



## Epigenetic regulation of noncoding RNA transcription by mammalian RNA polymerase III

RNA polymerase III (Pol III) synthesizes a range of medium-sized noncoding RNAs (collectively 'Pol III genes') whose early established biological roles were so essential that they were considered 'housekeeping genes.' Besides these fundamental functions, diverse unconventional roles of mammalian Pol III genes have recently been recognized and their expression must be exquisitely controlled. In this review, we summarize the epigenetic regulation of Pol III genes by chromatin structure, histone modification and CpG DNA methylation. We also recapitulate the association between dysregulation of Pol III genes and diseases such as cancer and neurological disorders. Additionally, we will discuss why in-depth molecular studies of Pol III genes have not been attempted and how *nc886*, a Pol III gene, may resolve this issue.

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### RNA polymerase III

For transcription of RNA from DNA, there are three RNA polymerases (Pol I, II and III) in eukaryotes (reviewed in [1]). Pol I transcribes all ribosomal RNA (rRNA) genes, except for 5S rRNA. Pol II transcribes all protein-coding genes and some noncoding RNA (ncRNA) genes including long ncRNAs, many small nuclear RNAs (snRNAs) and miRNAs. Pol III transcribes a subset of ncRNAs (hereafter termed 'Pol III genes'), including tRNA, 5S rRNA and U6 snRNA. Pol III is a well-characterized multi-subunit enzyme complex (reviewed in [2]) and synthesizes various types of medium-sized ncRNAs that are 100–300 nucleotides (nts) long. Although we have used the term 'medium-sized' because short RNAs usually refer to a 20-nt length, such as miRNAs, Pol III is specialized for short-length transcription when compared with Pol I or II. In case of the transcription of miRNA genes, although their mature forms are short, Pol II actually transcribes long primary tran-

scripts whose sizes are comparable to those of mRNAs.

Promoters of Pol III genes are classified into three types: type I (restricted to the 5S rRNA gene), type II (typical of tRNA genes) and type III (for which the U6 snRNA promoter is paradigmatic; **Figure 1**). Each type has distinct promoter architecture and assembly steps of Pol III transcription factors (TFs). In all the three types, positioning of the catalytic Pol III enzyme complex at the transcription start site (TSS) needs TFIIB consisting of BDP1 (B double prime 1), TBP (TATA-box binding protein) and BRF1 (B-related factor 1; for types I and II) or BRF2 (B-related factor 2; for type III). In the case of types I and II, TFIIB is recruited by TFIIC that recognizes the internal promoter elements box A and box B (or C in the case of type I). In type I, TFIIA binding to the intermediate element should precede TFIIC loading. In the case of type III, TFIIB is recruited by the upstream promoter elements with the aid of SNAPc (small nuclear RNA

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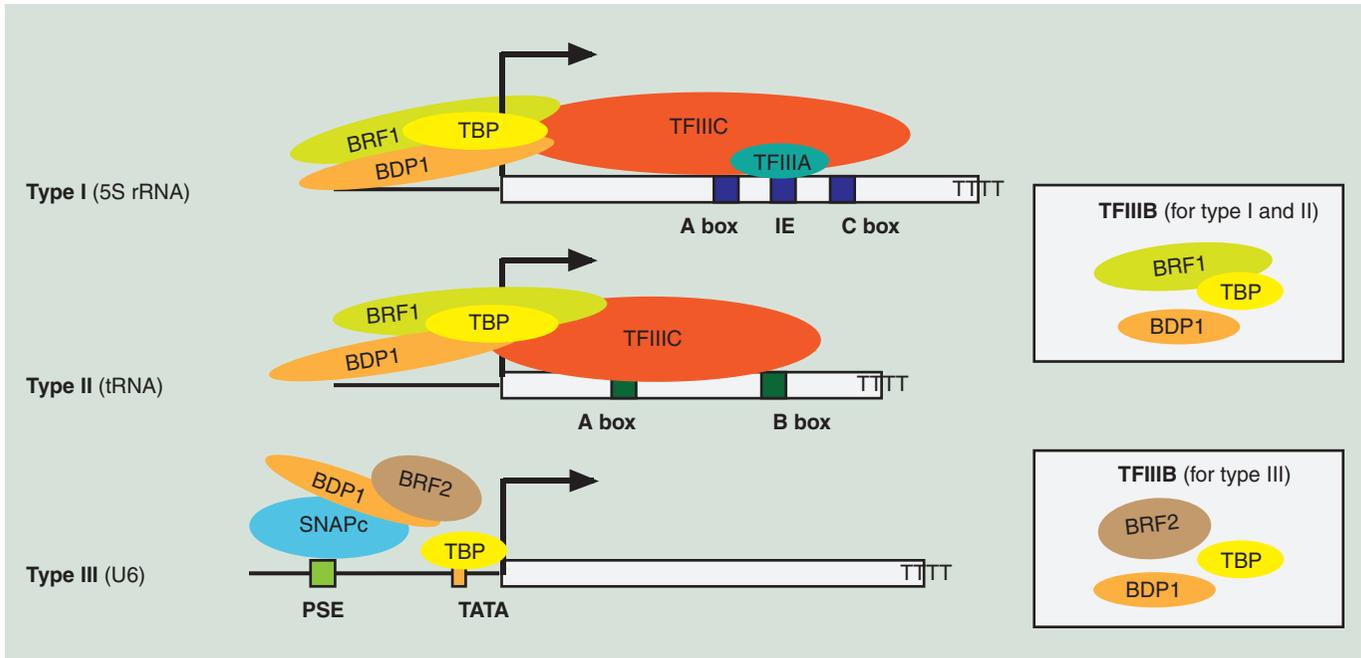
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**Figure 1. Three types of polymerase III promoters.** A cartoon illustrating three Pol III promoter types. DNA sequence elements and protein factors are depicted. Arrows indicate a TSS and direction. ‘TTTT’ is the Pol III termination sequence. TFIIB is the complex of three proteins and in the case of mammalian cells there are two different complexes with BRF1 or BRF2 (see boxes on the right). The relative positions of BRF1 and BDP1 are illustrated based on a ChIP-seq experiment in human cells [9]. BDP1 and BRF1 nearly coincide at approximately 20-nt upstream of TSS in the 5S rRNA gene (type I). In the case of tRNA genes (type II), BDP1 and BRF1 peaks are at -27 and -14 of TSS, respectively. Protein–protein interactions between TFIIC and the three TFII subunits have been reported and reflected in the cartoon (see [2] for details).

BDP: B Double Prime; BRF: B-Related Factor; ChIP: Chromatin immunoprecipitation; IE: Intermediate element; nt: Nucleotide; Pol III: Polymerase III; PSE: Proximal sequence element; TATA: TATA box; TF: Transcription factor; TSS: Transcription start site.

activating protein complex). Each Pol III promoter type and individual subunits of the Pol III machinery have been summarized in an excellent review [2] and are beyond the scope of this review. Therefore, we will not further elaborate on them, except that we intend to highlight different epigenetic regulations according to those Pol III types.

### Diverse roles of Pol III genes & their regulation

Many Pol III genes are abundantly expressed and were identified in early studies during 1970–1990. These classic Pol III genes include those coding for 5S rRNA, tRNAs, some snRNAs (U6 type), 7SK RNA, 7SL RNA, BC200, vault RNAs (vtRNAs), Y RNA, RNase P RNA, RNase MRP RNA (reviewed in [3]). In general, they are a component of a ribonucleoprotein complex to serve as a catalytic core or a guiding molecule for sequence-specific nucleic acid metabolism, which plays roles in protein synthesis, RNA splicing, tRNA and rRNA processing, protein trafficking, DNA replication, etc. These processes are so vital that the classic Pol III genes were considered to be house-keeping genes and therefore Pol III regulation was rather understudied.

