Lsh Mediated RNA Polymerase II Stalling at HoxC6 and HoxC8 Involves DNA Methylation

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Abstract

DNA cytosine methylation is an important epigenetic mechanism that is involved in transcriptional silencing of developmental genes. Several molecular pathways have been described that interfere with Pol II initiation, but at individual genes the molecular mechanism of repression remains uncertain. Here, we study the molecular mechanism of transcriptional regulation at Hox genes in dependence of the epigenetic regulator Lsh that controls CpG methylation at selected Hox genes. Wild type cells show a nucleosomal deprived region around the transcriptional start site at methylated Hox genes and mediate gene silencing via Pol II stalling. Hypomethylation in Lsh−/− cells is associated with efficient transcriptional elongation and splicing, in part mediated by the chromodomain protein Chd1. Dynamic modulation of DNA methylation in Lsh−/− and wild type cells demonstrates that catalytically active DNA methyltransferase activity is required for Pol II stalling. Taken together, the data suggests that DNA methylation can be compatible with Pol II binding at selected genes and Pol II stalling can act as alternate mechanism to explain transcriptional silencing associated with DNA methylation.

Introduction

DNA cytosine methylation is one of the major mechanisms involved in epigenetics since the pattern of DNA methylation is faithfully inherited by the next cell generation [1]. DNA cytosine methylation mediates transcriptional silencing and is crucial for the process of X inactivation, genomic imprinting and the control of developmental gene expression [2,3]. Since DNA cytosine methylation is tightly linked with tumor suppressor gene silencing in cancer cells, it is also thought to play a role in tumorigenesis. The prevalent cytosine methylation in mammals occurs in the CpG context in differentiated cells, however, a recent study reports DNA methylation in a non-CpG context in human embryonal stem cells, possibly a unique feature associated with pluripotency [4].

CpG methylation shows an inverse relationship with transcript levels, and multiple mechanisms have been implicated to explain gene silencing in dependence of DNA methylation [1]. Although an effect of DNA methylation at the elongation phase of transcription on reporter transgenes has been reported [5,6], most molecular mechanisms proposed to act on endogenous genes focus on the initiation phase of transcription. For example, DNA methylation can prevent binding of some transcriptional activators to their specific DNA sequences; furthermore, methyl-DNA binding proteins can recruit repressor complexes thus interfering with transcriptional initiation; moreover, DNA methyltransferases can interact with histone deacetylases rendering histones hypoa-cetylated. Histone acetylation in turn is part of the chromatin context that can facilitate the assembly of the Pol II and general transcription factor complex thus promoting transcriptional initiation [7]. In addition, a recent study demonstrated nucleosomal positioning at the transcriptional start site of the human MLH1 promoter in dependence of DNA methylation thus precluding Pol II binding [8]. These diverse mechanisms can interfere with transcriptional initiation, and for this reason, DNA methylation studies are frequently focused on promoter regions.

CpG methylation pattern are established and maintained by a family of DNA methyltransferases (Dnmt): Dnmt3a and Dnmt3b can act as de novo methyltransferases whereas Dnmt1 primarily maintains methylation pattern at the replication fork [1,9]. In addition, other factors have been identified that support the establishment of DNA methylation pattern during development [9], for example NP95 enhances Dnmt1 association to hemi-methylated DNA, and the SNF2 chromatin remodeling family members ATRX controls methylation at ribosomal DNA and some satellite sequences. Lsh (Lymphoid specific helicase, also known as Psg, HELLS, SMARCA6) is a SNF2 family member that controls DNA methylation during mammalian development. Likewise, the close A. thaliana homologue DDM1 (decrease in DNA methylation 1) affects DNA methylation in plants [10,11,12,13]. Lsh can interact with Dnm1, and part of its effect on DNA methylation may be explained by enhanced association of Dnm1 with genomic targets in the presence of Lsh [14,15,16,17]. Among the genomic targets of Lsh are repeat elements, such as minor and major satellite
sequences, Line and Sine elements and endogenous retroviral elements [10,13,18,19]. In addition, several specific genomic sites were identified as targets for Lsh, such as p57, Pu.1, and pluripotency genes, Oct4 or Nanog [14,17,20,21].

Previously, we reported that Lsh regulates the DNA methylation pattern and gene expression level of HoxA6 and HoxA7 genes in embryonic tissues and embryonal fibroblast lines [16]. Hox proteins are important developmental regulators that are controlled by tri-thorax group proteins, such as MLL (mixed lymphocyte leukemia), and by polycomb proteins [22]. Whereas MLL catalyzes methylation of histone H3 lysine 4 (H3K4me), the polycomb repressor complexes mediate H3K27 methylation and H2A ubiquitination. In addition, a functional link between Dnmts and polycomb proteins has been identified [23], and it is now well documented that DNA methylation occurs during normal cellular differentiation at Hox gene clusters [24,25,26,27]. Although DNA methylation is associated with Hox gene transcript levels, the mechanism of gene silencing remains uncertain, since polycomb mediated repression shows widespread Pol II stalling in Drosophila [28], but DNA methylation is thought to prevent Pol II binding and initiation [1].

To address the molecular mechanism of Hox gene silencing in dependence of Lsh and DNA methylation we examined nucleosomal positioning, RNA Pol II binding, RNA splicing and diverse histone modifications comparing wild type cells to hypomethylated Lsh−/− cells, or dynamically regulating DNA methylation levels in WT and Lsh−/− cells. Despite substantial differences in DNA methylation at HoxC genes we found similar nucleosomal positioning and abundant RNA Pol II binding in both Lsh−/− and wild type cells. Whereas methylated Hox genes in wild type cells show Pol II stalling, hypomethylated Hox genes have successful transcriptional elongation and efficient splicing. Moreover, dynamic modulation of DNA methylation in WT and Lsh−/− cells demonstrates a role for catalytically active Dnmt3b. Thus, our data suggests Pol II stalling as an alternate molecular pathway to induce transcriptional silencing at methylated genes.

