

## Perspectives

# Chromatin Turn Ons and Turn Offs of Ribosomal RNA Genes

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## KEY WORDS

DNA methylation, histone modification, chromatin, gene silencing, nucleolar dominance

## ABBREVIATIONS

rRNA	ribosomal RNA
NOR	nucleolus organizer region
ChIP	chromatin immunoprecipitation
PCR	polymerase chain reaction
FISH	fluorescence in situ hybridization
Aza-dC	5-aza-2' deoxycytosine
TSA	trichostatin A
FKBP	FK506 binding protein

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## ABSTRACT

Eukaryotes have hundreds (sometimes thousands) of ribosomal RNA (rRNA) genes whose transcription by RNA polymerase I helps establish the proliferative ability of cells by dictating the pace of ribosome production and protein synthesis. Interestingly, only a subset of the total rRNA gene pool is active at any one time, making rRNA genes attractive for understanding the dynamic balance between gene silencing and activation. However, the fact that rRNA genes are essentially identical in sequence in a pure species has been an obstacle to telling apart the active and inactive genes. Nature has provided one solution to this conundrum in the form of the epigenetic phenomenon, nucleolar dominance: the transcriptional silencing of one parental set of rRNA genes in a genetic hybrid. Parental genes in hybrids typically differ in sequence as well as expression, allowing a definition of the chromatin modifications of rRNA genes in the on and off states in vivo. By exploiting nucleolar dominance in plants, we recently showed that concerted changes in DNA methylation and histone methylation comprise an epigenetic switch that turns rRNA genes on and off. Independent studies using mouse and human cells have led to similar conclusions, implicating chromatin modifications as important components of the regulatory networks that control the effective dosage of active rRNA genes.

Several decades have passed since the electron micrographs of Miller and colleagues first provided evidence that some ribosomal RNA genes are transcribed and others are inactive.<sup>1,2</sup> In fact, though eukaryotes have hundreds (sometimes thousands) of rRNA genes, no more than half of the rRNA genes appear to be active at any one time,<sup>3-5</sup> indicating that eukaryotes control the effective dosage of their rRNA genes. Only recently has a role for chromatin modifications in regulating rRNA gene transcription become clear at a mechanistic level.

In a recent study,<sup>6</sup> we examined the density of methylated cytosines in the promoter regions of rRNA genes in the plant species, *Arabidopsis thaliana* following bisulfite treatment<sup>7</sup> and subsequent sequencing of 67 independent promoter clones. Importantly, a large subset of the rRNA genes were found to be hypermethylated on all promoter cytosines but a small subset was almost devoid of promoter methylation.<sup>6</sup> Meanwhile, chromatin immunoprecipitation (ChIP) experiments were revealing to us that rRNA genes associate with histone H3 dimethylated at lysine 9 (H3<sup>dimethyl</sup>K9), a mark of heterochromatin, but also associate with histone H3 trimethylated at lysine 4 (H3<sup>trimethyl</sup>K4), which is a mark of active genes. An assay we dubbed ChIP-chop-PCR<sup>6</sup> helped us integrate and make sense of these observations. The key to the ChIP-chop-PCR assay is the enzyme McrBC, which digests (chops) DNA having two or more purine-methylC motifs thereby preventing subsequent amplification of the template by PCR. This assay thus provides a means for discriminating between hypermethylated and hypomethylated DNA that is recovered by ChIP and was inspired by a clever, and conceptually similar assay devised in the Grummt lab using the methylation sensitive restriction endonuclease *Hpa* II.<sup>8</sup> Our initial results using ChIP-chop-PCR revealed that those rRNA genes immunoprecipitated with antibodies recognizing H3<sup>trimethyl</sup>K4 or RNA polymerase I are the genes whose promoters are hypomethylated, indicating that H3<sup>trimethyl</sup>K4 association and cytosine hypomethylation are characteristics of active rRNA genes.<sup>6</sup> By subtractive logic, we expected that the rRNA genes associated with H3<sup>dimethyl</sup>K9 and having hypermethylated promoter cytosines were likely to be the inactive set of rRNA genes, but we needed a way to test this prediction directly.

