019a). These experiments show, as expected, the association of nascent RNA with chromatin and the genomic target sites of short and long noncoding RNA molecules. These methods showed that the RNAs can be bound in *cis*, close to their site of synthesis, or in *trans*, at genomic loci distant from their site of synthesis.

The RNA molecules preferentially associated with chromatin can be grouped into long noncoding RNAs (lncRNAs), circular RNAs (circRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), enhancer RNAs (eRNAs), promoter-associated RNAs (paRNAs), antisense RNAs (asRNAs) and repeat RNAs, as described below.

3 caRNAs as regulators of transcription

Whereas the *Xist* RNA forms a repressive chromatin compartment, there is also growing evidence that noncoding, nascent RNAs mediate gene activation by changing the topology of chromatin. The enhancer-derived RNAs (eRNAs) are one such class of RNAs. These represent short (50 nt) to long (2000 nt) RNAs originating from enhancer elements (Arnold et al. 2020; Kim et al. 2010; Santa et al. 2010) and being suggested to bring the enhancer and promoter sequences into close proximity to promote gene activation (Arnold et al. 2020). eRNAs can loop DNA sequences by

interacting with transcription factors, or like the prostate-specific antigen (PSA) eRNA, by interacting with the positive transcription elongation factor TEFb that phosphorylates and activates RNA Polymerase II (Zhao et al. 2016). Also, non-enhancer transcripts like the chromatin enriched RNAs (cheRNA), nascent transcripts tethered to chromatin by RNA Polymerase II, promote gene-enhancer contacts in *cis* dependent on transcription factors (Werner et al. 2017; Yang et al. 2017). Furthermore, the chromatin association of asRNAs, paRNAs and repeat RNAs in *cis*, to the same locus, or in *trans* were shown to regulate transcription (Duda et al. 2021; Kuznetsov et al. 2018).

4 caRNAs organising the higher order structure of chromatin

Another group of caRNAs acts by stabilising actively transcribed regions. The chromosomes of higher eukaryotes are organised into topologically constrained functional domains (Belmont et al. 1989), correlating with transcriptional activity and gene density (Goetze et al. 2007; Naughton et al. 2013). Such a chromosomal organisation was shown to depend on chromatin-associated RNAs that interact with scaffold attachment factor A (SAF-A), a structural nuclear protein. SAF-A interacts with caRNA, forming a de-compacted chromatin mesh that is dependent on active transcription and required for genome stability (Nozawa et al. 2017). Also, the function of the CCCTC-binding factor, CTCF, organising chromatin loops depended on RNA molecules to maintain the interaction of specific domains (Saldaña-Meyer et al. 2019). In addition, caRNAs were shown to maintain the accessible structure of active chromatin compartments. RNase microinjection experiments showed that nuclear-retained coding RNAs retained the configuration of open chromatin, whereas heterochromatin domains showed no gross structural changes upon RNA depletion. The RNAs were found to be enriched long 3′-UTRs and suggested maintaining chromatin accessibility by stabilising RNAP II transcription factories (Caudron-Herger et al. 2011). caRNA in *Drosophila* cells was shown to be enriched in snoRNAs and, together with the decondensation factor 31 (Df31), enabling the reversible closing and opening of higher-order chromatin structures and regulating DNA accessibility (Schubert et al. 2012).

5 Tethering caRNAs to chromatin

RNA binding to chromatin occurs in different modes, such as the global, chromosome-specific binding, or sequence-specific recognition of genomic sites, as presented above, suggesting other mechanisms of RNA recruitment. One of the first studies suggested that very short chromosomal RNAs (about 40 nt in length) were covalently linked to histones, an observation that was controversially discussed (Pederson and Bhorjee 1979). Other modes of linking RNA to chromatin involve the interaction via a DNA-RNA hybrid duplexes, so called R-loops, the formation of RNA:DNA-DNA triplexes, as described in detail below, and non-covalent interactions with chromosomal proteins.