However, Pol III regulation is being recognized to be much more complicated especially in higher organisms. As opposed to yeast in which nearly all tRNA genes are actively transcribed [4–6], only a fraction of *in silico* predicted Pol III genes are occupied by the Pol III machinery and thus deemed to be active in mammalian cells [7–14]. Although the repertoire of active Pol III genes shows considerable overlap among cells of different tissue origins, cell-type specific Pol III-occupied loci do exist [7,8,13]. This observation indicates that a sophisticated regulatory mechanism for Pol III genes has emerged during evolution from simple eukaryotes to mammalian cells. Unlike Pol II genes, specific binding of TFs to upstream DNA sequences does not seem to be a prevailing mechanism to regulate individual Pol III genes. So, it has been speculated that epigenetic regulation could provide an alternative way [15]. Epigenetics is a broad term referring any inheritable trait caused by a change of genome functions that do not involve alteration of the DNA sequence. Variations in the chromatin structure caused by histone/DNA modification are the most common epigenetic mechanism and these will be discussed in this review. Results of studies from mammalian cells are mainly examined, but data from yeast (mostly

*Saccharomyces cerevisiae*, studies about which are well summarized in an excellent review [15]) and amphibians (*Xenopus laevis* for early biochemical studies) will be also introduced when informative.

The Pol III transcriptome has been expanding [3] and a growing number of studies uncovered unconventional roles for classic Pol III genes (reviewed in [16]). For example, some tRNAs and vtRNAs are cleaved to produce small regulatory RNAs that play a miRNA-like function ([17], reviewed in [18]). Another example is vtRNA2-1 (a.k.a, nc886) that binds to Protein Kinase R (PKR) and suppresses its activity, rather than acting as a component of the vault complex [19]. Such new functions suggest that the roles of Pol III genes are diverse and that their expression needs to be exquisitely regulated in many biological situations via an epigenetic mechanism in concert with TFs that usually regulate general Pol III activity. Such examples include: alteration of Pol III activity by Rb, p53 and MYC (to be elaborated later), suppression of Pol III transcription by MAF1 under stress conditions (reviewed in [20]), elevation of tRNA expression in pathogen-stimulated macrophages through the NF- $\kappa$ B pathway [21], induction of Pol III transcription by alcohol via estrogen receptor- $\alpha$  [22] and decrease/increase in transcription of some Pol III genes, respectively, by tumor-suppressive/stimulatory compounds [23,24]. In the last example, the chemical compounds (a polyphenol epigallocatechin gallate in [23] and a phytoestrogen in [24]) affected Pol III transcription interestingly in a gender-specific manner.

DNA methylation and histone modification are the most well-known mechanisms for gene silencing in mammalian cells. These epigenetic regulation mechanisms have been well studied in Pol II genes (reviewed in [25]), but not as extensively in Pol III transcription [15]. As mentioned above, classic Pol III genes are so essential that their silencing was beyond consideration. However, the uncovered diversity of Pol III genes and their biological roles suggests that epigenetic silencing is also important in some Pol III genes. One recent example is nc886 (a.k.a, vtRNA2-1) that is silenced by CpG DNA hypermethylation in several malignancies [26-29]. Besides cellular ncRNAs, Pol III transcribes some repeat elements, for example, short interspersed elements such as Alu and mammalian-wide interspersed repeats, whose silencing is thought to be important in maintaining genomic integrity. Furthermore, some viruses employ the host Pol III to transcribe their ncRNAs, such as EBER1/2 and VAI, which suppress PKR as a mean to evade the host innate immune response. Silencing of those ncRNAs might be an important cellular mechanism to counteract viral infection.

## Dysregulation of Pol III genes & diseases

As listed above, Pol III genes play fundamental cellular roles as well as other diverse functions. Therefore, their expression should be tightly controlled, as evidenced by the fact that the failure of such regulation is implicated in a number of diseases including cancer and neurological disorders. Until recently, this alteration was only attributed to the deregulated activity of TFs or the malfunction of Pol III transcription and processing machinery, which will be summarized here (Table 1). Knowledge of Pol III regulation is essential to understanding these diseases and may be of future clinical value to prevent and/or to treat them.

## Cancer

A hallmark of cancer cells is their increased growth rate that demands a higher rate of cellular metabolism. Since Pol III genes play fundamental roles in basic cellular metabolism, elevated Pol III transcription is expected and actually seen in transformed cell lines from a number of studies (see [30] and references therein). Expression of subunits of the Pol III machinery and Pol III genes has been found to be elevated also in clinical specimens; for example, TFIIC2 in ovarian cancer [31], BRF1 in hepatocellular carcinoma [32], BRF2 in several malignancies including non-small-cell lung cancer and esophageal squamous cell cancer ([33-35], reviewed in [36]), tRNA expression in breast cancer [37], the expression of BC200 RNA, Y RNA and nc886 in several tumors [38,39]. Among these, nc886 deserves special attention as it is the only Pol III gene, thus far identified whose epigenetic regulation is implicated in malignancies. The cellular effects and regulation of nc886 will be elaborated upon later in a separate section.

Pol III activity is repressed by tumor suppressors such as p53 and Rb, but is activated by oncogenes such as MYC. MYC is the most renowned oncogenic TF that amplifies the expression of a significant number (>15% of total) of genes when it is overexpressed in malignancies (reviewed in [40]). MYC activates the classic Pol III genes 5S rRNA and tRNA [41,42]. Rb and p53 are frequently inactivated in a number of malignancies (reviewed in [43]). The repression of Pol III transcription by Rb and p53 is well demonstrated in *Rb*- and *p53*-knockout mice in which Pol III activity is elevated [44-47].

Rather than recognizing DNA sequence elements, MYC, Rb and p53 regulate Pol III transcription via their interaction with TFIIB and thereby affect the subsequent steps of Pol III assembly [41,44-47]. Although this mechanism suggests that all Pol III genes should be similarly affected regardless of DNA sequence contexts, we speculate that epigenetic factors such as chro-

