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# Aberrant DNA Methylation of Ribosomal RNA Genes in Human Cancer

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## Abstract

Aberrant regulation of rRNA genes has been reported in human cancers. The aim of this study was to analyze the epigenetic regulation of rRNA genes in lung cancer and to correlate methylation levels of the promoter, 18S and 28S regions with different chromatin states. Here we report, that methylation of rDNA ranged from 10% to 30% at individual CpG sites in the promoter region. In primary lung cancers a 1.2-fold increased rDNA methylation was observed at 19 analyzed CpGs ($p < 0.001$). Moreover, we report an increased methylation level of rDNA towards the 28S region of rRNA genes. This hypermethylation was more pronounced in cancer cell lines compared to primary tissue. To analyze the methylation status of protein-enriched rDNA, we utilized a technique that combines ChIP and bisulfite sequencing. Pol I- and CTCF-associated rDNA exhibited reduced methylation levels compared to global rDNA. Histone H1-associated promoter regions showed a 1.5-fold increase in methylation levels compared to H3-associated rDNA. In DNMT1 knock out cells a strong reduction of methylation of 18S and 28S regions was found in comparison to wild type HCT116 cells (2.7- and 1.4-fold reduction, respectively). In double DNMT1 and DNMT3B knock out cells no substantial rDNA methylation was detected. Independent of this hypomethylation, 28S level and number of rDNA repeats were constant in wild type, single knock out and double knockout HCT116 cells. Our data suggest that aberrant methylation of rDNA occurs in human cancer and that rRNA gene activity can be modulated in a constant manner independent of the level of methylated rDNA.

**Keywords:** Ribosomal DNA; Cancer; rRNA gene; Epigenetics; DNA methylation; DNA methyltransferase

**Abbreviations:** rDNA/RNA: ribosomal DNA/RNA; DNMT: DNA Methyl Transferase; ChIP: Chromatin Immunoprecipitation; Pro: Promoter Region; CTS: CTCF Target Site; ETS: External Transcribed Spacer; wt: Wild Type.

## Introduction

In mammalians, three classes of RNA polymerases (Pol I, II and III) transcribe distinct sets of genes. About 70% of Pol II promoters are localized at GC- and CpG-rich sequences termed CpG islands. Active CpG island promoters are in an open chromatin configuration, which is devoid of cytosine methylation. DNA hypermethylation of CpG island promoters is associated with gene silencing and this epigenetic inactivation contributes to the pathogenesis of cancer [1,2]. Aberrant methylation of rDNA has been reported in cancer, but also in neuropsychiatric disorders [3-5]. Pol I transcribes the ribosomal RNA (rRNA) genes that are organized in multiple tandem repeats of about 400 copies in satellite regions of acrocentric chromosomes of the diploid human genome [6]. These repeats are organized in intergenic spacer and the rRNA gene that is transcribed as the 45S precursor (Figure 1). The rRNA gene contains the 5' external transcribed spacer (5'ETS), 18S rRNA, internal transcribed spacer 1 (ITS1), 5.8S rRNA, ITS2, 28S rRNA and the 3'ETS [7]. In eukaryotes only a subset of the ribosomal DNA (rDNA) is actively transcribed into rRNA and the other fraction of repeats is epigenetically silenced in heterochromatin [8-10]. We and others have shown that the inactive copies are packed into nucleosomes and contain methylated promoter sequences [11-13], whereas the transcribed rRNA genes are free from nucleosomes and the rRNA promoter is unmethylated [13-15]. It has been shown that loss of DNA methylation of rRNA genes enhances cryptic Pol II transcription of rDNA and therefore disrupts ribosomal RNA processing [16]. This indicates that DNA methylation positively influences rRNA synthesis and processing [16]. An important factor for the regulation of rRNA transcription is the rRNA transcription UBF [17]. Recently, it has been reported, that UBF interacts with the insulator binding protein CTCF and regulates the local epigenetic state of rDNA [18]. CTCF binds to a site upstream of the rDNA spacer promoter and prefers non-methylated over methylated rDNA [15,18]. It has been shown, that this spacer promoter stimulates transcription from the rRNA core promoter [19,20]. In cancer, aberrant methylation of rDNA has also been reported [3,21]. Hypermethylation of rRNA genes has been observed in breast, colorectal and endometrial cancers [3,21-23]. In prostate cancer overexpression of rRNA has been reported, but this was not linked to significant methylation changes of the rRNA promoter [24]. However, for hepatocellular carcinoma hypomethylation of the rDNA promoter has been revealed [25].

Here we analyzed the methylation levels and chromatin states of the distal promoter, 18S and 28S regions of rDNA in lung cancer cell lines and we report increased methylation towards the 3' end of rRNA genes. Pol I- and CTCF-enriched rDNA regions exhibit a reduced methylation when compared to the global rDNA. Histone H1-associated promoter regions showed an increase in methylation compared to global and H3-associated rDNA. In DNMT1 knock out cells strong reduction of methylation of 18S and 28S regions was found in comparison to wild type cells. In double DNMT1 and DNMT3B knock out cells no substantial methylation of rDNA was detected. Independent of the aberrant methylation, 28S levels and the number of rRNA genes was similar in wild type, single knock out and double knockout HCT116 cells. A significant aberrant methylation was observed in primary lung tumors relative to control samples.