Newly transcribed RNAs (nascent RNAs) may remain at their production site due to their association with the RNA polymerases, representing a *cis*-interaction. Similarly, R-loops, RNA-DNA hybrids formed during transcription, remain tethered to their production site even after synthesis. RNAs tethered by R-loops were shown to have a functional impact on genome integrity and are linked to transcriptional regulation (Chen et al. 2018b; Sollier and Cimprich 2015; Stork et al. 2016). R-loops are preferentially associated with promoters and enhancers exhibiting GC-skewed sequences (Chen et al. 2017; Ginno et al. 2012).

In contrast to *cis* targeting, *trans*-interactions occur by directing the RNA to a different genomic location after transcription and releasing the transcript from the RNA polymerase. Specific genomic binding sites of these RNAs can be determined by chromatin-bound proteins, specifically interacting with the RNA, or by the sequence-specific binding of the RNA to DNA via the formation of RNA:DNA-DNA triple helices. Even though triple helices have been known for 70 years, the *in vivo* role of these structures, their abundance and localisation still need to be studied (Felsenfeld and Rich 1957). In this review, we summarise direct biochemical and indirect evidence of their potential roles and existence, and we also shed light on their mechanism in regulating gene expression.

6 Triple helices

Double-stranded DNA (dsDNA) can form triple-helical structures by sequence-specific binding of a third nucleotide strand. Triple helical structure formation follows the Hoogsteen base-pairing rules, allowing the binding of a single-stranded DNA or RNA strand to the major grove of the Watson–Crick duplex (Figure 1A).

A dsDNA sequence that can form a triple helix is called a Triple Helix Targeting Site (TTS), specifically bound by a Triple Helix Forming Sequence (TFS). The TTS DNA sequence consists of a polypurine strand, serving for the specific interaction and Hoogsteen base pairing with three different kinds of TFS binding motifs (Felsenfeld et al. 1957; Hoogsteen

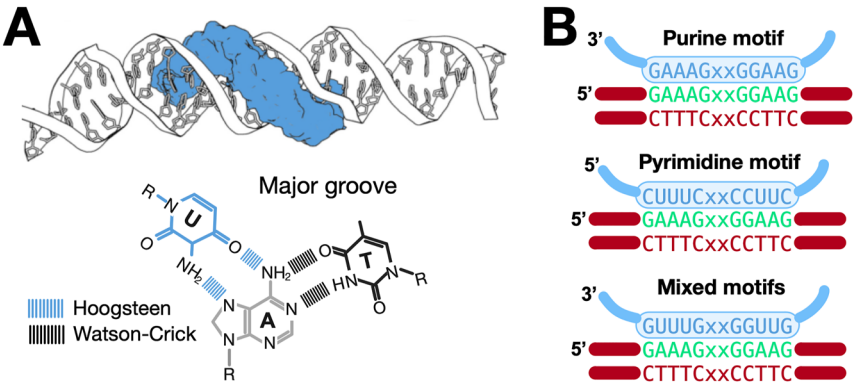

Figure 1: Schemes showing triple helix structures and motifs. (A) Model depicting the binding of a third strand of RNA to DNA and forming Hoogsteen base pairing. (B) Scheme showing the three different triplex forming motifs with exemplary sequences. The RNA strand is marked in blue, and the DNA strand forming the Hoogsteen basepairing is shown in green.

1959; Moser and Dervan 1987; Rajagopal and Feigon 1989). The TFS can be classified into Pyrimidine-, Purine-, and Mixed-motifs, consisting of TFS sequences with only pyrimidine, purine, or both types of nucleotides, respectively (Figure 1B). The sequence composition of the TFS directs the third strand's 5′ to 3′ orientation relative to the TTS purine strand, the Pyrimidine-motif binds in parallel, the Purine-motif bind anti-parallel, and the Mixed-motif can bind in both orientations (Beal and Dervan 1991; Morgan and Wells 1968).