Table 1. Disease associated with dysregulation of polymerase III genes and the polymerase III transcription/processing machinery.				
Gene	Description	Type of disease	Status	Ref.
<b>Dysregulation of Pol III genes and the Pol III transcription/processing machinery</b>				
TFIIIC2	Pol III TF (a subunit of TFIIIC)	Cancer	Overexpressed	[31]
BRF1	Pol III TF (a subunit of TFIIIB)	Cancer	Overexpressed	[32]
BRF2	Pol III TF (a subunit of TFIIIB)	Cancer	Overexpressed	[33–35], reviewed in [36]
tRNA	Decoding mRNAs into proteins	Cancer	Overexpressed	[37]
BC200 RNA	Translational modulator	Cancer	Overexpressed	[38]
Y RNA	Initiation of chromosomal DNA replication	Cancer	Overexpressed	[39]
MYC	Activation of Pol III transcription	Cancer	Overexpressed	Reviewed in [40]
p53	Repression of Pol III transcription	Cancer	Inactivated	Reviewed in [43]
Rb	Repression of Pol III transcription	Cancer	Inactivated	Reviewed in [43]
POLR3A/ POLR3B	Subunits of the catalytic Pol III complex	Syndromic hypomyelinating leukodystrophies	Mutated	[48–50]
POLR1C	A subunit of both Pol I and Pol III	Leukodystrophy	Mutated	[51]
BRF1	Pol III TF (a subunit of TFIIIB)	Cerebellar-dental-skeletal syndrome	Mutated	[52]
<i>n-Tr20</i>	A tRNA gene	Neurological defects	Mutated	[53]
TSEN	tRNA-splicing endonuclease	PCH	Mutated	[54]
CLP1	Maturation of tRNA and other RNAs	PCH-like neurodegeneration	Mutated	[55–57]
BDP1	Pol III TF (a subunit of TFIIIB)	Nonsyndromic hereditary hearing loss	Mutated	[58]
RNase MRP	Mitochondrial RNA processing	Cartilage-hair hypoplasia	Mutated	[59]
<b>Dysregulation of Pol III genes by epigenetic mechanisms</b>				
nc886	Suppression of PKR	Cancer	Silenced by CpG DNA hypermethylation	[26–29]
BDP: B double prime; CLP: Cleavage and polyadenylation factor; MRP: Mitochondrial RNA processing endoribonuclease; nc: Noncoding; PCH: Pontocerebellar hypoplasia; PKR: Protein kinase R; Pol: Polymerase; POLR: Polymerase (RNA) III Subunit; Rb: Retinoblastoma susceptibility protein; TF: Transcription factor; TSEN: tRNA-splicing endonuclease.				

matin structure and DNA methylation offer selectivity as a mechanism alternative to TF recognition of DNA sequence in the case of Pol II genes. For example, a subset of Pol III genes in heterochromatin might be excluded from MYC activation while all other Pol III genes are activated by MYC.

An outstanding question is whether the elevated Pol III activity in malignancies contributes to tumorigenesis. This elevated Pol III activity appears to be necessary to accommodate the increased cellular metabolism

of cancer cells (reviewed in [36]). However, MYC, Rb and p53 also regulate multiple Pol II target genes and have a great impact upon cell physiology; thus, it is difficult to discern that portion of Pol III regulation from other indirect effects. Another problem in defining the role of Pol III deregulation in cancer is that MYC, Rb and p53 can regulate Pol III genes globally; so, it is challenging to pinpoint a specific individual Pol III gene. A recently identified Pol III gene, *nc886*, is epigenetically silenced in cancer as opposed to most Pol

III genes. This case will be extensively discussed later. Collectively, Pol III transcription is definitely altered in cancer; further studies will be needed to answer its exact role in tumorigenesis.

### Neurological disorders & others

POLR3A and POLR3B are subunits of the catalytic Pol III complex. Several studies have indicated that syndromic hypomyelinating leukodystrophies are caused in patients with mutations in POLR3A and POLR3B genes [48–50]. More recently, mutations in POLR1C, a common subunit of both Pol I and III, were detected in leukodystrophy patients. Those disease-associated mutations affect Pol III assembly and transcription, but not that of Pol I [51].

A cerebellar–dental–skeletal syndrome is caused by mutations in BRF1, a subunit of TFIIB that loads Pol III to initiate transcription [52]. In this reference, the causal link was demonstrated unequivocally. Knock-out of *brf1* in zebrafish results in similar neurodevelopmental defects that were corrected by the wildtype human BRF1 gene, but not by the mutant BRF1 harboring the syndrome-associated mutations.

Defects in proper expression and maturation of tRNAs have been implicated in neurological disorders. After transcription by Pol III, precursor tRNAs should undergo removal of the 5'-leader and 3'-trailer sequences as well as base modifications (reviewed in [18]). A mutation at the stem of the T-loop of a tRNA gene, *n-T720* (a tRNA decoding the UCU anticodon to arginine), was identified in a mutagenesis screening of mice for neurological phenotypes. This mutation impairs the processing of the precursor tRNA and the consequent low level expression of the mature tRNA *n-T720* is the cause of neurodegeneration phenotypes [53]. Some tRNA genes (6% of the total tRNA genes in humans) have an intron, which should be removed by a tRNA-splicing endonuclease (TSEN). Inactivating mutations of TSEN have been identified in some subtypes of pontocerebellar hypoplasia [54]. A related observation is the mutations at CLP1, a multifunctional kinase implicated in the maturation of tRNAs and other RNAs in patients with neurodegeneration [55,56]. These mutations result in defective CLP1 lacking its kinase activity, leading to TSEN destabilization and consequently aberrant processing of tRNA [55–57].

Dysregulation of the Pol III machinery and Pol III genes is also causative to other diseases. A causative mutation for nonsyndromic hereditary hearing loss was identified at the *BDPI* gene encoding a subunit of TFIIB [58]. RNase MRP RNA is a Pol III gene that plays multiple roles in mitochondrial DNA replication and pre-rRNA processing. Mutations at this gene are causative to cartilage-hair hypoplasia that is character-

ized by short-limbed dwarfism due to skeletal dysplasia, variable level of immunodeficiency and predisposition to malignancies [59].

Collectively, several diseases are caused by alteration of Pol III transcription or dysregulation of the processing machinery as well as mutated Pol III genes. We anticipate that the repertoire of Pol III-associated diseases is more diverse than currently known and that more disease-causative alterations will be identified by virtue of cutting-edge technologies such as next-generation sequencing. However, the molecular link between Pol III genes and disease pathology remains unknown and its elucidation awaits future studies.

### Epigenetic regulation of Pol III genes: chromatin structure & histone modification

The effect of chromatin status on Pol III transcription as well as its importance in Pol III gene regulation has been shown in many previous studies (see also [15], which is a comprehensive review on this topic). In general, compact chromatin is repressive to transcription. In the case of budding yeast, Pol III genes are localized in nucleosome-free regions ([60], see also [15] and references therein), in agreement with the fact that most tRNA genes are active. In contrast, the situation in mammalian cells appears to be complicated. Individual Pol III genes are localized in different chromatin milieu. The effect of chromatin structure is different in the three Pol III subtypes and is likely variable among individual Pol III genes. Rather than being passively subject to epigenetic regulation, Pol III transcription plays an active role in determining chromatin structure.

Histones are a principle component of chromatin, and their modifications are a key determinant of chromatin structure and therefore of gene expression. Methylation and acetylation are two major types of histone modification and these are diversified according to the specific lysine residues at which the modification occurs as well as to the number of methyl groups at a given lysine. Histone modification is well studied in Pol II genes, but much less so in Pol III genes [61].

### Compact chromatin is generally inhibitory to Pol III transcription

Typically, the assembly of chromatin structure onto naked DNA involves sequential binding of the histone H3–H4 tetramer and two histone H2A–H2B dimers to form a histone octamer core particle. Then, the linker histone H1 is associated for chromatin compaction (reviewed in [62]). Early biochemical studies using yeast and *Xenopus* 5S rRNA as a model Pol III gene indicated that transcription is generally less efficient on compact chromatin rather than on naked

DNA [63–67] and that the repressive effect becomes greater as DNA/histones are assembled into more compact structures [67]. The histone octamer is more repressive than the H3–H4 tetramer [64,65]. H1 also directs further repression [68–72]. The repressive effect is more pronounced when the 5S rRNA gene exists in multiple tandem copies, likely because an extended array of nucleosomes forms a more compact high-order structure [65].

These observations suggest that the inhibition of Pol III transcription may be simply a physical consequence of chromatin compaction. In this model (termed ‘simple exclusion’), compact chromatin nonspecifically interferes with the binding of Pol III TFs or blocks the elongation of the polymerizing Pol III enzyme. Before making a general conclusion, it should be noted that 5S rRNA is a type I promoter and does not represent all the Pol III genes. In the next section, we will introduce several cases of chromatin-mediated regulation to highlight the differences between individual Pol III genes, which cannot be well explained by a simple exclusion model.