## Materials and Methods

### Human cell lines and primary samples

HeLa or A549 cells were cultured in DMEM. Lung cancer samples were previously described [26]. HCT116 cell lines: wild type (wt),

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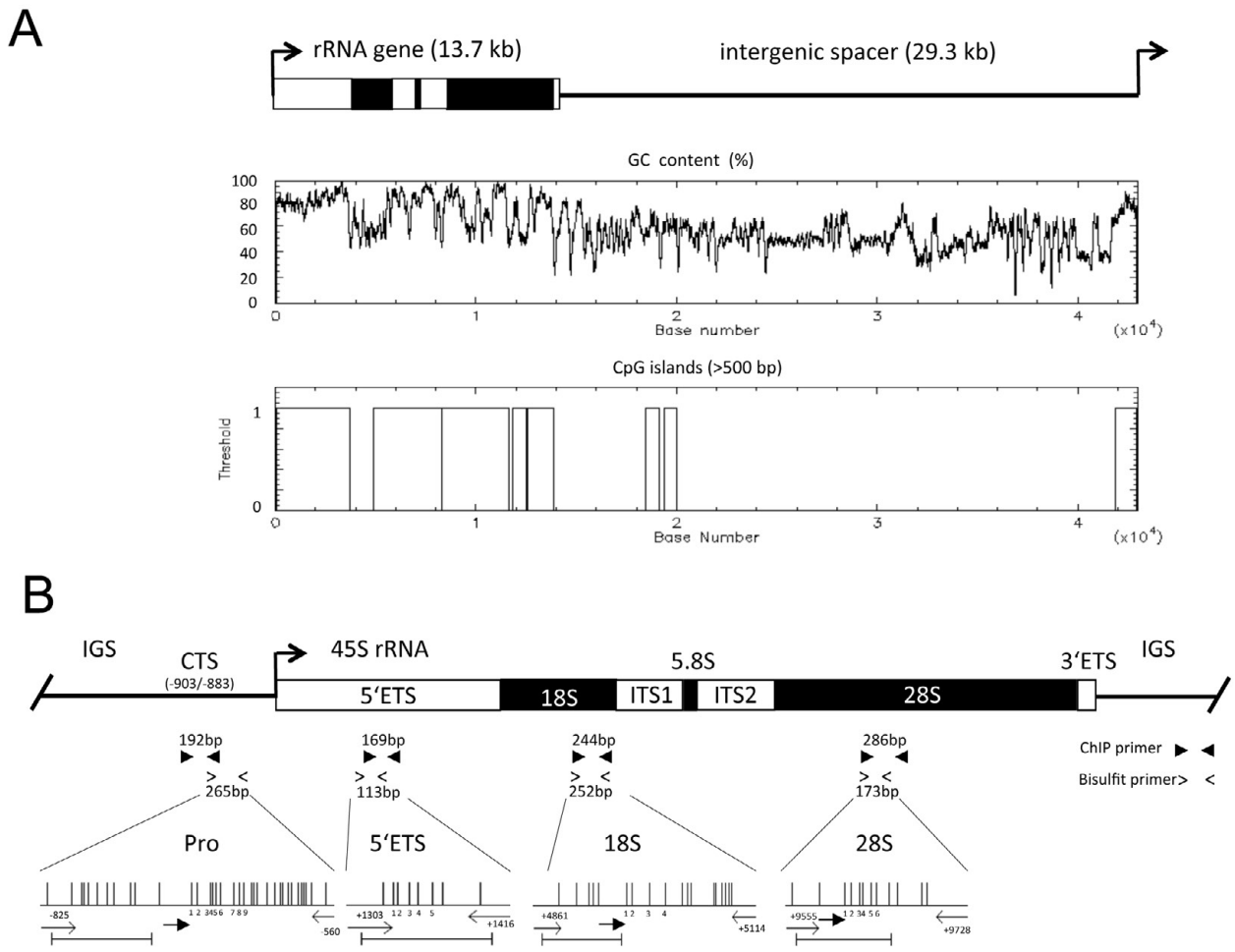


**Figure 1: Map of a human 43 kb rDNA repeat. A.** The transcription start sites of the rRNA gene repeat are marked with arrows. GC content and CpG islands (≥ 500 bp) of the rRNA gene and intergenic spacer (IGS) were determined by EMBOSS Cpgplot (www.ebi.ac.uk/emboss/cpgplot/). **B.** The rRNA gene is organized in 5' external transcribed spacer (5'ETS), 18S rRNA, internal transcribed spacer 1 (ITS1), 5.8S rRNA, ITS2, 28S rRNA and 3'ETS. The CTCF target site (CTS; GCGGCCGCCAGATGGAGCCC) is indicated relative to the transcription start site [15]. PCR products (e.g. Pro/promoter region) for ChIP and bisulfite sequencing are depicted. Individual CpGs are indicated by vertical lines and the analyzed CpGs for each region are numbered. Primers are marked with arrows. Scale bars indicate 100 bp.

DNMT1-/- (Clone 1C1), DNMT3B-/- (3bKO) and DNMT1-/-, DNMT3B-/- (DKO21) are a generous gift from Dr. Bert Vogelstein (Ludwig Center at Johns Hopkins University, Howard Hughes Medical Institute) and were cultured in McCoy's 5A [27]. All patients signed an informed consent at initial clinical investigation. This study was approved by local ethic committee (Ethik-Kommission am Fachbereich Medizin ML University Halle).