The structure of a triple helix has been described to be more rigid than the dsDNA, and the DNA exhibits an A-B intermediate conformation (Esguerra et al. 2014). Crystal structures of DNA:DNA-DNA triple helices showed that the axis of the third strand is perpendicular to the purine strand and exhibits sugar-phosphate backbone angles close to A-DNA-like values (Nunn et al. 1997). The A-B intermediate form of the DNA in the triplex exhibits a significant major groove widening, revealing structural changes that affect the stability of third-strand binding (Esguerra et al. 2014; Nunn et al. 1997).

The formation of triple helices was suggested to follow a nucleation-zipping model for pyrimidine triplexes, following a directional, 5′-to-3′ binding of the third strand, with respect to the purine strand of the TTS (Alberti et al. 2002).

7 Biophysical stability of triple helices

Initial studies described the triple helices as unstable structures in aqueous solutions, requiring acidic conditions and protonated cytosine to form stable triplexes (Lipsett 1964). Nevertheless, stable binding was demonstrated at physiological salt conditions in the presence of positively charged metabolites like spermine, spermidine and cobalt-hexamine that are abundant in cells (Tateishi-Karimata et al. 2014; Thomas and Thomas 1993). Positive charges counteract the repulsion forces generated between the negatively charged backbones of the TFS and the duplex, forming 4 specific triplets T-AT, G-GC, A-AT, and C^+-GC (Buske et al. 2012; Hoogsteen 1959; Malkov et al. 1993). Accordingly, ionic strength influences the formation of triple helices, where the triplet C^+-GC is the least sensitive Hoogsteen base pair to be affected by ionic variations (James and Fox 2003). Increasing salt concentrations tend to stabilise triplexes, with divalent cations being more effective (Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}) than monovalent cations (Na^+, K^+) (Maldonado et al. 2017; Malkov et al. 1993; Plum et al. 1990). These favourable conditions, in terms of stability, are explained by the affinity of the divalent cations for the phosphate backbone and their internal interactions within the triplex structure (Blume et al. 1999). Thermal dissociation studies suggest a higher affinity is obtained when the third strand is RNA instead of DNA due to a different sugar geometry within the major grove (Dagneaux et al. 1995; Maldonado et al. 2017).

The presence of high concentrations of positively charged polyamines in the cell suggests that these may counteract the instability of the Hoogsteen base pairing observed *in vitro*, enabling the formation and potential existence of stable triplexes *in vivo*. Currently, a web server that built up energy-minimized triplex helical structures allows the generation of triplex models designed by the user, with or without mismatches, creating the first steps for protein or drug interaction with the triplex of interest (Patro et al. 2017).

8 The impact of triple helix sequence on stability

Furthermore, the stability of the triplexes is affected by the type of motif, its actual sequence, and the length of the TFS.

The minimal requirement for the third strand to form a RNA:DNA-DNA triplex is a length of nine bases to reach significant binding affinities *in vitro*, matching the mean length of triplex motifs *in vivo*, with about 10–30 nt (Buske et al. 2011; Cheng and Pettitt 1992; Escudé et al. 1993; Frank-Kamenetskii and Mirkin 2003; Knauert and Glazer 2001; Morgan and Wells 1968; Roberts and Crothers 1996, 1992). The affinity between the RNA and DNA strands increases with the length of the third strand when the sequences perfectly match (Pasquier et al. 2017).