#### The effect of chromatin structure is more complicated than a simple exclusion model

There are two types of *Xenopus* 5S rRNA genes – oocyte and somatic. The effect of chromatin structure on these two types is different, although both are associated with histone octamers as well as the linker histone H1. The oocyte 5S rRNA gene is inactive when placed into the context of somatic tissues, and this repression is dependent upon H1 [71,72]. In contrast, the somatic 5S rRNA is active and unaffected by the binding of H1 [68–70,73,74]. The explanation for this discrepancy between the two types includes differences in nucleosome organization [73], histone modification [75] and TF binding [70]. This phenomenon provides an interesting case of biological complexity, as the developmentally regulated expression of 5S rRNA gene is quite surprising given that it plays a fundamental biological function and so is expected to be expressed constitutively.

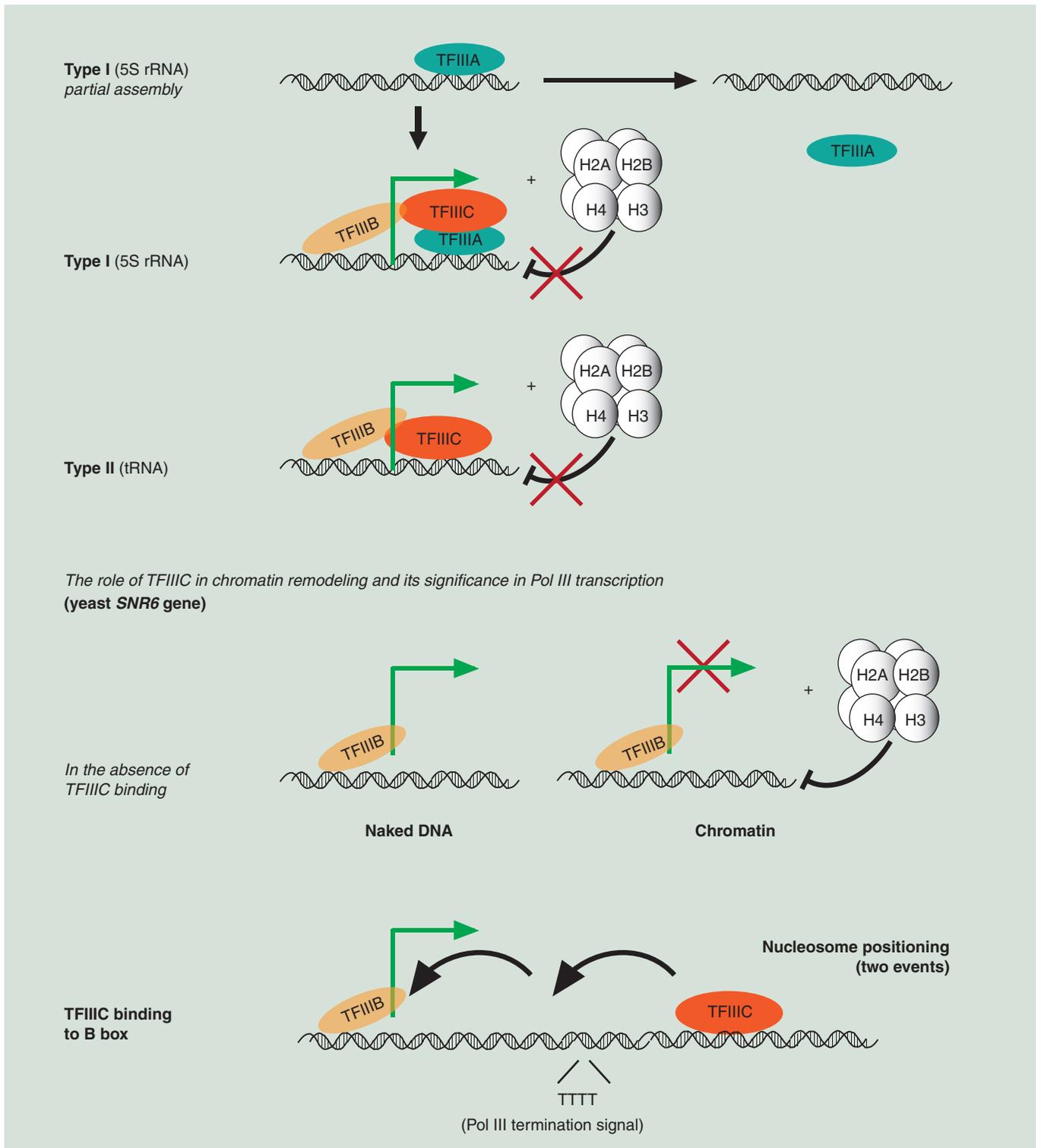
Besides the switching of 5S rRNA genes during development, the Alu repeat element is another intriguing example of chromatin-mediated regulation. Alu is present in more than 500,000 copies in the human genome, but is silenced in human cells *in vivo*, although it possesses Pol III promoter elements and is actually transcriptionally active in transfected cells and *in vitro* transcription assays. Certain biological conditions, such as adenovirus infection, relieve this suppression and robustly induce Alu transcription *in vivo* [76]. Alu is transcriptionally repressed upon nucleosome assembly *in vitro* with purified histones.

In this experimental condition, the repression does not require H1, unlike the case of 5S rRNA [66]. Recently, it has been shown that methylation at histone H3 is the underlying mechanism for the repression of Alu by compact chromatin structure [14].

The simple exclusion model predicts that chromatin compaction dominantly ejects the Pol III machinery from DNA. However, this seems to be largely incorrect. During the nucleosome assembly steps on the yeast and *Xenopus* 5S rRNA gene, the histone octamer formation displaces TFIIA when it alone is bound on DNA [64,65] (Figure 2). TFIIA is the Pol III initiation factor specific to the type I 5S rRNA gene and is required for subsequent recruitment of TFIIC and then TFIIB (Figure 1). Notably, once these TFs are assembled to form the complete Pol III machinery, transcription is resistant to the inhibition by the histone octamer [64,65] (Figure 2). Given that the transcription of the 5S rRNA gene should be mostly in the active state, the observation raises the possibility that continuous active transcription cycles might not allow histones to exert a repressive effect *in vivo*.