Results

To study the molecular mechanisms of Lsh mediated gene silencing, we first examined Hox gene expression in murine embryonal fibroblast (MEF) cell lines derived from Lsh−/− and wild type (WT) embryos by RT-PCR analysis and Real-time PCR analysis (Fig. 1A). Similarly to our previous report on HoxA6 and HoxA7 genes, HoxC6 and HoxC8 mRNA expression was evident in Lsh−/− MEFs and repressed in WT MEFs [16]. Using chromatin immunoprecipitation (ChIP) analysis and specific antibodies against Lsh, we found that Lsh was associated with upstream and downstream regions of HoxC6, HoxC8 and HoxA6 genes (Fig. S1A,B,C). This suggests that Lsh, at least in part, acts directly on Hox gene expression. Similarly to our previous findings at HoxA6 and HoxA7 genes [16], we detected profound DNA methylation differences at the promoter regions of HoxC6 and C8 genes using methylation sensitive restriction enzymes followed by PCR analysis (Fig. S2A,B) and MeDIP analysis (Fig. S2C,D). To generate a precise CpG methylation map, bisulfite sequencing analysis was performed evaluating a region of about 2000 bp upstream and 1000 bp downstream of the TSS at either HoxC6 or HoxC8 genes (Fig. 1B,C). The overall DNA methylation levels varied greatly between WT cells and Lsh−/− samples. In particular, the methylation level at the CpG islands in close proximity to the TSS was reduced at HoxC6 (10 fold) and HoxC8 (7 fold) genes in Lsh−/− MEFs. This demonstrates that Lsh regulates DNA methylation at HoxC6 and HoxC8 genes in WT MEFs.

To address the question whether Lsh controls HoxC6 and HoxC8 genes during embryonic development [29,30], we examined HoxC6 and HoxC8 gene expression by RT-PCR analysis in diverse tissues derived from day13.5 embryos. Forelimb, hindlimb and lung tissue showed signs of de-repression at HoxC6 and HoxC8 genes in the absence of Lsh, but HoxC8 expression in liver tissue was not affected by Lsh (Fig. 1D) suggesting that depletion of Lsh cannot re-activate specific Hox genes in every tissue, as has been previously described [16]. Bisulphite sequencing showed reduced DNA methylation in Lsh−/− hindlimb tissue compared to WT at HoxC6 and HoxC8 genes, similarly to the results in MEFs (Fig. 1E). Consistent with transcript levels, DNA methylation in liver tissue was not significantly changed comparing WT and Lsh−/− samples. Importantly, DNA hypomethylation was found at sites of active transcription, (HoxC6 in liver or Lsh−/− hindlimb), whereas DNA methylation was present at repressed sites (HoxC8 liver or WT hindlimb), supporting the idea that DNA methylation is closely associated with gene silencing. Overall, Lsh is associated with DNA methylation and is involved in gene silencing at HoxC6 and HoxC8 genes in embryo derived cell lines and in embryonal tissue at specific developmental stages.

Phased nucleosomal positioning at TSS or enhancer elements is associated with active transcription, and regular nucleosomal pattern have been found at Hox clusters [31,32,33,34]. Furthermore, Lsh is a homologue of SNF2 factors and may affect nucleosomal positioning at HoxC genes; although Lsh has not been demonstrated, as yet, to posses nucleosomal remodeling activity. To examine nucleosomal positioning at Hox genes, we performed a MNase protection assay [33] comparing mononucleosomes prepared from wild type or Lsh−/− samples by tiling PCR analysis (Fig. 2A). As shown in Fig. 2B,C, a similar pattern of MNase sensitivity around the promoter regions of HoxC6 and HoxC8 was observed comparing WT and Lsh−/− samples (for HoxA6 see Fig. S3A). The pattern suggested a fixed +1 nucleosome with a less pronounced −1 nucleosome and revealed a MNase unprotected region around the TSS. In further support, mononucleosomal DNA was subjected to MeDIP analysis to enrich for methylated DNA around the TSS of WT cells. Wild type samples revealed a MNase unprotected region around the TSS (Fig. 2D). Taken together, the results suggested that DNA hyper- or hypomethylation in WT or Lsh−/− cells had no detectable effect on nucleosomal positioning at those genes and wild type samples revealed a MNase unprotected region around the TSS similar to that in Lsh−/− samples.

Nucleosomal deprived regions can be associated with active promoter regions and engaged RNA Pol II [33]. Thus, we tested the idea whether the MNase unprotected region around the TSS of Hox genes in WT or Lsh−/− samples could be linked to Pol II binding. First, we confirmed the position of the major TSS sites at either HoxC6 and HoxC8 genes by using 5′RACE and site specific RT-PCR analysis (Fig. S4). Then, ChIPs analysis was performed using specific antibodies raised against the heptapeptide repeat of the carboxyterminal domain (CTD) of Pol II. Chromatin samples derived from Lsh−/− MEFs showed enrichment of Pol II at the TSS of HoxC6 and HoxC8 genes (Fig. 3A, and for HoxA6 Fig. S3B). Likewise, WT samples showed a peak of unphosphorylated Pol II binding at TSS consistent with the hypothesis that Pol II binding may interfere with nucleosomal occupancy and may determine phasing of the +1 nucleosome [33]. These findings suggest that CpG island methylation at HoxC6 and HoxC8 genes does not inevitably hinder Pol II binding. Moreover, repression of HoxC6 and HoxC8 transcription was not simply caused by a lack of Pol II binding but by a molecular mechanism following Pol II engagement.
Recent results of genome-wide analysis reported RNA Pol II occupancy at promoter regions of genes that were thought to be transcriptional silent [35,36]. At those promoters a Pol II initiation complex is assembled but the elongation process is repressed leading to Pol II stalling. To test for Pol II stalling in transcriptional repressed WT MEFs ChIPs analysis was performed for detection of Ser5 phosphorylated Pol II which is associated with transcription initiation and early elongation or promoter clearance [37]. The binding of Ser5 Pol II at the promoter region was compared to the binding at the gene body to assess successful Pol II elongation. Whereas WT and Lsh2/2 samples showed binding of Ser5 phosphorylated Pol II at the TSS of HoxC6 and HoxC8 (Fig. 3B) or HoxC8 (C) genes. Fifty of 53 CpG sites at the HoxC6 gene and 76 of 80 CpG sites at the HoxC8 gene were analyzed. Methylated CpGs are presented by black circles and unmethylated sites by open circles. The position of CpG sites is indicated by red lines in the graph and the light blue color indicates CpG islands according to http://www.urogene.org/methprimer/ (D) RT-PCR analysis for detection of indicated Hox genes in different tissues from Lsh+/+ and Lsh−/− embryos d13.5. (E). Genomic DNA derived from indicated tissues from Lsh+/+ and Lsh−/− embryos d13.5 was examined by bisulphite sequencing at regions indicated.