A direct comparison of purine and pyrimidine motifs with varying TFS and TTS GC content revealed qualitative and quantitative binding affinity and stability differences. Pyrimidine-motifs tend to bind with nanomolar affinity at physiological conditions *in vitro* when the GC-content of the TTS is low. In contrast, the Purine-motifs fail to bind under such conditions. This effect reverses with increasing GC-content of the TTS. At an average GC-content of 50 %, both types of TFS motifs bind with high affinity, whereas at higher GC-contents (>70 %), only the Purine-motifs form stable triplexes, and the Pyrimidine-motifs fail to do so (Maldonado et al. 2017). The stability of triplexes is highly sequence-specific, and single sequence mismatches have profound effects on their formation and stability. The location of these mismatches is relevant, with variations occurring close to the TFS ends having weaker effects than central ones (Colocci and Dervan 1995; Maldonado et al. 2017; Mergny et al. 1991). The kind of mutation severely impacts triplex stability, correlating with the finding that a thymidine and adenine in the TFS form more stable Hoogsteen hydrogen bonds with an adenine of the duplex. In contrast, guanine forms more stable hydrogen bonds with the guanosine of the TTS compared to other bases (Best and Dervan 1995). When comparing purine and pyrimidine TFSs against the same TTS, the pyrimidine motifs formed more stable triplets under slightly acidic conditions (C^+-GC) (Keppler and Fox 1997). Additionally, thermodynamic studies showed that alternating C^+-GC and T-AT triplets generate the most stable triple helices. TFSs must contain at least 19 nt to form a stable RNA:DNA-DNA triple helix in the presence of no more than two consecutive mismatches (James and Fox 2003; Kunkler et al. 2019). Nevertheless, it has been shown that depending on the sequence context, a single mutation within a 29 nt long TFS completely abolishes triple helix formation (Maldonado et al. 2017). Experimental approaches using modified nucleobases (LNAs and 2-thioU) revealed increased RNA:RNA-RNA triplexes stability. This was functionally validated on HeLa cells by inserting the corresponding TTS on a plasmid coding for GFP, where the transfection of modified TFSs was associated with GFP silencing (Szabat et al. 2018).

9 Potential triple helix binding sites in the genome

The discovery of the overrepresentation of polypurine and polypyrimidine sequences within regulatory elements of both prokaryotic and eukaryotic genes suggested a functional role of triplexes (Bucher and Yagil 1991). Initial genome-wide searches for triplex targeting sequences (TTS) in humans and mice identified about 1.9 million of these sequence elements, being uniformly distributed along the chromosomes, but highly enriched at regulatory elements (Wu et al. 2007). The TTSs are mainly located at promoters, suggesting their potential for gene expression regulation (Goñi et al. 2004). Furthermore, TTSs are predominantly increased in the regulatory regions of genes regulating physiological processes, being highly enriched in transcription factor genes (Goñi et al. 2006). The functionality of these TTSs was supported by potassium permanganate footprinting assays, which identified that non-canonical DNA structures (potential triplexes) are formed on these DNA elements (Kouzine et al. 2017).

The first computational pipeline for the prediction of TTS sites was *TRIPLEXATOR*, which included additional features to predict the TTS-TFS binding potential, minimal TTS sequence requirements, and provides the density of TTSs within the genome (Buske et al. 2012). This pipeline showed that chromatin-associated RNAs are enriched in TTS elements and can form triple helices at hundreds of human genes (Buske et al. 2012). The enrichment of TTSs in gene promoters and of TFSs in caRNAs suggests a potential regulatory link between both. After TRIPLEXATOR, *TTSMI* integrated the publicly available ENCODE data with the TTS sites to predict gene function correlations of triplex targeting sequences, adding an additional functional layer (Jenjaroenpun et al. 2015). Currently, different computational tools are available to predict the triplex formation (*LongTarget*, *TRIPLEXES*, *Triplex Domain Finder*, *TriplexFFP*, *and Fasim-LongTarget*), which use the basic Hoogsteen base-pairing rules or, in some cases, employ deep learning networks with validated TTS-TFS pairs to predict triplexes formation *in vivo* (Warwick et al. 2023). For example, a machine learning tool called *TriplexAligner* incorporated a kind of “new rules” based on RNA:DNA interactome studies not covered by the canonical Hoogsteen interaction rules. However, the biophysical data on TTS-TFS pairs and their binding affinities are scarce, not allowing us to predict *in vivo* binding affinities with high precision (Warwick et al. 2022).

The large number and specific genomic location of the TTSs and the enrichment of TFSs in caRNAs suggest a

regulatory function in chromatin and potentially other functions unknown to date (Pasquier et al. 2017).