## Chromatin immunoprecipitation

ChIP was done as described [28]. Cells were fixed using formaldehyde with a final concentration of 1% for 10 min at room temperature. Incubation of 1/7 volume of 1 M glycine for 5 min stopped the fixation process. Cells were washed two times with PBS and harvested in PBS. After centrifugation for 5 min at 2000 rpm at 4°C the supernatant was removed and cells were lysed using 1 ml SDS-lysis-buffer (1% SDS, 10 mM EDTA, 50 mM Tris/HCl pH 8.1) per $10^7$ cells for 10 min on ice. Chromatin was sheared by sonication to an average size ranging from 200–500 bp. After sonication the samples were centrifuged for 10 min at 4°C with maximal speed. The supernatant (chromatin) was diluted 1:10 with dilution-buffer (0.01% SDS, 1.1% Triton X, 1.2 mM EDTA, 16.7 mM Tris/HCl pH8.1, 167 mM NaCl) and 1.5 ml of the dilution was used for each chromatin immunoprecipitation. 10% of the chromatin used for one ChIP was preserved as an input sample and stored at -20°C. 1.5 ml of the dilution was pre-cleared with 5 µg IgGs and 30 µl ProteinG Plus/ProteinA Agarose (Calbiochem) by rotation at 4°C for 2 h. After centrifugation at 4°C for 1 min at 2000 rpm the supernatant was incubated with the corresponding antibody and rotated over night at 4°C. Binding of the immune-complexes occurs afterwards by incubation of the chromatin with 30 µl of ProteinG Plus/ProteinA Agarose (Calbiochem) for 2 h at 4°C. After the incubation beads were washed for 5 min rotating at 4°C one time with low-salt-buffer (0.1% SDS, 1% Triton x100, 2 mM EDTA, 20 mM Tris/HCl pH 8.1, 150 mM NaCl), one time with high-salt buffer (0.1% SDS, 1%Triton X, 2 mM EDTA, 20 mM Tris/HCl pH 8.1, 500 mM NaCl), one time with LiCl-buffer (0.25 M LiCl, 1% NP40, 1% Deoxycholat, 1 mM EDTA, 10 mM Tris/HCl pH 8.1) and two times with TE-buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Chromatin bound to the beads and input material were resuspended in 100 µl TE, 1 µl of 10mg/ml RNAse A was added and incubated for 30 min at 37°C to digest the remaining RNA. Next 7.5 µl of 10% SDS, 4 µl of 20 mg/ml Proteinase K were added

and incubated for further 2-4 h at 37°C. The reverse crosslink was performed by incubation at 65°C overnight. DNA was recovered using the Nucleo Spin Gel and PCR clean up Kit (Machery-Nagel Düren, Germany) according to the manufacture's protocol and eluted in 40 µl $H_2O$. For PCR control analysis 2 µl of precipitated DNA was amplified in a reaction buffer containing 0.2 mM dNTP mix, 1.5 mM $MgCl_2$ and 1.5 U Taq polymerase for 27 cycles for CTS and ETS and 30 cycles for 18S and 28S, respectively with 10 pmol of forward and reverse primers (Supplementary Table 1).

## Antibodies

For ChIP H1 (Santa Cruz, sc8030), Pol I (Santa Cruz, sc28714), CTCF (Millipore, 07-729), H3 (Abcam, ab1791) and normal rabbit control IgG (Abcam, ab46540) antibodies were used.

## Methylation analysis

DNA methylation of four rDNA regions (promoter, ETS, 18S and 28S region) was determined by bisulfite pyrosequencing [29,30]. Bisulfite treatment of genomic DNA was done as described previously [30]. Bisulfite treatment after ChIP was carried out with EpiTect Kit (Qiagen, Hilden, Germany) according to the manufacture's protocol. For pyrosequencing 5 to 100 ng of bisulfite treated DNA was amplified in a reaction buffer containing 0.2 mM dNTP mix, 1.5 mM $MgCl_2$ and 1.5 U Taq polymerase for 40 cycles with 10 pmol of forward and biotinylated reverse primers and sequenced with an internal primer (Supplementary table 1). Pyrosequencing was performed in PyroMark Q24 according to the PyroMark Gold Q24 Reagents Handbook (Qiagen, Hilden, Germany). Pyrosequencing was done for two to three independent bisulfite reactions and the average methylation frequency for each CpG site and rDNA region was calculated.

## Expression analysis

Cell lines were treated with 5-Aza-2'-deoxycytidine for 4 days and RNA was isolated using Isol-RNA lysis procedure [31]. RNA from cell lines