doi:10.1371/journal.pone.0009163.g001

Figure 1. DNA methylation at silenced HoxC6 and HoxC8 genes. (A) RT-PCR analysis for detection of indicated Hox genes using total RNA derived from Lsh+/+ and Lsh−/− MEFs (Top panel) and Real-time PCR analysis (Bottom panel). Primers were employed in exon1 and exon2 thus detecting spliced transcripts. Gene expression levels were normalized against the housekeeping gene Gapdh and are represented as fold increase in Lsh−/− cells compared to Lsh+/+. Error bars represent the mean standard deviation of three independent experiments. (B, C) Genomic DNA derived from Lsh+/+ and Lsh−/− MEFs was examined by bisulphite sequencing at regions 2000 bp upstream and 1000 bp downstream of TSS at the HoxC6 (B) or HoxC8 (C) genes. Fifty of 53 CpG sites at the HoxC6 gene and 76 of 80 CpG sites at the HoxC8 gene were analyzed. Methylated CpGs are presented by black circles and unmethylated sites by open circles. The position of CpG sites is indicated by red lines in the graph and the light blue color indicates CpG islands according to http://www.urogene.org/methprimer/ (D) RT-PCR analysis for detection of indicated Hox genes using total RNA derived from Lsh+/+ and Lsh−/− MEFs (Top panel) and Real-time PCR analysis (Bottom panel). Primers were employed in exon1 and exon2 thus detecting spliced transcripts. Gene expression levels were normalized against the housekeeping gene Gapdh and are represented as fold increase in Lsh−/− cells compared to Lsh+/+. Error bars represent the mean standard deviation of three independent experiments. (B, C) Genomic DNA derived from Lsh+/+ and Lsh−/− MEFs was examined by bisulphite sequencing at regions 2000 bp upstream and 1000 bp downstream of TSS at the HoxC6 (B) or HoxC8 (C) genes. Fifty of 53 CpG sites at the HoxC6 gene and 76 of 80 CpG sites at the HoxC8 gene were analyzed. Methylated CpGs are presented by black circles and unmethylated sites by open circles. The position of CpG sites is indicated by red lines in the graph and the light blue color indicates CpG islands according to http://www.urogene.org/methprimer/ (D) RT-PCR analysis for detection of indicated Hox genes in different tissues from Lsh+/+ and Lsh−/− embryos d13.5. (E). Genomic DNA derived from indicated tissues from Lsh+/+ and Lsh−/− embryos d13.5 was examined by bisulphite sequencing at regions indicated.

doi:10.1371/journal.pone.0009163.g001
transcriptional elongation in Lsh hypomethylated repeat elements in previously, we reported increased H3K4 methylation levels at been shown to promote transcriptional elongation [41,42]. The chromodo- main protein Chd1 recognizes the lysine methyl mark and has trimethylation (H3K4me3) and binding of Chd1. The chromodo- main protein Chd1 associates with elongation factors as well as splicing components, and consequently, depletion of Chd1 impairs the splicing efficiency [41,42]. Efficient immunoprecipitation was detected in cells that had been labeled with Br-UTP indicating the specificity of the assay (Fig. S6A,B). Using nascent transcripts, the ratio of pre-mRNA to H3K4me3 levels or due to slightly higher overall Chd1 protein levels in Lsh−/− cells (Fig. S5). To test whether Chd1 is functionally involved in HoxC6 and HoxC8 gene expression, Lsh−/− cells were specifically depleted of Chd1 mRNA (Fig. S6B) and Chd1 protein (Fig. 4G) using small interfering RNA (siRNA). Within 48 hrs of siChd1 RNA treatment, HoxC6 and HoxC8 mRNA showed a significant suppression (46% and 67% respectively) compared to control siRNA treated Lsh−/− cells (Fig. 4H). This suggests that Chd1 plays a functional role in the transcription of HoxC6 and HoxC8 genes in Lsh−/− cells.

To delineate further the role of Chd1 in transcription, we examined the maturation of pre-mRNA to spliced RNA since Chd1 associates with elongation factors as well as splicing components, and consequently, depletion of Chd1 impairs the splicing efficiency [41,42]. Primers were designed to detect transcripts at HoxC6 and HoxC8 genes: for detection of ‘total’ transcripts (at the first exon),a, for pre-mRNA (at the exon/intron boundary),b, or for spliced RNA (c, spanning both exons)(Fig. 5A). The ratio of total transcripts to spliced RNA (a/c) and pre-mRNA to spliced RNA (b/c) was lower in Lsh−/− samples compared to WT samples suggesting that Lsh−/− cells were relatively more efficient in splicing than WT cells (Fig. 5B). Nuclear RNA samples derived from WT cells showed some evidence of transcripts close to the TSS (site a) and transcripts of pre-mRNA (site b) but barely detectable spliced transcripts (site c) in contrast to Lsh−/− samples (Fig. 5C). To rule out effects of RNA long-term stability and to quantify transcription rates, a nuclear run-on assay was performed using Br-UTP labeling for detection of nascent RNA [44]. Efficient immunoprecipitation was detected in cells that had been labeled with Br-UTP indicating the specificity of the assay (Fig. S6A,B). Using nascent transcripts, the ratio of pre-mRNA to