10 Functional triplet targeting sites

Triple helices were shown to exert biological functions, like a synthetic TFS targeting a conserved purine tract of the HIV genome that serves as a start site for reverse transcription. This TFS inhibits HIV retrotranscription *in vitro* and retrovirus replication in infected cells (Volkmann et al. 1995). Similarly, the Purine-motif TFS targeting the interferon-responsive elements inhibited transcription *in vitro* and from reporter genes in transfected cells (Faria et al. 2000; Roy 1993). These studies show the formation of functional triple helices in cells, and far more pieces of evidence arose from studies on lncRNAs. The formation of RNA:RNA-RNA triple helix structures was shown to inhibit the endonucleolytic degradation of the *MALAT1* and *NEAT1* lncRNAs (Brown et al. 2014, 2012; Wilusz et al. 2012). Moreover, by placing these motifs downstream of an ORF, it was demonstrated that this intramolecular triplex acts as a translational enhancer in the absence of a poly(A) tail (Wilusz et al. 2012).

During early mouse embryogenesis, *in vitro* and *in vivo* experiments suggested that the reactivation (expression) of LINE-1 transposons at the 2-cell stage, which are then down-regulated again in mature stages, is modulated by a triple helical structure formed by LINE-1-derived short RNA triplexes regulating the LINE-1 ORF1 (Fadloun et al. 2013). Evidence for the genomic function of RNA:DNA-DNA triplexes comes from studies in mouse spleen. The chromosomal integration of multiple TTS sites in the mouse genome and the injection of the corresponding TFO induced double-strand brakes and activation of apoptosis in an XPD-dependent manner (Tiwari and Rogers 2013). Besides other RNA biotypes, microRNAs (miRNAs) were reported to regulate gene expression at the transcriptional level, in addition to their genuine role. The miR-223 localises inside the nucleus and targets the NFI-A promoter region at miR-223 complementary DNA sequences, recruiting the polycomb complex (PcG) to induce NFI-A transcriptional silencing (Zardo et al. 2012). Due to their length and sequence composition, a subset of miRNAs has been proposed to form triplexes and to bind to regulatory elements in different species (Paugh et al. 2016; Toscano-Garibay and Aquino-Jarquin 2014). Recent studies indicate that microRNAs may also play a role in up-regulating mRNA transcription levels, potentially acting through triple helix formation. An algorithm designed to determine the miRNA-triplex formation revealed that genes containing sequences favouring microRNA triplex formation are markedly enriched 3.3 fold for genes whose expression is positively correlated with the expression of microRNAs targeting these triplex binding sequences (Paugh et al. 2016). Further studies supporting a role for miRNA-triplexes in gene regulation show similar effects on cancer-relevant targets, like KRAS, TCF7L2, and EGFR (Fadaka et al. 2019).

11 lncRNA mediated triplexes and gene regulation

Besides the short-noncoding RNAs, many long-noncoding RNAs are tethered to chromatin and shown to regulate gene expression, chromatin density and nuclear architecture (Mercer et al. 2009; Rinn and Chang 2012). Due to their length, and sequence composition, the lncRNAs present complex three-dimensional structures recognised by specific proteins, to accomplish their regulatory functions (Chen 2016; Li and Fu 2019). One mechanism for lncRNAs-dependent gene expression regulation involves forming triple helices at regulatory elements. For example, in quiescent cells, transcription from an alternative promoter of the DHFR gene generates a lncRNA that directly interacts with the gene promoter through triple helix formation, resulting in dissociation of the pre-initiation complex and transcriptional repression (Martianov et al. 2007). In a similar manner, the mouse rDNA promoter-derived lncRNA, called pRNA (promoter RNA), interacts with the binding site of the transcription factor TTF-I via triple helix formation, inducing RNA-dependent DNA methylation and transcriptional silencing by tethering the DNA methyltransferase DNMT3b to the gene promoter (Schmitz et al. 2010).