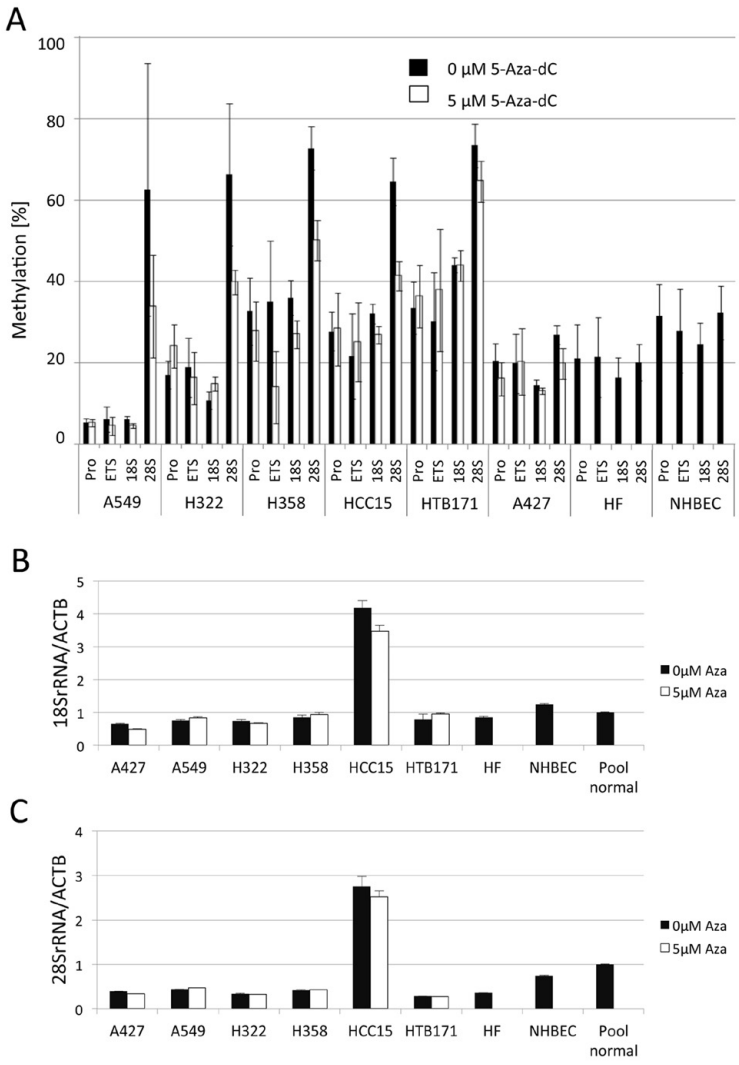


**Figure 2: Methylation and expression analysis of rRNA genes in lung cancer cell lines. A.** Mean methylation levels of nine CpGs located in the promoter region (Pro), five CpGs in the 5' external transcribed spacer (ETS), four CpGs in the 18S and six CpGs in the 28S region were obtained by bisulfite pyrosequencing in normal bronchial epithelial cells (NHBEC), human fibroblasts (HF) and six lung cancer cell lines (A549, H322, H358, HCC15, HTB171 and A427) after 0 and 5 µM 5-Aza-2'-deoxycytidine (5-Aza-dC) treatment for four days. **B**. RNA from cell lines as well as normal human controls (pool of breast, lung, kidney and liver RNA) were DNAse treated and 1 µg reversely transcribed. For Realtime PCR 8 ng of cDNA was used for 18S rRNA or ACTB and analyzed in triplicate. 18S rRNA level was normalized to ACTB and mean expression with according SD is shown. **C.** 28S rRNA level was analyzed by quantitative RT-PCR and normalized to ACTB levels.

as well as normal human control RNA (breast, lung, kidney and liver) were DNAse (Fermentas) treated and 1µg of RNA was reversely transcribed using poly-dT primers and random hexamers in 25 µl of RT-mix with MMLV reverse transcriptase (Promega, Heidelberg, Germany) for 1 h at 42°C. Resulting cDNA was diluted with 100 µl $H_2O$. For realtime PCR (SybrSelect Applied Biosystems; Rotor-Gene 3000; Corbett Research) 1µl of samples was used for ACTB, 28S and 18S rRNA analysis and performed in triplicate. All primers are listed in Supplementary Table 1.

### Statistical evaluation

Statistical analysis was performed with the two-tailed, paired t-test using Excel (Microsoft, Redmond, USA). All reported p-values are considered significant for $p \leq 0.05$.

## Results

### Epigenetic regulation of rDNA in lung cancer

Previously, it has been reported that an aberrant methylation of rDNA exists in different cancer entities (e.g., hypermethylation in breast tumors [3] and a hypomethylation was reported in hepatocellular carcinoma [25]). Here we aimed to reveal the methylation status of different rDNA regions in lung cancer cells by bisulfite pyrosequencing.

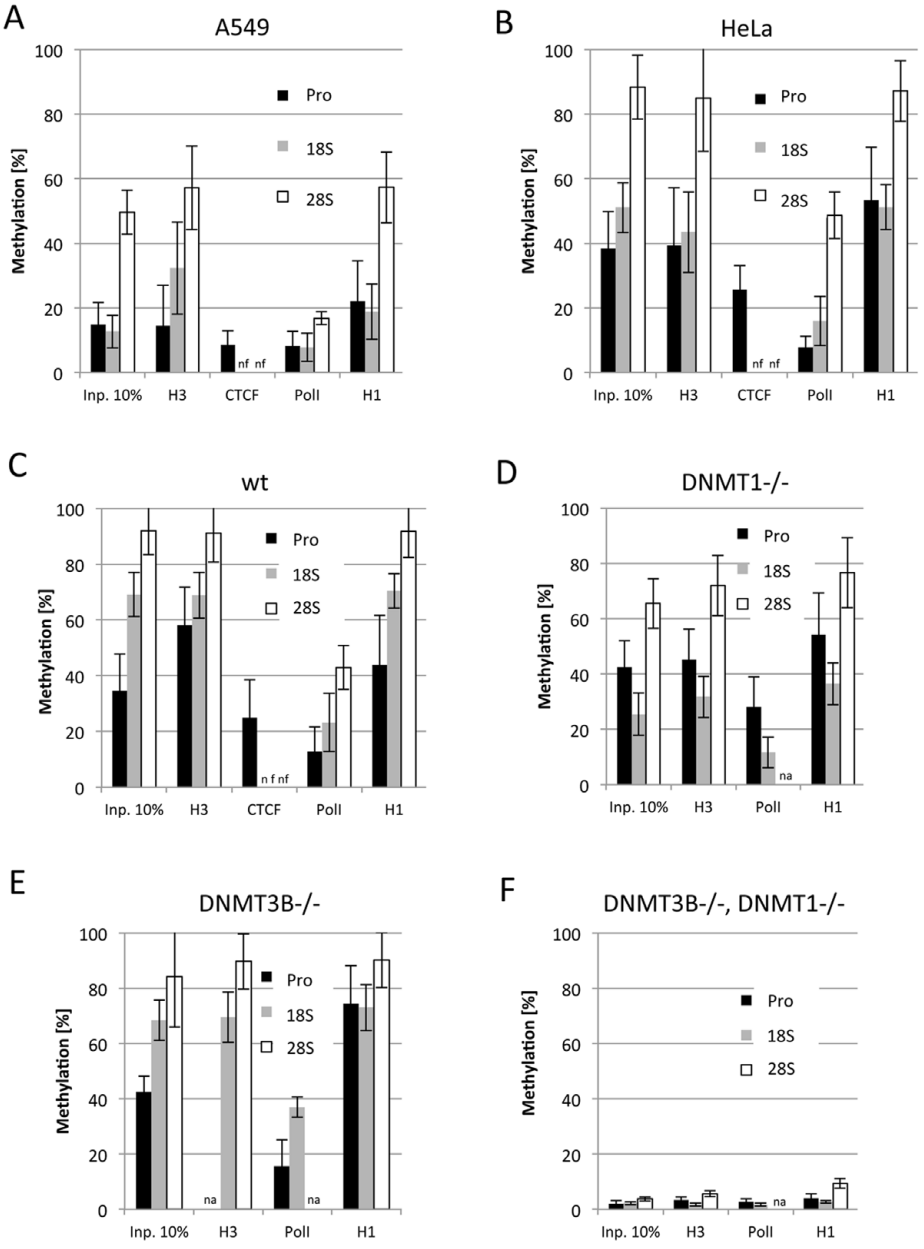


**Figure 3: Chromatin-associated methylation level of cancer cell lines.** Chromatin of A549 (**A**), HeLa (**B**), wildtype/wt HCT116 (**C**), DNMT1-/- HCT116 (**D**), DNMT3B-/- HCT116 (**E**) and DNMT1-/-, DNMT3B-/- HCT116 (**F**) cell lines was precipitated with histone H3-, H1-, CTCF- and Pol I-specific antibodies. After ChIP with the indicated antibodies, DNA was purified and bisulfite treated. The methylation at the promoter region (Pro: nine CpGs), 18S (four CpGs) and 28S (six CpGs) regions were analyzed by quantitative pyrosequencing and compared to the 10% input control. Methylation analysis of CTCF-specific chromatin at 18S and 28S regions was not feasible (nf) or for other samples not analyzed (na).