Figure 2. MNase protected sites are similar at methylated and hypomethylated Hox genes. (A) Diagram depicting the procedure for the MNase assay and the PCR amplicons used for the detection of Hox genes. The chromatin derived from Lsh+/+ and Lsh−/− MEFs was treated with MNase. The DNA was then gel-purified and fragments less than 200 bp were used for real-time PCR. The amplicons were about 100 bp in size and were spaced 60±5 bp apart. The MNase protected profile of HoxC6(B) and HoxC8(C) genes was determined by normalizing the amount of the MNase derived PCR products to those of untreated DNA. Error bars represent the mean standard deviation of three to four independent experiments. (D) MeDIP analysis to enrich for methylated DNA at HoxC6 (left) and HoxC8 (right) genes of Lsh+/+ and Lsh−/− samples after MNase treatment.

doi:10.1371/journal.pone.0009163.g002
spliced transcripts was less than one in Lsh$^{-/-}$ samples indicating efficient splicing (Fig. 5D). In contrast, WT samples showed a 3 to 8 fold higher ratio in relation to Lsh$^{-/-}$ when either total transcripts to mature mRNA ($a/c$) or pre-mRNA to spliced mRNA ($b/c$) were compared. Therefore, the data suggests that WT samples compared to Lsh$^{-/-}$ samples are impaired in converting HoxC6 and HoxC8 pre-mRNA templates into mature transcripts. To further support the notion that Chd1 is involved in splicing in Lsh$^{-/-}$ cells, we examined Chd1 depleted Lsh$^{-/-}$ MEFs (as shown in Fig. 4G). Upon Chd1 depletion, the ratio of pre-mRNA to spliced transcripts reverted back to ratios similar to those in WT samples pointing to a functional role for Chd1 in splicing (Fig. 5E). Taken together, the data suggests an inadequacy in transcriptional elongation and generation of spliced mature RNA in WT cells, and successful elongation and splicing of pre-mRNA in Lsh$^{-/-}$ cells that involves Chd1.

To address the question, whether changes in DNA methylation by other means than Lsh depletion can affect Pol II stalling, we treated WT MEFs with 5-Azacytidine. Bisulphite sequencing indicated reduced CpG methylation at the TSS region (region1) and further downstream at the second exon comprising the splicing signal (region2) after 5-Azacytidine treatment (Fig. 6A and Fig. S7). Also, hypomethylated WT cells showed re-activation of repressed HoxC6 and HoxC8 transcripts (Fig. 6B). Moreover, the Pol II stalling index changed 3 to 4 fold at HoxC6 and HoxC8 genes after 5-Azacytidine treatment (Fig. 6C). In addition, ChIPs...
analysis revealed an increase of Chd1 binding at HoxC6 and the HoxC8 genes after 5-Azacytidine treatment, similar to enhanced Chd1 binding observed in Lsh2/2 samples (Fig. 6D). Finally, the ratio of total RNA to spliced transcripts (a/c) and pre-mRNA to spliced transcripts (b/c) were reduced after treatment of WT cells with 5-Azacytidine suggesting an improved conversion of pre-mRNA into mature transcripts similar to that seen in Lsh2/2 samples (Fig. 6E). Taken together, the data suggested that 5-Azacytidine treatment can overcome Pol II stalling in WT cells.

To understand whether dynamic regulation of DNA methylation in MEFs can cause Pol II stalling in hypomethylated Lsh2/2 cells, we attempted to restore Lsh function. Lsh was introduced into Lsh2/2 MEFs using an inducible tetracycline dependent expression system. As shown in Fig. 7A, Lsh protein levels were enhanced within 24 hrs of tetracycline treatment. Also, HoxC6 and HoxC8 mRNA expression was reduced after Lsh induction, as demonstrated by RT-PCR analysis (Fig. 7B). This change in gene expression was accompanied by some gain in CpG methylation at the TSS regions of HoxC6 and HoxC8 genes (from 11 to 35% at HoxC6 and from 8 to 47% for HoxC8) and at the second exon next to the splicing signal (Fig. 7C and Fig. S8A,B). This result was consistent with our previous observations that Lsh can directly associate with Hox genes (Fig. S1) and that Lsh is required for targeting of Dnmt3b to HoxA genes [16]. Moreover, Lsh re-expression in Lsh−/− cells increased the Pol II stalling index (Fig. 7D), further supporting a close connection of Lsh mediated DNA methylation and Pol II stalling.

To demonstrate that restoration of Pol II stalling does require Dnmt3b we performed Dnmt3b depletion (Fig. 7E,F) by siRNA interference in Lsh−/− cells that expressed tetracycline inducible Lsh. As shown in Fig. 7G, only control siRNA treated Lsh−/− cells showed HoxC6 and HoxC8 gene suppression after Lsh induction, whereas Dnmt3b depleted cells failed to do so. Bisulphite sequencing and methylation sensitive PCR analysis indicated increased CpG methylation after Lsh induction when Dnmt3b was present, but not in Dnmt3b depleted cells (Fig. 7H and Fig. S9 Fig. S10). This suggests that Dnmt3b plays a role in Lsh mediated DNA methylation at those sites. In addition, only control siRNA treated Lsh−/− cells showed a shift in the ratio of pre-mRNA to spliced transcripts towards the WT phenotype after tetracycline induced Lsh expression, but not the Dnmt3b depleted Lsh−/− cells (Fig. 7I). Thus, Lsh function is not simply ‘associated’ with DNA methylation levels, but does indeed require Dnmt3b to mediate gene silencing and Pol II stalling at Hox genes.