To define the epigenetic marks of silent rRNA genes, we turned to *A. suecica*, the allotetraploid hybrid of *A. thaliana* and *A. arenosa* in which the *A. thaliana*-derived rRNA genes are subjected to nucleolar dominance and are transcriptionally silenced.<sup>9</sup> Exploitation of

this phenomenon allowed us to definitively discriminate between active and inactive rRNA genes and define their chromatin modification states. Using ChIP, ChIP-chop-PCR, and FISH followed by immunolocalization, we verified that the silent *A. thaliana* rRNA genes associate exclusively with H3<sup>dimethyl</sup>K9 and are enriched with hypermethylated promoter cytosines.<sup>6</sup> We also took advantage of the fact that silencing in nucleolar dominance is reversible, such that the normally silent *A. thaliana*-derived rRNA genes in *A. suecica* can be derepressed with chemical inhibitors of DNA methylation (5-aza-2' deoxycytosine; aza-dC) or histone deacetylation (trichostatin A; TSA).<sup>6</sup> By manipulating the on or off state, we were thus able to examine the changes in DNA methylation and histone modification that correspond to the alternate transcriptional states. Interestingly, when rRNA genes are derepressed with either aza-dC or TSA, the genes become enriched with H3<sup>trimethyl</sup>K4 and lose their promoter cytosine hypermethylation. Thus far, we have been unable to uncouple the loss of H3<sup>dimethyl</sup>K9 association from the acquisition of H3<sup>trimethyl</sup>K4 association and the loss of cytosine methylation, indicating that these are concerted changes integral to the rRNA gene "on" and "off" switch.<sup>6</sup>

Given that normally silent *A. thaliana*-derived rRNA genes in *A. suecica* can be derepressed by chemically inhibiting histone deacetylation with TSA, we sought to identify which histone deacetylases might be required for repressing the *A. thaliana*-derived rRNA genes. By expressing double-stranded RNAs from transgenes targeting specific HDACs for RNAi-mediated suppression, we found that *A. thaliana*-derived rRNA genes in *A. suecica* were derepressed in RNAi lines targeting *HDT1* (its name according to www.chromDB.org, see ref. 10; it is also known as *AtHD2a*<sup>11</sup>). *HDT1* is a nucleolar-localized member of a plant-specific HDAC family first identified following biochemical purification and sequencing of a maize HDAC activity, HD2 by Loidl and colleagues.<sup>12,13</sup> ChIP and ChIP-chop-PCR experiments revealed that in *HDT1*-RNAi lines the *A. thaliana*-derived rRNA genes in *A. suecica* are enriched with H3<sup>trimethyl</sup>K4 and lose promoter cytosine methylation, indicating that *HDT1* is a key component of the "off" switch.<sup>6</sup>

In *A. thaliana*, four homologs of *HD2* exist,<sup>10,11</sup> *HDT1-4*. Of these, only *HDT1* has been found to have a definitive role in rRNA

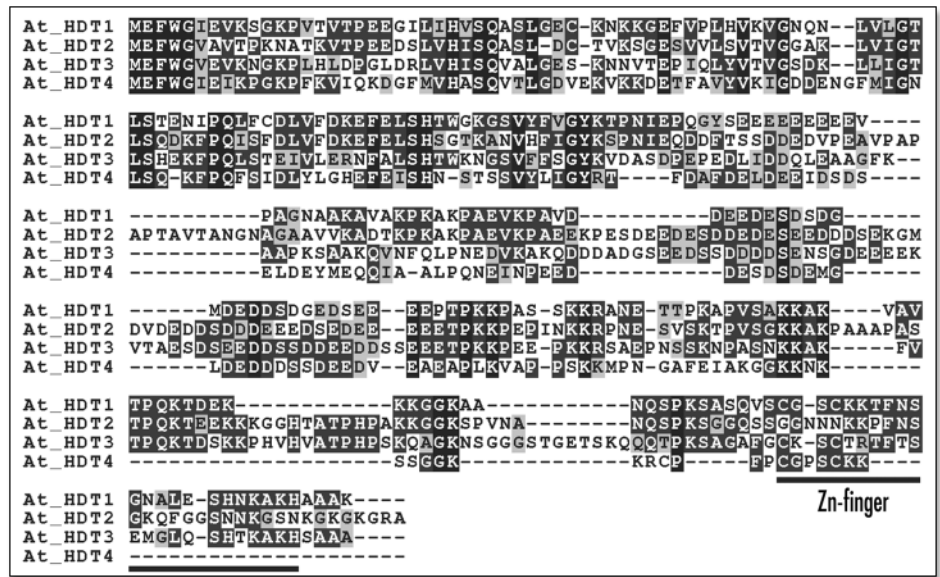


Figure 1 (Above). The *A. thaliana* HDT-type histone deacetylase family. A multiple alignment of the known HDT-type histone deacetylases from *A. thaliana* is shown. The Cys2His2 Zn-finger motif present in HDT1 and HDT3 is indicated by the black bar.