The human rDNA contains hundreds of repeats of CpG-rich rRNA genes (Figure 1A) and we analyzed the methylation of nine CpGs located in the promoter region (Pro) between the CTCF-target site (CTS) and the transcription start site, four CpGs in the 5' external transcribed spacer (5'ETS), four CpGs in the 18S region and six CpGs in the 28S region (Figure 1B and Figure 2). In normal bronchial epithelial cells (NHBECs) and human fibroblasts (HF) we found similar levels of methylation at these regions (29% and 20%, respectively; Figure 2). When we compared these methylation levels to six lung cancer cell lines (A549, H322, H358, HCC15, HTB171 and A427) we observed that methylation of Pro, ETS and 18S was again at similar levels, however methylation at the 28S region was drastically increased (≥ 2.2-fold compared to Pro) in five out of six cell lines (in A427 only 1.3-fold; Figure 2A). Additionally, in A549 cells a pronounced hypomethylation of Pro (5%), ETS (6%) and 18S (6%) was observed when compared to the normal cell lines. However, this hypomethylation was not found in the other five lung cancer cell lines (Figure 2A).

To analyze if aberrant methylation of rDNA correlates with significant changes in rRNA levels, we treated these cancer cells with 5 μM of 5-Aza-2'-deoxycytidine (5-Aza-dC) for four days and analyzed methylation and rRNA levels (Figure 2). The 28S region was the only region that was consistently demethylated (1.1- to 1.8-fold reduction) in all lung cancer cell lines (Figure 2A). However, this demethylation was not observed in the promoter region (average 1.01-fold reduction; Figure 2A). 5-Aza-dC inhibits the maintenance of DNA methylation during replication and therefore the 5-Aza-dC-induced demethylation depends on the DNA synthesis rate and cell cycle progression. Since some of the cells proliferate slower upon 5-Aza-dC treatment, DNA demethylation of the 28S region is less pronounced in these cells (e.g., HTB171 and A427, Figure 2A). Due to the high toxic effect of 5-Aza-dC, treatment of NHBEC and human fibroblasts (HF) with this DNMT inhibitor was not feasible.

| Cell line | rDNA region | Global methylation[a] (in %) | Pol I-associated methylation (in %) | Reduction in methylation (Global/Pol I) |
|---|---|---|---|---|
| A549 | Pro | 15 ± 7 | 8 ± 5 | 1.9 |
| | 18S | 13 ± 5 | 8 ± 4 | 1.6 |
| | 28S | 50 ± 7 | 17 ± 2 | 2.9 |
| HeLa | Pro | 38 ± 11 | 8 ± 3 | 4.8 |
| | 18S | 51 ± 8 | 16 ± 8 | 3.2 |
| | 28S | 88 ± 10 | 49 ± 7 | 1.8 |
| wt HCT116 | Pro | 35 ± 13 | 13 ± 9 | 2.7 |
| | 18S | 69 ± 8 | 23 ± 10 | 3.0 |
| | 28S | 92 ± 9 | 43 ± 8 | 2.1 |
| DNMT1-/- | Pro | 42 ± 10 | 28 ± 11 | 1.5 |
| | 18S | 26 ± 8 | 12 ± 6 | 2.2 |
| | 28S | 66 ± 9 | na[b] | - |
| DNMT3b-/- | Pro | 42 ± 6 | 16 ± 10 | 2.6 |
| | 18S | 69 ± 7 | 37 ± 4 | 1.9 |
| | 28S | 84 ± 18 | na[b] | - |

[a]methylation level of input rDNA
[b]not analyzed

**Table 1:** Summary of methylation levels of global and Pol I-specific rDNA.

We also analyzed rRNA levels after 5-Aza-dC treatment and observed only weak changes in the 18S and 28S levels (Figure 2B and C, respectively). In HCC15, that exhibit high rRNA levels, a 1.2- and 1.1-fold reduction was found for the 18S and 28S levels. However, there was no consistent trend between demethylation and induced or reduced rRNA levels. As a control we have analyzed the expression of the lung tumor suppressor RASSF1A, which is epigenetically inactivated in A427, A549 and H322 cells [1,26]. As previously reported reexpression of RASSF1A was observed after 5 μM 5-Aza-dC treatment (Supplementary Figure S1).

## Different chromatin-associated factors correlate with distinct methylation levels of rDNA

To further dissect the impact of chromatin state on the regulation of rRNA genes in cancer we performed ChIP of rDNA with Pol I-, CTCF-, histone H3- and H1-specific antibodies in cell lines, that exhibit low or high rDNA methylation (Figure 3 and Supplementary Figure S2). Subsequently, the methylation level of protein-enriched regions was investigated by bisulfite pyrosequencing (Figure 3). The promoter region (Pro), that contains the CTCF-target site, was precipitated with CTCF-specific antibodies (Figure 3 and Figure S2). CTCF binding at the ETS, 18S and 28S regions was absent (Figure 3 and Figure S2). In contrast with Pol I-, H3- and H1-specific antibodies all four regions (CTS, ETS, 18S and 28S) were precipitated. Next we compared the