Finally, we examined the role of catalytically active Dnmt3b in HoxC6 and HoxC8 gene silencing. We transfected WT MEFs with a mutant form of Dnmt3b (a kind gift of Dr Hsieh) that had been previously shown to be catalytically inactive [45]. RT-PCR analysis showed an increase in HoxC6 and HoxC8 mRNA in WT cells that had been transfected with mutant Dnmt3b, in contrast to control WT cells (mock transfected or transfected with wild type Dnmt3b) (Fig. 8A). Bisulphite sequencing indicated effective reduction of CpG methylation at HoxC6 and HoxC8 TSS regions after introduction of mutant Dnmt3b (Fig. 8B). Calculation of the Pol II stalling index showed that transfection of mutant Dnmt3b reduced Pol II stalling in WT cells at HoxC6 and HoxC8 genes (Fig. 8C). Furthermore, the ratio of pre-mRNA to spliced...
transcripts shifted towards a profile comparable to that generated by Lsh/-/- deletion (Fig. 8D). Taken together, the data suggests that catalytically active Dnmt3b is functionally involved in Pol II stalling at HoxC6 and HoxC8 genes.

**Discussion**

**Nucleosomal occupancy and DNA methylation at Hox genes**

DNA methylation is a stable epigenetic mark that is generally associated with repressed, compact and less ‘accessible’ chromatin. Several studies that assessed nucleosomal distribution identified some genomic sites that were influenced by DNA methylation as well as sites that were unaffected (reviewed in [46]). The effect of DNA methylation was enhancing as well as preventing nucleosomal occupancy (for example at the chicken beta globin locus) making it necessary to examine nucleosomal position at the individual promoter level [8,46]. In the current study we found no evidence by MNase profiling that the level of DNA methylation (Fig. 1, 2) influences the position of nucleosomes at HoxC6 and HoxC8 genes. While our MNase assay may be limited to determine subtle changes (less than 60 bp) in nucleosomal positioning, the comparable binding pattern of Pol II at TSS in WT and Lsh/-/- samples further supports the notion of a similar molecular mechanisms, it is difficult to predict nucleosomal positioning, and it is therefore advisable to examine individual methylated promoter regions. Our study indicates in principle that DNA methylation at CpG islands can be compatible with accessibility and does not inevitably exclude Pol II engagement.

**Pol II stalling versus elongation**

We identified here the mechanism of gene silencing, and found in ‘methylated’ WT cells Pol II stalling and in hypomethylated cells all the hallmarks of transcriptional elongation, including histone modifications and Ser2 Pol II transition and effective splicing. Pol II stalling was originally demonstrated to control the expression of heat shock genes and a number of proto-oncogenes, but recent reports suggest widespread regulation of Pol II elongation, including developmentally regulated Hox genes [28,34,36]. A genome wide study in human cells found Pol II promoter stalling in embryonic stem cells as well as somatic B cells and hepatocytes [35]. In murine macrophages, LPS inducible genes are in part regulated by transition of the pre-associated Ser5 phosphorylated Pol II into the elongation phase [48]. In addition, unstimulated genes showed low levels of pre-mRNA that were quickly degraded. After stimulation, Ser2 Pol II transition and full length mature transcripts were detected indicating that splicing was effectively regulated [48]. As for transcriptional regulation of Hox genes we provide evidence that Chd1 is involved, a chromodomain protein that associates with elongation as well as splicing factors. The in vivo relationship of DNA methylation and H3K4me3 may serve as recruitment signal for Chd1 that recognizes directly the trimethylation mark. Chd1 can bridge H3K4me3 with FACT or PAF that are engaged in transcriptional elongation [41,42]. In
addition, Chd1 physically associates with spliceosomal components (U2 snRNP subunits) [42]. Consequently, depletion of H3K4me levels (using siRNA interference of the HMT Ash2) results in reduced association of Chd1 to specific chromatin targets and a decrease in the recruitment of splicing components (SF3A1 and SF3A2) to selected genomic sites. Furthermore, depletion of splicing factors such as SC35 reduces transcriptional elongation suggesting a close relationship between elongation and splicing [44].

Figure 6. 5-Azacytidine treatment overcomes Pol II stalling. (A) Summary of bisulphite sequencing results derived from ten clones (Figure S8) to illustrate the percentage of reduced CpG methylation after 5-Azacytidine treatment at the first 10 (HoxC6, Up) or 11 (HoxC8, Down) CpG sites downstream of TSS (region 1) or 10 (HoxC6) or 11 (HoxC8) CpG sites at the second exon (region 2) (B) Real-time PCR analysis for detection of HoxC6 and HoxC8 genes in Lsh+/− MEFS before and after five days of 5-Azacytidine (Aza) treatment (1 μM). The mock treated samples were set to one for better comparison. (C) Ratio of Ser 5 Pol II ChIP signals of the TSS region over the downstream region and the ratio of 5′ and 3′ regions of exon1 comparing chromatin derived from Lsh ++ MEFS of mock and 5-Azacytidine treated cells. (D) ChIPs analysis for detection of Chd1 in the TSS region comparing chromatin derived from WT MEFS of mock and 5-Azacytidine treated cells. IgG serves as a negative control. (E) Real time RT-PCR analyses detecting the ratio of unspliced to spliced mRNA of the HoxC6 and HoxC8 gene from Lsh +/+ and Lsh −/− MEFS after the treatment of 5-Azacytidine.
doi:10.1371/journal.pone.0009163.g006
The role of DNA methylation