In contrast to the 5S rRNA gene that is repressed by compact chromatin unless TFIIC is preloaded, tRNA genes are refractory to repression by histones [77] (Figure 2). tRNA is a type II gene and has internal promoter elements box A and box B, which are recognized by TFIIC. Upon DNA binding, TFIIC recruits TFIIB (Figure 1). So, unlike 5S rRNA, it avoids the requirement of type I-specific TFIIA that is evicted by histone octamers [64,65]. In a yeast study, nucleosome assembly was abrogated in a transcriptionally active tRNA gene; but it was resumed when a mutation was introduced at the promoter elements and thereby transcription was inactivated [77]. This observation corroborates the fact that most yeast tRNA genes are nucleosome-free and active [4–6,60]. Conceivably, transcription elongation may displace nucleosomes and thereby reverse the repression by compact chromatin. However, this simple mechanism is unlikely because TFIIC plays an active role in remodeling chromatin assuring efficient transcription to occur, according to several reports on the yeast U6 snRNA gene (SNR6).

snRNAs are paradigmatic type III genes whose transcription is driven by external promoter elements including a TATA box (see Figure 1). The yeast snRNA SNR6 is an intriguing case. Unlike usual snRNA genes from other organisms, SNR6 has a box B sequence downstream of Pol III termination signal. This B box element is essential to transcription of chromatin templates (Figure 2), but is dispensable for naked DNA [78]. Actually, it has been well demonstrated that TFIIC binding to the B box of SNR6 leads to chromatin remodeling in an ATP-dependent manner [79]. Notably,



**Figure 2. Differential effect of histones on three polymerase III promoter types. (A)** In the 5S rRNA gene, the binding of histone octamers is dominant over TFIIIA before TFIIIC loading and displaces TFIIIA to inhibit transcription (the top image). In contrast, histone octamers cannot inhibit transcription after TFIIIC loading in the type I and type II Pol III promoters (2nd and 3rd images, respectively). See the text for details. **(B)** The role of TFIIIC in the case of the yeast SNR6 gene that harbors a TFIIIC-binding site (a box B) downstream of the Pol III termination signal. TFIIIC binding mediates two consecutive nucleosome positioning events for optimal binding of TFIIIB to ultimately activate transcription. See also the text for further details. SNR6: U6 snRNA; TF: Transcription factor.

the TFIIC effect is not simply to relieve the repressive effect of chromatin, because the transcription of the chromatin template in the presence of TFIIC is more efficient than that of naked DNA. TFIIC binding to the B box leads to a positioned nucleosome between the A and B boxes, which are consequently juxtaposed for the optimal binding of TFIIC. Following this initial nucleosomal positioning, TFIIC mediates another nucleosome re-arrangement by shifting the nucleosome between the boxes A and B to approximately 40-nt upstream. This allows efficient loading of TFIIB and ultimately high-level transcription [80]. *In vivo* probing of chromatin structure has indicated that the second rearrangement is critical for the modulation of SNR6 transcription under stress conditions [81]. These SNR6 data argue against the simple exclusion model by providing a remarkable example of the discriminating interplay between Pol III TFs and chromatin. It appears that TFIIC plays a critical role in chromatin remodeling (Figure 2) and this will be discussed in the next section, in regard to its potential histone acetyltransferase (HAT) activity.

Collectively, the effect of chromatin structure on Pol III transcription seems to be very complicated. Chromatin structure has different effects on each Pol III promoter type (Figure 2) and is influenced by many other factors: DNA sequence contexts, the Pol III machinery and TFs. Early studies employed classic ncRNA genes that are transcribed from multiple genomic loci, leading to highly abundant and constitutive RNA expression. For those RNAs, what is the significance of regulation at individual loci? We hypothesize that an epigenetic change at a Pol III locus under some conditions might alter the expression of nearby Pol II genes by affecting the chromatin milieu. This hypothesis has been supported by several reports on yeast Pol III genes and one report on mouse short interspersed repeat elements (reviewed in [82]).

### A new era of Pol III study evolves due to advanced techniques

Classic biochemical studies have been highly valuable to the initial understanding on epigenetic regulation of Pol III transcription. However, these studies were done *in vitro* and it was questionable whether the data reflect *in vivo* situations. This issue has been elucidated by advanced techniques. Chromatin immunoprecipitation (ChIP) assays are a commonly used method to assess the binding of the Pol III machinery in the original chromosomal context. One hurdle in studying Pol III regulation is the repetitive nature of many Pol III genes that makes it challenging to assess the contribution of each genomic locus among multiple genomic loci as well as to examine global Pol III expression pro-

files. For this reason, ChIP of Pol III (Pol III enzyme or TFs recruiting it), which captures unique flanking sequences in addition to the transcripts themselves, is commonly used as a proxy indicator rather than the level of RNA. In yeast and *Drosophila*, ChIP in concert with array hybridization (ChIP-chip) defined the Pol III transcriptome mainly consisting of tRNA genes [4–6,83]. The advent of next-generation sequencing technologies provides a single nucleotide resolution and ChIP combined with this technique (ChIP-seq) has been exploited to identify the Pol III transcriptomes in mammalian cells.

Data from ChIP-seq of the Pol III machinery have provided an amazing wealth of information. Pol III ChIP-seq experiments have been done in several cancer cell lines, fibroblasts, stem cells, T-cells and mouse liver tissue ([7–14]; summarized in Table 2). These data clearly have proven that all Pol III genes are not active in mammalian cells and that they are expressed in a cell-type-specific manner. Pol III ChIP-seq captures some previously unidentified Pol III-bound loci and contributes to defining a complete Pol III transcriptome [7–11]. For example, nc886 was identified as a novel Pol III gene in this way [9,12]. It also confirmed the requirement of different Pol III TFs according to three promoter types (e.g., BRF1 for type I and II vs BRF2 for type III) *in vivo* [9]. Lastly, but most importantly, Pol III ChIP-seq data have been analyzed in comparison to genomic features (such as DNA sequence, histone marks, expression of nearby Pol II genes, the presence of CpG islands, etc.), providing insights about Pol III gene expression and regulation.

Several pieces of information from ChIP-seq data analysis are as followings. TFIIC-occupied loci include but outnumber Pol III-occupied loci [7,10,11,13], indicative of additional roles of TFIIC besides Pol III transcription. In yeast, similar TFIIC-only sites were identified and found to act as a barrier between hetero- and eu-chromatin [84]. Sequence comparison between Pol III-occupied or unoccupied tRNA genes indicates that the internal promoter elements boxes A and B share similar consensus sequences. So, promoter sequences, although they are required for transcription, do not appear to determine the expression level of tRNA genes [7,9]. ChIP-seq data have also shown a relation between global chromatin status and Pol III transcription. A remarkable case is the comparison of adipose-derived stem cells (ADSCs) to the ADSC-derived pluripotent stem cells [13]. ADSC-derived pluripotent stem cells are more pluripotent than ADSCs and also have Pol III binding at more loci, most likely because they have more open chromatin structure than ADSC. The relation to histone modifications and nearby Pol II genes will be discussed in the next section.

Table 2. Summary of chromatin immunoprecipitation-seq experiments for polymerase III and modified histones.

Cell lines or tissues	ChIP-seq with:	Relation to histone modifications and nearby Pol II (and brief description about results)	Ref.
Human cell lines (HeLa, HEK293T, human foreskin fibroblasts, Jurkat)	– TFIIIB (BRF1 and BRF2) – TFIIIC (TFIIIC63) – Pol III (RPC32)	– H3K4me1/3, H2A.Z, H3K27ac – H3K27me3 – Pol II: (+) correlation to Pol III peaks – DNase I hypersensitive sites: (+) correlation to Pol III peaks	[7]
Human CD4 <sup>+</sup> T cells (and compared with HeLa)	– TFIIIB (BRF1) – TFIIIC (TFIIIC220) – Pol III (RPC155) – Pol II (S2P) – H3K4me3	– H3K4me1/2/3, H2A.Z, H3K9ac, H3K4ac, H3K23ac, H3K27ac, H3K36ac, H2AK9ac, H3K18ac, H3K27me1, H4K16ac, H4K20me1 – H3K27me2/3, H3K9me3 – H3K79me2, H3K36me3 – Pol II: (+) correlation to Pol III peaks	[8]
IMR90hTert fibroblasts	– TFIIIB (BDP1 and BRF1) – SNAPc (SNAPC2) – Pol III (RPC4)	No comparison	[9]
K562 (immortalized myelogenous leukemia line)	– TFIIIB (BDP1 and BRF1) – TFIIIC (TFIIIC110) – Pol III (RPC155)	– H3K4me2/3, H3K27ac, H3K9ac – Pol II: (+) correlation to Pol III peaks	[10]
Mouse ES cells	– TFIIIB (BRF1 and BRF2) – TFIIIC (TFIIIC220/110/90) – Pol III (RPC1/4)	No comparison	[11]
Mouse liver tissue	– Pol III (RPC1/4, RPB2) – Pol II (RPB2) – H3K4me3, H3K36me3	– H3K4me3 – H3K36me3	[12]
Human ES cells	– TFIIIC (TFIIIC63) – Pol III (RPC155)	– H3K4me1/2/3, H2A.Z, H3K9ac, H3K27ac – H3K36me3, – H3K27me3 (cf. H3K27me3 in HeLa [8]) – Pol II: (+) correlation to Pol III peaks	[13]
HeLa cells and A31 fibroblasts	– TFIIIB (BRF1) – TFIIIC (TFIIIC110/220) – Pol III (RPC155) – H3K9me3	– H3K9me3	[14]