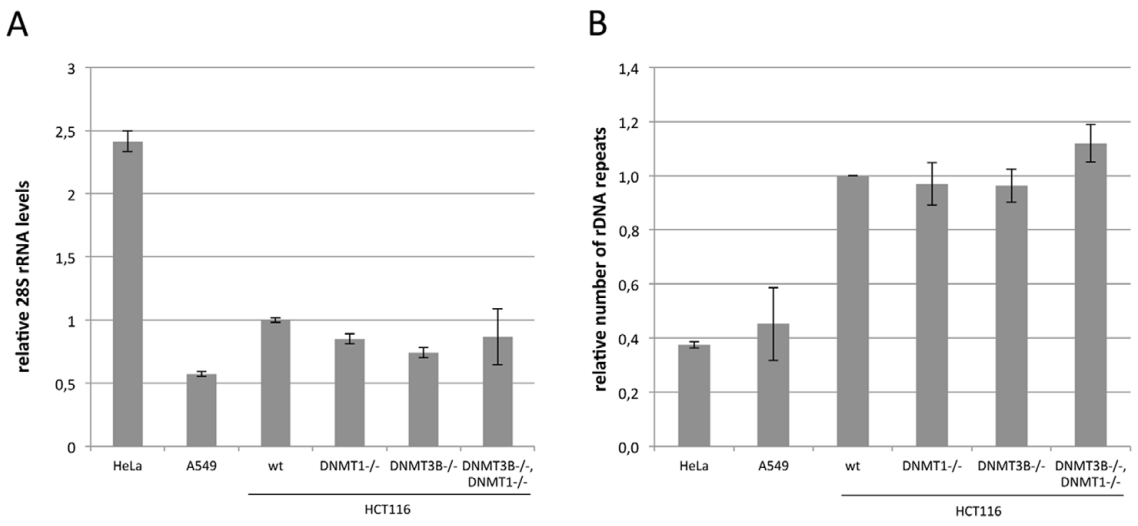


**Figure 4: 28S level and number of rRNA genes**. **A.** RNA was isolated from the indicated cancer cells. 28S rRNA level was analyzed by quantitative RT-PCR and normalized to GAPDH. Levels of 28S were plotted relative to wildtype HCT116 (wt) cells (=1). **B.** Genomic DNA was isolated and numbers of rDNA repeats were quantified by real time PCR. rDNA repeats were normalized to GAPDH DNA signal and plotted relative to wt cells (=1).

methylation of protein-associated rDNA with the methylation of global (input) rDNA (Figure 3). Once again we observed an increased methylation (>2-fold) in A549 and HeLa cells at the 28S region when compared to Pro. Pol I- and CTCF-associated rDNA exhibited a reduced methylation in comparison to global levels (Figure 3A and B and Table 1). Thus in HeLa, the Pol I-associated Pro showed only 8% methylation as compared to 38% for all promoter regions (4.8-fold reduction, Table 1). This drastic decrease in methylation levels for Pol I-associated rDNA was also observed for 18S and 28S regions (3.2- and 1.8-fold, respectively). Interestingly, the Pol I-associated 28S region exhibited still 50% of methylation in HeLa cells. In A549 cells, we also observed this strong reduction (1.6- to 2.9-fold) in methylation of Pol I-associated rDNA (Figure 3A and Table 1).

CTCF-associated Pro showed a 1.7- and 1.5-fold reduction of methylation compared to global rDNA in A549 and HeLa cells, respectively (Figure 3A and B). Since no proximal CTCF target sites (CTS) exist in the 18S and 28S region [15], analysis of methylation after ChIP is not feasible (Figure 1 and 3). In principal histone H3-associated methylation of rDNA shows a similar methylation as compared to global (input) levels (Figure 3A and B). H1-associated promoter regions exhibited a slight increase in methylation levels when compared to global levels (Figure 3A and B).

## The impact of DNA methyl transferases on the epigenetic regulation of rRNA genes

To analyze the influence of different DNA methyl transferases (DNMT) on the methylation and expression of rRNA genes, we utilized the HCT116 colon cancer cell line and DNMT-deficient derivatives [27]. From the wild type (wt) HCT116 cell line, clones had been generated that harbor single homozygote deletion of DNMT1 (DNMT1-/-) or DNMT3B (DNMT3B-/-) or a double knockout (DNMT1-/-, DNMT3B-/-) [27]. For these four cell lines we analyzed the methylation level of chromatin-associated regions (Pro, 18S and 28S) (Figure 3 and Table 1). For wt-HCT116 cells we observed an increased global methylation from the distal promoter (Pro) region (35%) towards the 18S (69%; 2-fold) region and 28S (92%; 2.6-fold) region (Figure 3C and Table 1). It is interesting to note that methylation of individual rDNA molecules is homogenous (Supplementary Figure 3). In DNMT1-/- cells, the methylation of the 18S region (26%) and the 28S region (66%) was strongly reduced as compared to wt (2.7- and 1.4-fold, respectively) (Figure 3D). Interestingly methylation of the promoter region (42%) was not affected. For DNMT1-/- cells the 18S region showed a reduced methylation (1.6-fold) when compared to the promoter region The methylation levels of the DNMT3B-/- cells were similar to wt cells for all analyzed rDNA regions (Figure 3E). For the double knock out cells no substantial methylation of rDNA was detected (Figure 3F).