We describe here that Pol II promoter stalling at HoxC6 and HoxC8 genes in WT cells depends on Lsh and is tightly associated with DNA methylation. There are multiple lines of evidence for a functional role of DNA methylation beyond the observation of Pol II stalling in WT cells and elongation in hypomethylated Lsh cells. 1. Lsh and Dnmt3b bind directly to Hox genes and Lsh regulates Dnmt3b binding [16]. 2. Dynamic regulation of DNA methylation in WT cells by 5-Azacytine treatment or modulating Dnmt3b activity overcomes Pol II stalling. 3. Likewise, restoration...
of DNA methylation mediated by wild type Lsh in $Lsh^{-/-}$ cells restores Pol II stalling. 4. Restoration by Lsh requires the presence of Dnmt3b. 5. Pol II stalling requires the catalytic site of Dnmt3b and thus requires DNA methylation. Though Pol II stalling at the HoxC6 and HoxC8 genes involves DNA methylation, clearly not all Pol II stalling is attributed to DNA methylation and Pol II stalling is found at unmethylated promoter regions [2]. For Hox genes there may be multiple levels of control of how DNA methylation contributes to Pol II stalling; for example, a site specific increase of H3K4me3 may follow reduced DNA methylation [6,27,43,49,50] and the level of subsequent Chd1 recruitment may influence elongation and splicing. Also, DNA

Figure 8. The catalytic domain of Dnmt3b is required for Pol II stalling. (A) RT-PCR analysis for the detection of HoxC6 and HoxC8 mRNA from $Lsh^{+/+}$ and $Lsh^{-/-}$ MEFs transfected with wild type Dnmt3b (Wt 3b) and Mutant Dnmt3b (Mut 3b) for 72 hrs. (B) Bisulphite sequencing results to illustrate reduced CpG methylation at the first 10 (HoxC6, Left) or (HoxC8, Right) in samples prepared from WT MEF cells transfected with Mut 3b for 72 hrs. (C) Ratio of Ser 5 Pol II ChIP signals for the TSS region over the downstream region and the ratio of Ser 5 Pol II signal using a primer set located 5’ and 3’ of exon1 comparing chromatin from WT MEFs after the transfection with Mut 3b. (D) Real time RT-PCR analysis detecting the ratio of unspliced to spliced mRNA of the HoxC6 (left) and HoxC8 (right) gene in WT MEFs after the transfection with Mut 3b.

doi:10.1371/journal.pone.0009163.g008
methylation levels are involved in the modulation of polycomb protein binding and their subsequent histone modifications [16,23]. The silencing effect of polycomb proteins has been linked to altered chromatin environment and Pol II stalling and, recently, it was proposed that a steric hindrance excluding Pol II elongation may mediate polycomb silencing [20]. Whether methylated CpG island genes, other than at HoxC6 and HoxC8 genes, can also show Pol II stalling requires further investigation. In particular, the position of CpG sites (upstream, around or downstream of TSS) deserves further attention since it had been shown that DNA methylation downstream of TSS can impact transcriptional elongation at integrated transgenes or episomal plasmids [3,6]. A recent genome wide DNA methylation analysis observed a strong association of methylation peaks with the translational initiation codon [51]. Furthermore, a lack of DNA methylation peaks at translational initiation codons correlated well with Pol II elongation efficiency, consistent with the idea that DNA methylation can regulate Pol II elongation.

Lastly, aberrant DNA methylation patterns at tumor suppressor genes and at Hox genes have been found in cancer cells, but the mechanism of transcriptional silencing for many sites remains uncertain [16,24,26,52,53,54,55]. Our study provides evidence that DNA methylation at some TSS can be compatible with Pol II binding. Therefore Pol II stalling may serve as an alternate mechanism for gene silencing at some methylated tumor suppressor genes and may suggest the possibility of novel strategies to interfere with gene silencing in cancer cells.

Materials and Methods

Cell culture, tissues, plasmids and siRNA

The mouse embryonic fibroblasts (MEF) [56] were treated with 1 μM 5-Azacytidine (Sigma) for 5 days. Embryonic tissues were derived from embryos of day 13.5 of gestation. Dnmt3b plasmids Wt3b (pMT3b) and Mut3b (pMt3bMut, that contains a single point mutation at position 657 converting cysteine to serine Wt3b (pMT3b) and Mut3b (pMt3bMut, that contains a single point mutation at position 657 converting cysteine to serine) [45] were a kind gift from Dr. Hsieh (University of Southern California). The pT-REX-Lsh plasmid was constructed by subcloning Flag-tagged Lsh [57] into the pcDNA4/TO vector (Invitrogen). Lsh+/− MEFs were transfected with pcDNA6/TR (Invitrogen) and pT-Tex-Lsh and Blasticidin. Lsh protein was induced by adding 6 μg/ml of the pcDNA4/TO vector (Invitrogen). The cDNA was diluted 1:10, and 2 μl were used for analysis. Results were normalized for expression of the housekeeping gene Gapdh. For methylation-sensitive PCR analysis [58] DNA was extracted by using Qiagen DNeasy kit and 1.0 μg was digested with MspI (Roche) or BstII I (NEB). PCR analysis was carried out as follows: 3 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, and finally 5 min at 72°C. For Real-time PCR analysis as described by [16] a standard curve was generated by serially diluting input samples. For MNase digestion the fold difference was calculated [59] between MNase-treated and untreated samples. Values used were collected from the linear range of amplification. Primers were listed in the supplemental Table S1.

MNase protection analysis

The assay was carried out as described previously [60]. In brief, after lysis of single cell suspensions, chromatin was digested with MNase, DNA extracted by phenolchloroform and separated on a 1.5% agarose gel. This procedure yielded ~80% DNA from mononucleosome (~150 bp) and ~20% DNA from di- and trimonucleosomes (~300 and ~450 bp, respectively). The mononucleosomal DNA was excised, gel-purified, and used as a PCR template.

ChIP and MeDIP analysis

ChIPs were performed as previously described (Xi et al., 2007). Sonication yielded fragments between 300 to 500 bp in average. For detection of H3 modifications data was normalized by performing simultaneously ChIP analysis using anti-H3 antibodies. Before carrying out MeDIP, genomic DNA was digested with MspI or and Pst I to produce random fragments ranging in size from 200 to 500 bp. One μg of gel purified DNA was used for a MeDIP assay as described previously [2]. Real-time PCR analysis was performed, for each primer the amplification efficiency was calculated and the data expressed as enrichment related to Input.