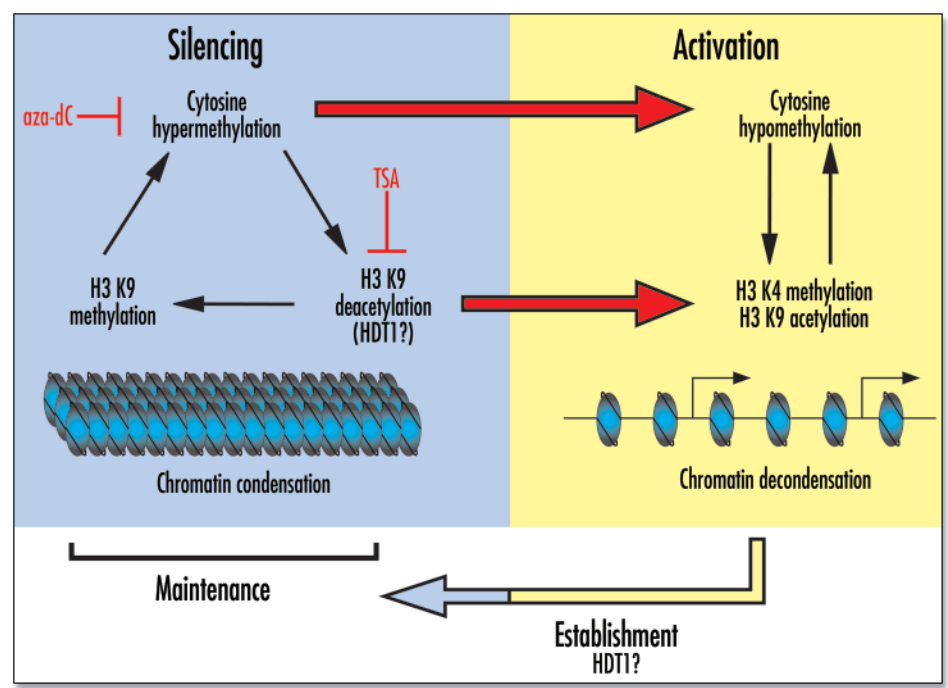


Figure 2 (Above). A model for rRNA gene transcriptional silencing and activation. In a self-reinforcing repression cycle (at left), H3 lysine 9 (H3K9) methylation can specify DNA methylation which then specifies histone deacetylation due to association of HDACs with methylcytosine binding domain proteins. Deacetylation of H3K9 is a prerequisite for H3K9 methylation, thus completing the cycle. Inhibiting DNA methylation with aza-dC, or inhibiting histone deacetylation with TSA or by targeted knockdown of HDAC activity switches the rRNA genes to an active state and leads to H3K4 methylation as well as DNA hypomethylation. Histone deacetylase HDT1 may be required during the maintenance or the establishment phases of transcriptional silencing, or both.

gene silencing. For instance, in *HDT3* and *HDT4*-RNAi lines, the mRNAs encoding the targeted HDACs were substantially reduced without affecting the silencing of *A. thaliana*-derived rRNA genes subjected to nucleolar dominance (Lawrence RJ, Pikaard CS,

unpublished). However, results for *HDT2* were less clear-cut. Several RNAi lines targeting *HDT2* caused the derepression of *A. thaliana*-derived rRNA genes in *A. suecica*. However, subsequent analysis of mRNA levels in these lines revealed that both *HDT1* and *HDT2* were being knocked down (Lawrence RJ, Pikaard CS, unpublished). Therefore, it is possible that the knockdown of *HDT1* is responsible for the loss of nucleolar dominance in these lines. Nonetheless, we cannot definitively rule out the possible involvement of *HDT2* in rRNA gene silencing.