A genome-wide analysis of the *MEG3* lncRNA interactions with chromatin identified the regulation of TGF-β related genes by forming triple helices on distal regulatory elements. The triple helices occur due to a GA-rich sequence close to the 5′ end of *MEG3*, allowing the recruitment of PRC2 and establishing H3K27me3 histone marks to repress the TGF-β related genes (Mondal et al. 2015). In contrast, the antisense lncRNA *Khps1* activates expression of the SPHK1 gene through triple helix formation on its promoter region and the recruitment of the transcriptional activator E2F1 (Postepska-Igielska et al. 2015). This kind of molecular mechanism, based on triple helix formation at regulatory elements and the tethering of chromatin-effector molecules, was described for several lncRNAs, like *PARTICLE* regulating the *MAT2A* gene through PRC2 (O'Leary et al. 2015), the *WWOX* gene (O'Leary et al. 2017), or the lncRNA *Fendrr* regulating genes associated with heart and body wall development in mice through PRC2 and TrcG/MLL (Grote et al. 2013), *HOTAIR* regulating senescence-associated genes through PRC2 on mesenchymal stem cells (Kalwa et al. 2016), *LNMAT1* promoting *CCL2* expression by the recruitment of hnRNPL to

the promoter region (Chen et al. 2018a), *cis*-acting ncRNAs derived from the *FAU* and *ß-globin* genes inhibiting the expression of their source genes (Zhou et al. 2019b), *CDKN2B-AS1* repressing *CDKN2B* gene expression through triple helices formation on the promoter region and tethering CTCF and EZH2 (Ou et al. 2020), HIFα-AS1 repressing *EPHA2* and *AMD* genes through the triplex formation on regulatory elements and the recruitment of the HUSH complex (Leisegang et al. 2022). These many examples led to the development of computational tools to detect and characterise lncRNA-dependent triplex formation with DNA for genome-wide detection of triplex target genes (Jalali et al. 2017; Kuo et al. 2019; Wang et al. 2018). Additionally, a newly defined class of super-lncRNAs are suggested to target super-enhancers through triple helix formation. The group identified 442 super-lncRNA transcripts with Purine- and Pyrimidine-motifs, originating in 27 different human cell and tissue types, with 70 % of these super-lncRNAs being tissue-restricted (Soibam 2017). Data from ChIRP (Chromatin Isolation by RNA purification) assays for 12 different lncRNAs in humans, mice, and *Drosophila* were used to feed a convolutional neural network, leading to the identification of novel triplex forming domains for all of these lncRNAs (Wang et al. 2018). Some of these results have been challenged by a method that isolates RNA associated with DNA in the form of triple helices. This method did involve protein removal and, therefore, discards the possibility of protein-dependent tethering of the RNA. The results suggest that caRNAs form triple helices by transcripts originating from coding and noncoding regions and that triplexes are formed at accessible and transcriptionally active chromatin domains (Sentürk Cetin et al. 2019).

A study aimed to quantify the binding landscape of the TFS of 23.898 lncRNAs genome-wide. They identified more than 700 targeting sites on promoter regions, suggesting a role in chromatin organisation together with the architectural proteins CTCF and NSRF (Jalali et al. 2017). In *Drosophila melanogaster*, a survey for potential TFSs on immature transcripts (13.919 on pre-mRNAs and 2470 on pre-lncRNAs) found an average TFS length of 24 nt, which were matched against the genome. The results showed that potential triplexes formed by both pre-mRNAs and pre-lncRNAs are enriched on genes related to development and morphogenesis (Pasquier et al. 2017).