Bisulphite sequencing

Genomic DNA (1 μg) was subjected to bisulphite treatment by using MethylDetector Bisulphite Modification kit (Active Motif). Amplified fragments were subcloned into the pCR2.1-TOPO vector (Invitrogen). Independent clones were sequenced by using the M13 F primer and only sequences with individual fingerprint selected for analysis.

Nuclear run-on assays

Nuclear RNA was extracted from the nuclei using RNasea mini kit (Qiagen). The nuclear run-on assay was has been previously described [44]. In brief, a NTP mix plus BrUTP was used whereas the control reactions for monitoring nonspecific binding omitted NTPs. The run-on reaction was performed at 25°C for 15 min on about 107 permeabilized cells and DNase I–treated total RNA was extracted and immunoprecipitated using anti-BrU antibody (B2531, Sigma) and anti Protein G Dynabeads (Invitrogen). Recovered RNA was reverse transcribed and analyzed by Real-time PCR.

Supporting Information

Figure S1 Lsh association with Hox genes. ChIP analysis using specific antibodies against Lsh (or control IgG) followed by PCR analysis to detect association of Lsh with promoter regions, TSS, and gene body regions of HoxC6 (A), HoxC8 (B) and HoxA6 (C) genes comparing Lsh+/+ and Lsh−/− MEFs. Enrichment is represented as a percentage of input. Error bars represent

PCR analysis

For RT-PCR total RNA was extracted using Qiagen RNeasy Mini Kit and 2 μg were reverse transcribed with 50 ng random primers and 10U Reverse Transcriptase (Superscript III Kit, Invitrogen). The cDNA was diluted 1:10, and 2 μl were used for analysis.
the standard deviation for the mean of three independent experiments. 

Found at: doi:10.1371/journal.pone.0009163.s001 (0.50 MB TIF)

**Figure S2** DNA methylation analysis at HoxC6 and HoxC8 genes. (A,B) Methylation sensitive PCR to determine DNA methylation level at HoxC6 (A) and HoxC8 (B) genes. The map on top illustrates the position of methylation-sensitive restriction enzyme (Mac II), as well as the primers used for the methylation-sensitive PCR analysis (Top). PCR analysis using genomic DNA derived from Lsh+/+ and Lsh−/− MEFs after digestion with Mac II. The undigested DNA served as a control for equal input of DNA (Uncut). (C,D) MeDIP analysis for detection of CpG methylation at the HoxC6 (C) or HoxC8 (D) genes. Region 1 (deviod of CpG sites) was amplified as negative control. Genomic DNA was digested with Mse I (C) or Mse I and Pst I (D), immunoprecipitated with anti-5-methylcytosine antibodies and specific Hox locations assessed by real-time PCR. Binding was expressed as a percentage of the input DNA. The bar graphs represent the mean of two independent MeDIP experiments. 

Found at: doi:10.1371/journal.pone.0009163.s002 (0.39 MB TIF)

**Figure S3** MNase protection profile at HoxA6. (A) The MNase protected profile of HoxA6 was determined by normalizing the amount of the MNase derived PCR products to those of the untreated DNA. (B) Enrichment of unphosphorylated Pol II (left) and Ser 5 Pol II (right) at HoxA6. (C) Ratio of Ser 5 Pol II ChIP signals at the TSS region over the downstream region of HoxA6 and the ratio of Ser 5 Pol II signal using a primer set located within exon1. 

Found at: doi:10.1371/journal.pone.0009163.s003 (0.43 MB TIF)

**Figure S4** Identification of major transcriptional start sites (TSS) at HoxC6 and HoxC8 genes. (A) Identification of HoxC6 TSS using cDNA derived from Lsh−/− MEFs and primer specific for TSS region 1 and region 2. RT-PCR analysis suggest that regions 2 (as marked in Fig. 1 and Fig. 2 as TSS) is indeed the major TSS for HoxC6 since no signal was detected fro region 1. (B) Identification of HoxC8 TSS using cDNA derived from Lsh−/− MEFs and primer specific for TSS region 1. (C,D) Identification of HoxC6 (C) and HoxC8 (D) TSS using RLM-RACE. RNA is treated with tobacco acid pyrophosphatase (TAP) to remove the cap structure from the full-length mRNA leaving a 5’-monophosphate which is subsequently ligated to a synthetic RNA adaptor. After reverse transcription nested gene specific primers and adaptor specific primers are used for gene specific amplification. Results indicate a major amplification product about 320 bp for HoxC6 and 290 bp for HoxC8 confirming the preferential use of the predicted TSS for both genes. The major TSSs are reported in the Mouse Genome database (MGI) at chr13:102,839,993 for HoxC6 and at chr15:102,820,970 for HoxC8. 

Found at: doi:10.1371/journal.pone.0009163.s004 (0.63 MB TIF)

**Figure S5** Protein analysis in the absence of Lsh. (A) Western analysis of extracts derived from Lsh+/+ and Lsh−/− MEFs using the indicated antibodies. (B) Real-time RT-PCR analysis for detection of Chd1 mRNA from Lsh−/− MEFs treated with control siRNA (siControl) or siRNA targeted against Chd1 (siChd1) for 48 hrs. 

Found at: doi:10.1371/journal.pone.0009163.s005 (0.46 MB TIF)

**Figure S6** Nuclear run on assay. (A) Schematic diagram for the nuclear run-on assay based on the use of BrUTP. The nascent transcript snRNA detection was demonstrated by analyzing total RNA from Lsh+/+ and Lsh−/− MEFs labeled with BrU, Uridine (U) and BrU in the presence of α-amanitin. (B) PCR products with reverse transcriptase (+RT) or without reverse transcriptase (-RT) from nascent RNA that were immunoprecipitated by anti-Br-UTP antibody for detection of nascent RNA generated in the nuclear run on assay. The WT sample generated with RT served as positive control. Data indicate the purity of the nascent RNA used for the assay. 