We also analyzed the methylation levels of Pol I-associated rDNA in these cells (Figure 3 and Table 1). As already observed for A549 and HeLa cells, Pol I-associated rDNA exhibited a drastically reduced methylation in the wt HCT116 as well as in the DNMT1-/- and DNMT3B-/- cells (1.5- to 3.0-fold; Table 1). Once again an increase in methylation towards the 3' end of Pol I-associated rDNA was revealed in wt and DNMT3-/- cells. An increased methylation was also found for histone H1-associated promoter regions in wt (1.3-fold), DNMT1-

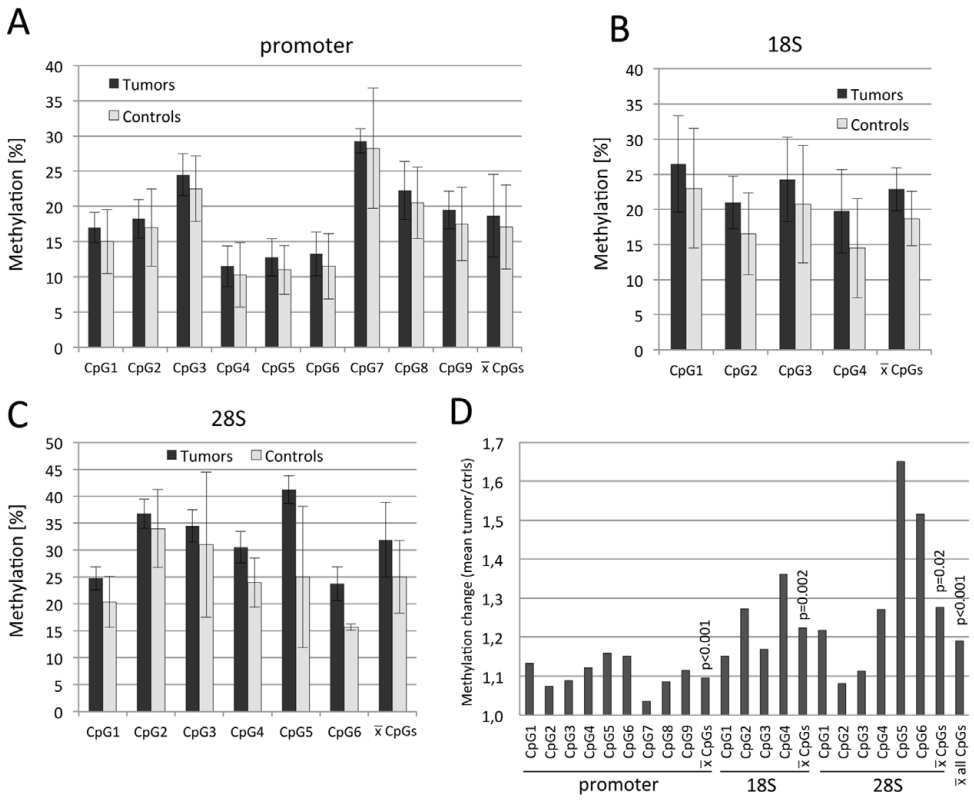


**Figure 5: Methylation analysis of rRNA genes in primary lung tissues.** Methylation levels of single CpG in the promoter region (**A**), 18S (**B**) and 28S (**C**) regions were analyzed in four human lung tumors and matching controls by bisulfite pyrosequencing and plotted. CpGs indicate the mean methylation of the analyzed region. **D.** The quotient between mean methylation levels at individual CpG sites and combined sites for promoter, 18S and 28S in tumors and controls (ctrls) were calculated and plotted. Statistical analysis was performed with the two-tailed, paired t-test comparing the mean methylation of matching CpG sites in a specified region.

/- (1.3-fold) and DNMT3B-/- (1.8-fold) cells compared to global methylation levels of the promoter region (Figure 3).

Since no substantial methylation was observed for the double knock out cells, we were interested to analyze if this correlates with an aberrant rRNA level or altered number of rRNA genes (Figure 4). When we compared rRNA levels of double knock out (DNMT1-/-, DNMT3B-/-) to wt-HCT116 cells we did not observe significant changes in the 28S level (Figure 4A). Similar levels of 28S were also found for single knock out DNMT1-/- or DNMT3B-/- cells. However, in HeLa an increase of 28S (2.4-fold) and in A549 a 50% decrease relative to wt-HCT116 was revealed (Figure 4A). Since methylation levels were higher in HeLa in comparison to A549 we were interested to reveal the relative number of rDNA repeats in these cell lines (Figure 4B). Interestingly, we observed cells comparable number of repeats in HeLa and A549. Furthermore similar numbers of rDNA repeats were found in the HCT116 cell lines. However, for A549 and HeLa cells we observed 60% less rDNA repeats in comparison to HCT116 cells (Figure 4B).

### Hypermethylation of rDNA in primary lung cancer

Subsequently, we analyzed the methylation of nine CpGs located in the promoter, 18S and 28S regions in primary lung cancer by bisulfite pyrosequencing (Figure 5). Due to inter- and intra-individual variations in rDNA methylation a large variability was observed (Figure 5). Interestingly, methylation of the promoter region was significantly increased (1.1-fold; $p<0.001$) in lung tumors (19%) compared to matching controls (17%) (Figure 5A and D). This increased methylation (1.04- to 1.16-fold) was found at all nine analyzed CpGs (Figure 5A and D). Significant hypermethylation was also present at the 18S (1.2-fold; $p = 0.002$) and 28S (1.3-fold; $p = 0.02$) region in lung tumors compared to controls and was again present at all analyzed CpGs (Figure 5B, 5C and 5D). Taken together, this data indicates that a significant 1.2-fold increased methylation of the analyzed rDNA regions occurred in primary lung tumors relative to controls ($p < 0.001$; Figure 5D). Moreover, an increased methylation was detected at the 28S region in tumors (32%; 1.7-fold) and in controls (25%; 1.5-fold) compared to the promoter region in tumors and controls (19% and 17% respectively, Figure 5).

## Discussion

Previous studies have indicated that an aberrant methylation of rDNA exists in different cancer entities including breast, cervix and endometrial cancer [3,4,22,23]. Here, we report significantly increased methylation of primary lung tumor samples compared to matching normal tissues at all analyzed CpG sites (Figure 5). It will be important to validate these results in larger data sets. In lung cancer cell lines (A427, H322, H358, HCC15, HTB171) a similar methylation of the promoter, 5'ETS and 18S regions was observed (Figure 2). Methylation of the analyzed promoter region is similar to the methylation levels of the core promoter in HCT116 cells [16,24,32]. Interestingly, methylation increased drastically at the 28S region in most cancer cell lines (A549, H322, H358, HCC15, HTB171, HeLa and HCT116) (Figure 2 and Figure 3). This indicates a hypermethylation of rRNA genes towards its 3' end in cancer cell lines, an observation that has not been reported previously. This increase was also observed in primary tissues; however it was less pronounced (Figure 5). It will also be interesting to analyze the methylation level at the 5.8S region, which is located between the 18S and 28S regions (Figure 1A). A427 cells, that display an epithelial morphology, exhibit a similar methylation as normal cells (Figure 2A). At the promoter region rather low methylation levels were found for A549 cells and a high methylation was observed for HeLa and HCT116 cells (Figure 3). This difference could be either due to inter-individual variability or due to long-term cell culture conditions. It has been reported that cell culturing can cause gradual changes in DNA methylation pattern [33-35].