Red- and blue-colored histone modifications indicate stimulatory and repressive functions to Pol III transcription.  
BDP: B double prime; BRF: B-related factor; ChIP: Chromatin immunoprecipitation; ES: Embryonic stem; Pol: Polymerase; RPB: RNA Polymerase II Subunit B; RPC: RNA Polymerase III Subunit C; SNAPc: Small nuclear RNA activating protein complex; TF: Transcription factor.

### Histone acetylation

In general, histone acetylation is associated with open chromatin structure and active transcription [85]. For example, histone H3 acetylated at lysine residues 9 and 27 (H3K9ac and H3K27ac) is present at actively transcribed Pol II genes. Especially, H3K27ac is found in the enhancer region of Pol II genes. The positive effect of acetylation is similarly seen also in Pol III genes [75,86–88]. When acetylated *in vitro*, histone octamers lose their capability to interfere with TFIIIA binding to the *Xenopus* 5S rRNA gene [87], most probably because they become deficient in forming high-order structures [89]. A ChIP experiment revealed that acetylated histone H4 is associated

with the transcriptionally active *Xenopus* somatic 5S rRNA gene [75], which corroborates aforementioned *in vitro* data. Recent mammalian ChIP-seq data are also in agreement with this concept. Pol III-occupied loci tend to be marked with H3K9ac and H3K27ac ([7,8,10,13]; Figure 3A & Table 2). Some acetylated histone marks display peaks relative to the TSS [8,10]. For example, H3K4ac, H3K9ac, H3K18ac are enriched at the -1 nucleosome of active Pol III genes (Figure 3A), suggesting that these histones contribute to defining the TSS of Pol III genes. Interestingly, these acetylated histones show peaks at the +1 nucleosome position in the case of Pol II genes. Other acetylated histones including H2AK9ac and H4K16ac are enriched in

active Pol III genes but no peaks are found near TSS. It should be noted in some mammalian ChIP-seq data that histones are depleted at the TSS [8,10] similarly to yeast tRNAs [4–6], probably due to the fact that active transcription displaces nucleosomes [77].

So, how are the histones acetylated? As mentioned in the previous section, TFIIC, a Pol III initiation factor composed of multiple subunits, can overcome nucleosome structure. TFIIC was shown to possess intrinsic HAT activity in the 110 and 90 kDa subunit (TFIIC110 and TFIIC90 in humans) [90,91]. However, a more recent study [92] indicated that TFIIC has a very weak HAT activity as compared with p300, a potent HAT. When a Pol III gene in chromatin is transcribed *in vitro* with purified proteins, TFIIC acts as a recruitment factor for p300 rather than as a *bona fide* HAT (Figure 3A). Nonetheless, TFIIC may function as a HAT *in vivo*, possibly aided by some factors missing in the purified system. Another mechanism for histone acetylation is via MYC. Not only by interacting with TFIIB, MYC activates Pol III transcription by acetylation. MYC recruits GCN5, a HAT, as well as the cofactor TRRAP at tRNA and 5S rRNA genes (Figure 3A). This MYC-directed complex does not contain TIP60, although it is a HAT that acetylates histones in many Pol II genes [93]. As a result of p300 and GCN5 actions, histone H3 is hyperacetylated and Pol III recruitment is stimulated [88]. Based on a report [94], p300 and GCN5 are likely to be HATs for H3K27ac and H3K9ac, respectively (Figure 3A).

### Histone methylation

Histone methylation is associated with either repressed or active chromatin [61]. Histone H3 trimethylated at lysine residues 9 and 27 (H3K9me3 and H3K27me3) is associated with repressive chromatin, whereas histone H3 that is mono-, di- or trimethylated at lysine residue 4 (H3K4me1/2/3) and is trimethylated at lysine 36 (H3K36me3) indicates open chromatin [95,96]. In the case of Pol II genes, H3K4me1 is a marker for the enhancer region, H3K4me3 is for active promoters and H3K36me3 is for transcription elongation.

Similarly to what has been observed in Pol II genes, Pol III ChIP-seq peaks are positively/negatively correlated with those open/repressive histone marks ([7,8,10–14]; Figure 3B & Table 2). However, there are some differences between Pol II and Pol III genes. H3K4me3 displays a peak at the -1 nucleosome relative to TSS in Pol III genes, but at the +1 nucleosome in Pol II genes [8,10,12]. H3K27me3, as repressive chromatin mark, is enriched in both of inactive Pol II and Pol III genes. However, there is a sharp peak at the TSS in the case of Pol III genes (Figure 3B), but no peaks are

seen in Pol II genes [8]. H3K79me2 and H3K36me3, which are present in active Pol II genes, are not enriched in active Pol III genes ([7,8,10,12,13]; Figure 3B). In the case of H3K36me3, it can be explained by its methylation mechanism. The recruitment of a histone methyltransferase for H3K36me3, Set2, onto target chromatin is guided by its binding to the phosphorylated C-terminal domain tail of elongating Pol II [97]. This may be the reason for the lack of H3K36me3 in Pol III genes.