A multiple alignment of HDT1-4 protein sequences is shown in Figure 1. HDT1 and HDT2 are most similar overall, though HDT2 lacks a predicted Cys<sub>2</sub>His<sub>2</sub> zinc-finger domain that is present at the carboxyl termini of both HDT1 and HDT3 (underlined in Fig. 1). It is not clear if these zinc fingers are used for DNA-binding, but an attractive hypothesis is that HDT1 exerts its repressive effects on *A. thaliana*-derived rRNA genes in *A. suecica* through direct DNA binding. Interestingly, though HDT-family HDACs are found only in plants, Aravind and Koonin noted that the N-terminal two-thirds of the HD2/HDT family of histone deacetylases shares sequence homology with the FKBP class of peptidyl-prolyl cis-trans isomerases (PPIases), a class of proteins that enzymatically assist the folding of other proteins.<sup>14</sup> The regions of similarity include the putative HDAC domain at the amino terminus as well as a central acidic domain. Though the FKBP C-terminal PPIase domain is missing in HDT family histone deacetylases, an *S. pombe* FKBP, SpFkbp39p, was recently shown to localize to the nucleolus and be required for silencing of a protein-coding reporter gene integrated among the rRNA genes.<sup>15</sup> Moreover, SpFkbp39p displays histone chaperone activity in chromatin assembly reactions and the histone chaperone domain is distinct from the PPIase domain. Furthermore, in *S. cerevisiae* an FKBP interacts with the histone deacetylase RPD3a, which has been implicated in mediating the transition from an open to closed chromatin conformation among rRNA genes as cells enter stationary phase.<sup>16</sup> Collectively, these intriguing observations inspire the speculation that multi-functional complexes combining histone deacetylase with histone chaperone activities may play fundamental roles in rRNA gene silencing in plants, yeast and possibly other eukaryotes.

Our findings studying chromosomal rRNA genes in plants agree nicely with several studies focused on human and mouse rRNA genes. For instance, a correlation between decreased promoter cytosine methylation and increased rRNA gene transcription in human liver carcinomas was recently noted by Jacob and colleagues.<sup>17</sup> Subsequent experiments by these authors using transfected rRNA gene constructs revealed that methylation at any of several cytosines in the promoter region can decrease promoter activity. Furthermore, methylcytosine binding protein MBD2, which localizes to the nucleolus as well as throughout the nucleus, was implicated in the transcriptional repression induced by methylation at these cytosine positions. In other studies, Grummt and colleagues showed that co-transfection of a rRNA gene construct together with a construct overexpressing a subunit of the nucleolar chromatin remodeling complex, NoRC would bring about the silencing of the rRNA gene reporter.<sup>8</sup> Silencing was shown to be accompanied by de novo cytosine methylation of the transfected rRNA gene promoter as well H3 lysine 9 methylation and Heterochromatin Protein 1 recruitment, indicating that NoRC plays a key role in rRNA gene silencing via establishment of repressive chromatin modifications.<sup>8</sup> Subsequent biochemical experiments showed that NoRC can physically interact with the DNMT1 and DNMT3b DNA methyltransferases and with

the Sin3-HDAC1/2 complex,<sup>18</sup> suggesting that the inter-connections between DNA methylation and histone deacetylation observed in both mammals and plants may be due primarily to NoRC and its related activities. Thus it will be interesting to determine if a NoRC-like chromatin remodeling activity plays a role in rRNA gene silencing in nucleolar dominance in plants.

The available evidence concerning rRNA gene silencing is consistent with a circular model in which H3 lysine 9 methylation can specify DNA methylation and likewise, DNA methylation can recruit histone deacetylases (most importantly H3 lysine 9 deacetylases) to facilitate H3 lysine 9 methylation, thereby comprising a self-reinforcing cycle (Fig. 2).<sup>19-23</sup> Our results indicate that the cycle can be disrupted by inhibiting either DNA methylation or histone deacetylation, either of which has the same consequences: transcriptional activation, loss of promoter cytosine methylation, loss of histone H3K9 methylation, gain of histone H3K4 methylation and gain of histone H3K9 acetylation (Fig.2).<sup>6</sup> We speculate that loss of one or more histone H3 lysine 9 methyltransferase or methylated DNA binding proteins should also disrupt the repression cycle and experiments are underway to try to identify these activities. Exactly where HDT1 fits into this model remains to be determined. Likewise, determining whether HDT1 function in the maintenance or establishment phases of silencing, or both, is an important question to guide future research.

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