Yan et al. have utilized methylation sensitive southern blot assays with a probe that covers the whole repeat and therefore were not able to dissect methylation levels of different rDNA regions in detail [3]. Bacalini et al. have analyzed the promoter, 18S and 28S regions by the 'MassARRAY EpiTYPER' assay in breast cancer and reported hypermethylation of rDNA [22]. However no increased methylation towards the 28S region was detected. Moreover, Bacalini et al. reported a considerable inter-individual variation in methylation levels [22], which was also seen in lung tumors and control samples and therefore a considerable standard deviation was observed (Figure 2 and Figure 5). Mean intra-individual promoter methylation of tissues ranges from 10% to 30% at the nine analyzed CpG sites (Figure 5). Since unmethylated rRNA promoter regions are associated with Pol I (Figure 3), our and other data suggest that an average of 80% of the human rDNA repeats are in an active epigenetic state [22]. Interestingly, in A549 cells we observed an increase in unmethylated promoter sequences (2.5-fold) and 18S regions (3.9-fold) when compared to HeLa cells. However, rRNA levels are five times higher in HeLa cells (Figure 3 and 4A) and relative numbers of rDNA repeats are similar (Figure 4B). This indicates that the activity of rRNA genes is further modulated by other mechanisms, including Pol I load. Gagnon-Kugler *et al.* have reported that loss of methylation induces cryptic Pol II transcription of rRNA genes and reduces level of processed rRNA [16]. This could indicate that an increased methylation level of rDNA may result in an induction of matured rRNA. Overexpression of rRNA has been reported in prostate cancer, but was not linked to aberrant promoter methylation [24]. Hypermethylation of Pol II transcribed tumor suppressor genes (e.g. RASSF1A) is frequently observed in prostate cancer [36,37].

In lung cancer an increased expression of DNMT1 and DNMT3B expression has been reported [38], and this aberrant expression was linked to increased promoter methylation [39,40]. Thus we were interested in analyzing the impact of DNMT1 and DNMT3B on rRNA regulation in more details. As already previously reported [24,41], no substantial methylation of rDNA was found in the DNMT1 and DNMT3B double knock out HCT116 cells (Figure 3). Independent of this lack of methylation the number of rDNA repeats and the level of 28S rRNA were constant (Figure 4) [41]. After the inhibition of DNMTs by 5-Aza-2'-deoxycytidine we also observed, that levels of 18S and 28S RNA were not affected in most cell lines (Figure 2). This is also seen when we analyzed the single DNMT1 or DNMT3B knock out cells (Figure 4). However, Majumder et al. have reported an increase in rRNA level in DNMT1-/- or DNMT3B-/- cells, which was not observed by us or by several others [16,32,41]. It has been shown that in DNMT1-/- single knock out cells rRNA gene promoter methylation is reduced [24,32]. Here we also observed that in DNMT1-/- cells methylation levels at the 18S and 28S region are reduced when compared to wt HCT116 (2.7- and 1.4-fold, respectively; Figure 3). It has been reported that hypomethylation of rRNA genes enhances cryptic Pol II transcription that counteracts rRNA synthesis [16]. All these data indicate that in dependence of the number of unmethylated rDNA, rRNA levels can be modulated in a constant manner by transcriptional and epigenetic mechanisms.

We have utilized a technique that combines chromatin immuno-precipitation and bisulfite sequencing and has been termed *BisChIP-*

*seq* [42]. With this method we have analyzed the methylation level of protein-enriched rDNA (Figure 3). We observed that H3-associated rDNA methylation resembles global methylation levels, whereas H1-associated promoter regions are rather methylated (Figure 3). It has been reported that histone H1 co-localizes with methylated rDNA clusters [43]. Previously, it has been found that the insulator binding factor CTCF is associated with the promoter region of human and mouse rDNA [15,18]. It has been reported that CTCF augments the pre-rRNA level and knockdown of CTCF reduces pre-rRNA transcription in HeLa cells [44]. Huang *et al.* also reported that CTCF positively regulates rRNA transcription in a Pol I-dependent manner. Our findings show that CTCF-enriched promoter sequences exhibit a reduced methylation when compared to global levels (Figure 3) and therefore co-localize with Pol I-associated rDNA in human cancer cell lines. Others have reported that CTCF interacts with UBF and unmethylated rDNA and thereby activates spacer promoter transcription by Pol I [18]. In cancer, alteration of CTCF by mutations or by aberrant PARylation has been observed and this was linked to aberrant methylation of CTCF target sites [45-47]. We have recently reported that CTCF induces hypomethylation of its binding site at a tumor suppressor gene promoter [48].

In summary, we observed a significant aberrant hypermethylation of rDNA in lung cancer samples in comparison to controls. Moreover, we observed that general methylation levels of rDNA are higher at the 28S region compared to the 5' region. Hypomethylation of rDNA occurs predominantly at the 3' end of rRNA genes after depletion of DNMTs by an inhibitor or by a genetic disruption. Our data indicates that rRNA levels can be kept constant, independent of the number of methylated rDNA repeats.

## Authors' Contributions

RD has created the study. ST and RD participated in the design of the study. ST, AMR and BL acquired data. ST, AMR, BL and RD controlled analyzed and interpreted data. RD, AMR, BL and ST prepared the manuscript. ST, AMR, BL and RD read, corrected and approved the final manuscript.

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