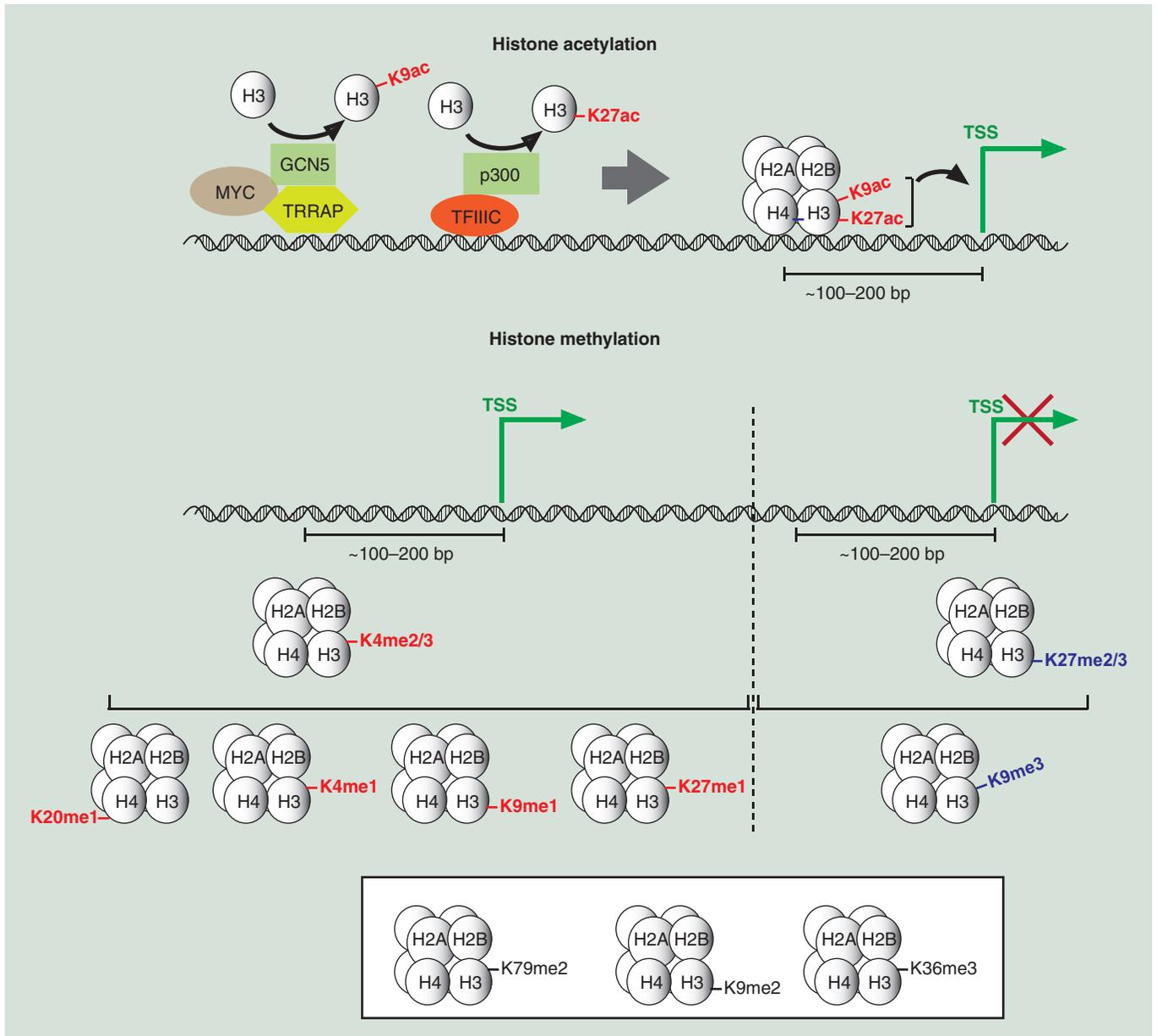
In the case of H3K27me3, its methylation appears to be executed by the histone methyltransferase EZH2, a component of the Polycomb repression complex 2 (PRC2) [98]. The recruitment of PRC2 to Pol III loci is mediated by physical interaction between PRC2 components (EZH2 itself and another component SUZ12) and TFIIC subunits. This report again highlights the role of TFIIC as a determinant for chromatin structure.

### Histone phosphorylation

Besides histone acetylation and methylation, histone H3 is also phosphorylated at serine residues 28 and 10 (H3S28ph and H3S10ph) and this phosphorylation is implicated in mitotic chromosome condensation and regulation of gene expression (reviewed in [99]). A couple of reports have indicated the role of histone phosphorylation in Pol III transcription [100,101]. Treatment with an oncogene and a chemical carcinogen leads to the association of H3S28ph and H3S10ph with some Pol III loci to activate their transcription. This suggests that histone phosphorylation and the resultant activation of Pol III genes might contribute to the transformation process. However, the mechanistic link among histone phosphorylation, chromatin structure and Pol III transcription has not been explored and needs further investigation.

### Collaboration with neighboring Pol II genes

Collectively, chromatin status dictated by histone modification is an important determinant for Pol III transcription. Therefore, it is not surprising that Pol II and Pol III genes are coregulated when closely located. This notion has been confirmed by comparisons between Pol II and Pol III ChIP-seq data. Pol II binding is enriched at approximately 300-nt upstream of U6 snRNA loci that are actively transcribed [102]. This close localization of Pol II is also seen in other Pol III genes including 7SK RNA, vtRNAs, RNase MRP RNA and many tRNA genes [8]. Several ChIP-seq data consistently indicated that Pol III occupancy is positively correlated with the active transcription of nearby Pol II genes ([7,8,10,12,13]. Also, active Pol III loci have enriched Pol II binding ([7,8,10,13]; Table 2).



**Figure 3. Summary of histone acetylation and methylation in polymerase III transcription.** (A) Acetylation of histone H3. Histone H3 acetylation by GCN5 and p300 is shown on the left. The deposition of H3K9ac and H3K27ac at the indicated position relative to TSS stimulates Pol III transcription, as schematically shown on the right. See the text for details. (B) Roles of methylated histones in Pol III transcription. Red, blue and black letters indicate stimulatory, repressive and neutral methylation to Pol III transcription, respectively. H3K4me2/3 and H3K27me2/3 display a peak in indicated positions relative to TSS. Histones below horizontal brackets are enriched in the active/inactive Pol III loci, but display no prominent peak. Neutral histone marks are in a box below. See also Table 2. GCN: General control of amino acid synthesis protein; Pol: Polymerase; TRRAP: Transformation/transcription domain-associated protein; TSS: Transcription start site.

### Epigenetic regulation of Pol III genes: CpG DNA methylation

The most common epigenetic modification of DNA is methylation at the 5-carbon position of the pyrimidine ring of cytosine in CpG dinucleotides. Some gene promoters have a CpG island, a block of CpG-rich sequences that is found at the promoter region of housekeeping genes or tumor-suppressor genes that are usu-

ally unmethylated and expressed in normal conditions (reviewed in [103]). In some aberrant conditions such as malignant transformation, CpG hypermethylation occurs and the gene is silenced via chromatin remodeling (reviewed in [25]). This gene-silencing mechanism has been extensively studied in Pol II genes. In contrast, reports about CpG methylation and Pol III regulation are surprisingly scarce.

### Early studies

The first report of CpG DNA methylation was the measurement of Pol III transcription upon injection of *in vitro* methylated DNA templates into *Xenopus* oocytes [104]. Since then, similar experiments in mammalian cells and *in vitro* transcription assays have been performed in a handful of articles [26,27,105–107]. In general, Pol III transcription is inhibited by CpG methylation in these studies, but a couple of points should be addressed therein. First, the DNA templates were ectopically added and so might be considered in a non-chromosome context. However, they were expected to be in a chromatin context because they probably associated with histones soon after transfection into *Xenopus* oocytes, mammalian cells and *in vitro* transcription reactions employing crude extracts. Second, the three Pol III promoter types appear to be differently regulated: type II (for tRNAs) and type III (for U6 snRNA) were inhibited by methylation, whereas type I (for 5S rRNA) was unaffected [104,108].

### Silencing by CpG hypermethylation: what is the mechanism?

CpG DNA hypermethylation can lead to gene silencing via two possible mechanisms. One is the inhibition of Pol III accessibility through the remodeling of chromatin structure by histone modification ('indirect chromatin-remodeling mechanism'). This mechanism is well studied in Pol II genes (reviewed in [25]) and is presupposed to be true for Pol III genes. Actually, a number of ChIP-seq data have supported this model by showing that Pol III binding is positively correlated with histone markers for open chromatin and with the active transcription of neighboring genes [7,8,10–14]. However, this model is somewhat contradictory to data from the early studies that were discussed in the previous section. The indirect chromatin mechanism is hardly justified by the inefficient transcription from ectopically introduced CpG-methylated DNA templates. Moreover, if heterochromatin formation by hypermethylation is the simple mechanism, the three Pol III promoter types should be equally affected.