12 Proteins stabilising triple helix structures

The existence of triple helix binding proteins further supports their presence in the cell. A large list of known triple helix binders was reviewed before (Buske et al. 2011) additions suggest also a role for HP1 in triple helix formation and maintaining the epigenetic silencing of repeat sequences (Zhang et al. 2022). The abundant and chromatin-associated high mobility group proteins HMG1 and HMG2 were shown to bind triplexes (Jain et al. 2005; Reddy et al. 2005; Suda et al. 1996). These proteins are involved in the maintenance of the chromosome architecture and the distribution of euchromatin and heterochromatin domains (Jain et al. 2005; Reddy et al. 2005; Suda et al. 1996). Even sequence-specific DNA binding factors, like the GAGA transcription factor, were shown to bind a Pyrimidine-motif triplex that inhibits the expression of downstream genes (Orozco et al. 1998). While in humans, the tumour suppressor P53 was demonstrated to bind triple helical structures constituted only by T-AT triplets, specifically through its C-terminal DNA binding domain (Brázdová et al. 2016). The centromere binding protein CDP1 binds a Purine-motif triplex with high affinity, whereas the corresponding Pyrimidine-motif is bound three orders of magnitude worse (Musso et al. 2000). Mutation of CDP1 mutants show defects in chromosome segregation during mitosis and a high level of chromosome fragmentation (Musso et al. 2000). Sites of triple helix formation in the genome have been reported as possible sites for genomic instability (Kaushik Tiwari et al. 2016). Therefore, intensive research has focused on proteins able to resolve these non-canonical structures. The human *DDX11* gene codes for a DNA helicase (superfamily 2 with XPD, FANCJ, and RTEL1) essential for sister chromatid cohesion. Mutation of this helicase was found to be associated with the Warsaw breakage syndrome, a genetic disorder characterised by high levels of genomic instability (Guo et al. 2015; Parish et al. 2006). Biochemical and cellular studies showed that the preferential substrates of DDX11 are triplexes, dissolving inter- and intra-molecular triplexes in an ATP dependent manner (Guo et al. 2015). Deletion of DDX11 in cells results in an accumulation of triple helical structures and genomic instability (Guo et al. 2015).

13 Chromatin architecture and triplex formation

Chromatin is the natural substrate of RNA-DNA triple helix formation and presents a major obstacle to its sequence-specific recognition. The basic packaging unit of chromatin, the nucleosome, is capable of associating with almost any DNA sequence. However, clear data is showing that nucleosomes adopt well-defined positions on DNA, preferentially at regulatory regions of the genome (Ramsay 1986; Schones

et al. 2008; Schwartz et al. 2018). About 30 million nucleosomes are arranged on the human genomic DNA, like "pearls on a string", separated by a short linker sequence. This nucleosomal chain is compacting the DNA and presents a significant barrier to sequence-specific access. Nucleosomal DNA is not only in-accessible for proteins but also inhibits the binding of triple helices (Goñi et al. 2006). The interaction of DNA with nucleosomes presents a problem for triplex formation as the TFS needs to wrap around the DNA duplex, which is already wrapped around the histone octamer. If the third strand spans more than ten bp, then it would have to thread between the histone octamer and DNA surface to continually access the DNA major groove. Initial studies have shown that triplex formation inhibits nucleosomal assembly on the RNA-bound TTS and functions as a nucleosomal barrier (Espinás et al. 1996; Westin et al. 1995). Furthermore, it was shown that nucleosomes inhibit triplex formation when located inside the realm of the nucleosome. However, target sites towards the exit/entry site of nucleosomal DNA can still be accessed by TFSs (Brown and Fox 1998, 1996; Maldonado et al. 2019). The reduced accessibility of chromatin for TFS binding was also shown by using biotinylated- and psoralen-modified TFOs in a capture assay, comparing naked DNA versus nuclei and whole cells (Besch et al. 2004). Similarly, LNA-TFSs, used to target specific genes, showed that triplex formation was more efficient on active genes with an accessible chromatin structure (Brunet et al. 2006).

These findings leave only the linker DNA in between the nucleosomes as potential binding sites of triple helices, requiring a special arrangement of the TTS in the nucleosomal landscape. The size of the linker DNA is highly variable in higher eukaryotes, with about 20 bp in *Saccharomyces cerevisiae* and a mean length of about 35 bp in a typical vertebrate nucleus, albeit with great variations between cell types (Compton et al. 1976; Woodcock and Ghosh 2010). Therefore, linker regions are sufficiently long to accommodate stable triple helix binding, requiring the precise localisation of the TTS between two nucleosomes.

A recent study addressed the genome-wide localisation of TTS concerning nucleosome positions, showing a preferential localisation of TTS adjacent to nucleosomes. A newly developed TRIP-seq method was used to show that the corresponding TTS were bound by RNA and the neighbouring nucleosomes enriched in activating histone marks (Maldonado et al. 2019). Furthermore, the detailed analysis of TTS positioning relative to the nucleosome revealed a triplex stabilising effect of the nucleosome (Figure 2A). Triplexes located at the entry/exit site of the nucleosome are specifically stabilised by the histone H3 tail, providing clear evidence for the regulatory role of chromatin in triplex formation (Maldonado et al. 2019). In line with these results, molecular dynamics simulations show that the binding of an RNA:DNA-DNA triple helix next to a nucleosome increases the attraction of the H3 tails, resulting in the stabilisation of this structure (Kohestani and Wereszczynski 2023).

14 Future perspective

The studies above suggest that the binding of TFS to chromatin is regulated on different levels. First, the nucleosomal landscape and the positioning of nucleosomes adjacent to a TTS is a pre-requisite for TFS binding. The size of the linker DNA is, in general, large enough to accommodate stable TFS binding. However, the post-translational code of the histones is still an unexplored regulatory level in TFS binding that could determine the stability and half-life of TFS-TTS complexes in chromatin (Figure 2C). Over one-third of the H3-tail amino acids have been shown to be post-translationally modified and recognised by various reader proteins (Musselman et al. 2012). The interaction of the TFS with the

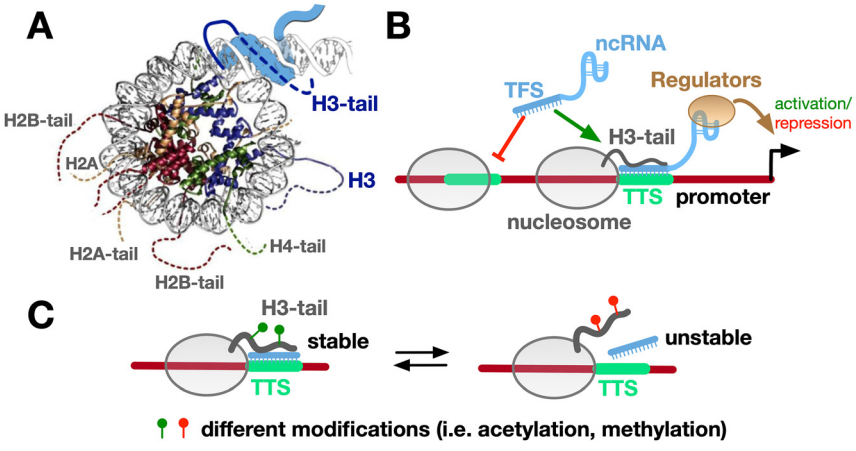

Figure 2: Models describing the binding and function of triple helices in chromatin. (A) 3D model of the nucleosome, the linker DNA with bound RNA and marking the location of the N-terminal histone tails. (B) Model showing the role of nucleosome positioning and the histone H3-tail in stabilising triple helix formation. It is suggested that sequence-specific RNA binding recruits, for example, transcriptional regulators. (C) Post-translational modifications of the histone H3 tail could either stabilise or destabilise the triple helical structure.

histone H3 tail could change the accessibility of the tail for the reader proteins and change epigenetic signaling. Alternatively, or in combination, the modifications could also change the stability of the TFS-TTS interaction and determine the half-life of triplex-chromatin complexes. It can be envisioned that histone H3 methylation, retaining the positive charge of lysine, would maintain stable binding, whereas histone acetylation would weaken these interactions. Interestingly H3K4methylation is associated with active chromatin states, whereas H3K9 is found at inactive chromatin domains. These modifications could stabilise the specific binding of noncoding RNAs targeted by their triplex-forming sequences and direct RNA-bound effector proteins to the associated genomic loci (Figure 2B).

In summary, the chromatin landscape and its changes in nucleosome positions with development would determine the accessible TTS pool and thereby determine the sequence-specific targeting of noncoding RNA to the genome. More pieces of the puzzle have still to be uncovered to understand triplex formation in the nucleus of a cell, as we envision a cooperative effect with the histone post-translational modifications that mark the activity of particular chromatin genomic domains.

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