An alternative mechanism is that CpG hypermethylation *per se* renders the promoter less preferable for Pol III binding ('direct DNA-binding mechanism'). This model is favored by the aforementioned circumstantial evidence and also is directly supported by experimental data from an *in vitro* screening of proteins that bind to methylated DNA [109]. In this work, several proteins comprising TFIIC bind efficiently to an unmethylated DNA fragment, but this binding is abrogated upon CpG methylation. Interestingly, the methylation effect on TFIIC binding is seen in naked DNA, but disappears when the DNA is assembled

into nucleosomes. In another study, CpG methylation inhibits binding of Pol III-activating TFs [106].

Further elucidation is needed as to which model is correct. Nonetheless, the two mechanisms are not necessarily mutually exclusive. Toward this goal, an in-depth mechanistic study is necessary, but such an investigation has been hampered by lack of a suitable model Pol III gene. Classic Pol III genes such as 5S rRNA, tRNAs and U6 snRNA are not good models, because their transcription units are either identical or highly similar sequences that are scattered throughout the genome in multiple copies. Therefore, it is nearly impossible to correlate the transcription rate from a single genomic locus to the steady-state RNA level and also impractical to introduce a mutation into all genomic copies. Although some Pol III genes are encoded at a unique locus or a few loci, their silencing by CpG hypermethylation has not been reported until recently. In this regard, an interesting Pol III gene has been recently identified and will be discussed below.

### nc886: a model Pol III gene that is epigenetically regulated by CpG methylation

As described in the previous section, some Pol III genes were shown to be suppressed by CpG DNA methylation. However, the biological significance is obscure in most cases. nc886 is the first and so far only Pol III gene which is silenced by CpG DNA hypermethylation and some functions in disease have been identified [26–29].

nc886, a 101-nt long ncRNA, was originally known as a precursor miRNA or a vtRNA. Actually, it was registered in the miRNA database (miRBase; [www.mirbase.org/](http://www.mirbase.org/)). Additionally, VTRNA2-1 is listed as the official name (in HUGO Gene Nomenclature Committee; <http://www.genenames.org/>), because nc886 shows considerable sequence homology to canonical vtRNAs that are RNA components of a large ribonucleoprotein complex called the vault complex. However, strong experimental evidence has demonstrated that nc886 neither produces mature miRNAs nor exists in the vault complex [19]. The same report discovered that nc886 binds to a protein called PKR that plays critical biological roles in innate immunity and cancer (reviewed in [110]). Upon binding, nc886 suppresses PKR activity. Epigenetic silencing of nc886 during tumorigenesis leads to PKR activation that appears to serve as an intrinsic pro-inflammatory signal to provoke oncogenes. So, nc886 is a putative tumor suppressor.

nc886 has a strong CpG island at its upstream region. Among the three Pol III promoter types, nc886 seems to belong to type II because it has the well-conserved internal promoter elements boxes A and B, which are recognized by TFIIC and so is crucial for transcription

[LEE YS, UNPUBLISHED DATA; see also (Figure 1)]. The nc886 CpG island encroaches into the transcribed region and encompasses boxes A and B. Interestingly, each box contains a CpG dinucleotide. In nc886 and any other Pol III genes, it is completely unexplored whether CpG methylation on these residues is necessary and/or sufficient to interfere with TFIIC recognition. Such a study might provide a clue to discern between the two mechanisms: ‘indirect chromatin-remodeling mechanism’ versus ‘direct DNA-binding mechanism.’

### The significance of nc886 silencing in malignancies & other diseases

Clinical data in gastric cancer, esophageal squamous cell carcinoma, small cell lung cancer and acute myeloid leukemia strongly supported the idea that nc886 is a tumor suppressor. In these malignancies, CpG hypermethylation of nc886 is associated with poor survival of patients [26–29]. It should be underscored again that such epigenetic silencing of a Pol III gene in malignancies was unprecedented until the identification of nc886. nc886’s role in cancer is well summarized in a recent review [111].

Besides its silencing in cancer, the methylation of nc886 has several uniquely intriguing aspects. In normal human individuals, it is methylated in one allele, specifically of maternal origin [28,112]. Also, it is a metastable epiallele whose degree of methylation in the offspring is determined by maternal nutrient status during the periconceptual period [113]. Given that nc886 suppresses PKR, the variable expression of nc886 according to the degree of methylation is expected

to determine an individual’s differential response to inflammatory signals and thus susceptibility to viral infection. So, nc886 is hypothesized to be implicated in infection-related diseases, but this awaits further investigation. Apart from the biological consequence, those observations on nc886 are also scientifically interesting by themselves and raise several questions. How does maternal diet influence offspring’s methylation status? When the maternal allele is methylated during early development, how does the methylation machinery distinguish it from the other alleles and specifically methylate it? Is the molecular mechanism of methylation (i.e., methylation enzymes) different between the normal developmental program and tumorigenesis?

### Future perspective

Classic biochemical studies laid the foundation of our knowledge on epigenetic regulation of Pol III transcription. Following this, recent genome-wide studies are providing ample information in an unprecedented way. Now, we appreciate that detailed regulatory mechanisms for individual Pol III genes seem to be much more complex than has been previously thought. We anticipate that accumulation of global ChIP-seq and RNA-seq data will be instrumental in elucidating how individual genes are epigenetically regulated. However, this should be complemented by in-depth molecular studies that have fallen behind because of lack of a suitable model Pol III gene. Toward this goal, nc886 could be an ideal Pol III gene to investigate molecular mechanisms, because it is encoded at a unique locus in the human chromosome unlike many repetitive

#### Executive summary

##### Background of RNA polymerase III

- Polymerase III (Pol III) transcribes medium-sized noncoding RNAs (Pol III genes) with fundamental and also diverse unconventional cellular functions.
- Pol III genes are tightly controlled via epigenetic mechanisms.

##### Deregulation of Pol III genes & diseases

- Pol III expression is elevated in cancer. In this case, MYC, Rb and p53 regulate the Pol III machinery to alter Pol III genes globally.
- Mutations in genes coding the Pol III polymerase and processing enzymes are causative to some neurological disorders.
- Epigenetic silencing of nc886 plays a role in tumorigenesis and hypothetically in infection-related diseases.

##### Epigenetic regulation of Pol III genes: chromatin structure & histone modification

- Compact chromatin is generally repressive in Pol III transcription; however, its effects on individual Pol III genes are diverse and furthermore, it is in turn influenced by the Pol III machinery.
- Pol III transcription is activated and repressed by histone modifications indicative of open chromatin and heterochromatin, respectively. Consistently, Pol III transcription is positively influenced by active transcription of nearby Pol II genes.

##### Epigenetic regulation of Pol III genes: CpG DNA methylation

- CpG methylation appears to differently affect three subtypes of Pol III genes.
- The suppression of Pol III genes by CpG hypermethylation involves heterochromatin formation; however, its direct effect on the Pol III machinery to bind to promoter sequences is also a possibility.
- A Pol III gene called nc886 has been recently found to be silenced by CpG hypermethylation.

Pol III genes and so is experimentally manageable *in vivo*. Investigation of a model for Pol III transcription will certainly provide useful information in studying other Pol III genes, even though nc886's molecular mechanism would probably not represent all Pol III genes because epigenetic regulation of individual Pol III genes is extremely diverse as has been emphasized throughout this review. Finally, we expect that more cases of Pol III genes will be identified as being epigenetically regulated in various biological conditions, such as differentiation, human diseases, virus infection and malignancies.

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