Found at: doi:10.1371/journal.pone.0009163.s006 (0.43 MB TIF)

**Figure S7** DNA hypomethylation after 5-Azacytidine treatment. Genomic DNA derived from Lsh+/+ MEFs before and after treatment of 5-Azacytidine was examined by bisulphite sequencing at region 1 (top) and region 2 (2nd exon, bottom) at the HoxC6 (A) and the HoxC8 (B) genes. 

Found at: doi:10.1371/journal.pone.0009163.s007 (0.72 MB TIF)

**Figure S8** Re-introduction of Lsh in Lsh−/− MEFs. Genomic DNA derived from Lsh+/+ and Lsh−/− MEFs before and after 24 hours of tetracycline treatment to induce Lsh in Lsh−/− cells. The map on top illustrates the position of methylation-sensitive restriction enzyme (Mac II in HoxC6 and BssH II), as well as the primers used for methylation-sensitive PCR analysis (Top). PCR analysis using genomic DNA derived from Lsh−/− MEFs after digestion with Mac II and BssH II. The digested DNA without enzyme sites served as a control for equal input of DNA (Bottom). 

Found at: doi:10.1371/journal.pone.0009163.s008 (0.74 MB TIF)

**Figure S9** DNA hypomethylation after Dnmt3b depletion. Methylation sensitive PCR to determine methylation level at HoxC6 (A) and HoxC8 (B) after targeting by Dnmt3b siRNA or control siRNA before and after 24 hours of tetracycline treatment to induce Lsh in Lsh−/− cells. The map on top illustrates the position of methylation-sensitive restriction enzyme (Mac II in HoxC6 and BssH II), as well as the primers used for methylation-sensitive PCR analysis (Top). PCR analysis using genomic DNA derived from Lsh−/− MEFs after digestion with Mac II and BssH II. The digested DNA without enzyme sites served as a control for equal input of DNA. 

Found at: doi:10.1371/journal.pone.0009163.s009 (0.11 MB TIF)

**Figure S10** Dnmt3b is required for re-methylation at HoxC6 and HoxC8. Genomic DNA derived from Lsh−/− MEFs after targeting by Dnmt3b siRNA or control siRNA before and after 24 hours of tetracycline treatment to induce Lsh in Lsh−/− cells. The map on top illustrates the position of methylation-sensitive restriction enzyme (Mac II in HoxC6 and BssH II), as well as the primers used for methylation-sensitive PCR analysis (Top). PCR analysis using genomic DNA derived from Lsh−/− MEFs after digestion with Mac II and BssH II. The digested DNA without enzyme sites served as a control for equal input of DNA. 

Found at: doi:10.1371/journal.pone.0009163.s010 (1.39 MB TIF)

**Table S1** Primers for PCR, ChIP, Nuclear Run on and real-time PCR 

Found at: doi:10.1371/journal.pone.0009163.s011 (0.16 MB DOC)

**Acknowledgments** 

We are grateful to Drs. Nancy Colburn and Peter Johnson for critical comments on the manuscript. We are grateful to Dr. Chih-Lin Hsieh for her generous gift of Dnmt3 mutants. We thank Jennifer Waters and Jenny Mercado for technical assistance. 

NCI-Frederick is accredited by AAALAC International and follows the “Guide for the Care and Use of Laboratory Animals” (National Research Council; 1996; National Academy Press; Washington DC). 

**Author Contributions** 

Conceived and designed the experiments: YT SX VB KM. Performed the experiments: YT SX VB. Analyzed the data: YT SX VB KM. Contributed reagents/materials/analysis tools: YT SX VB KM. Wrote the paper: YT SX VB KM.
References

1. Klose RJ, Bird AP (2006) Genomic DNA methylation: the mark and its mediators. Trends Biochem Sci 31: 89–97.

2. Mohn F, Weber M, Rebhan M, Roloff TC, Richter J, et al. (2008) Lineage-specific polycomb targets and de novo DNA methylation define restriction and potential of neuronal progenitors. Mol Cell 30: 755–766.

3. Latham T, Gilbert N, Ramsdoyre B (2008) DNA methylation in mouse embryonic stem cells. Curr Stem Cell Res Ther 3: 31–53.

4. Lister R, Pelizzola M, Down RH, Hawkins RD, Hon G, et al. (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 462: 315–322.

5. Lorenz MC, Dickerson DR, Schmitt M, Groudine M (2004) Intragenic DNA methylation alters chromatin structure and elongation efficiency in mammalian cells. Nat Struct Mol Biol 11: 1086–1075.

6. Oktue CY, Hesch CL (2007) DNA methylation dictates histone H3K4 methylation. Mol Cell Biol 27: 2746–2757.

7. Conaway RC, Sato S, Tomomori-Sato C, Yao T, Conaway JW (2005) The mammalian Mediator complex and its role in transcriptional regulation. Trends Biochem Sci 30: 250–255.

8. Lin JC, Jeong S, Liang G, Takai D, Fatemi M, et al. (2007) Role of nucleosomal positioning in human HOX gene clusters. Genome Res 18: 196–222.

9. Sims RJ 3rd, Belotserkovskaya R, Reinberg D (2004) RNA polymerase II: the short and long of it. Genes Dev 18: 1049–1061.

10. Seila AC, Calabrese JM, Levine SS, Yeo GW, Rahl PB, et al. (2008) Divergent transcription from active promoters. Science 322: 1049–1053.

11. Simic R, Lindstrom DL, Tzan HG, Reineck KL, Costa PJ, et al. (2003) Chromatin remodeling protein ChlI interacts with transcription elongation factors and localizes to transcribed genes. EMBO J 22: 1846–1856.

12. Strathdee G, Holyoake TL, Sim A, Parker A, Oscier DG, et al. (2007) HOXA5 is a candidate gene for myelodysplasia. Clin Cancer Res 13: 5048–5055.

13. Strathdee G, Holyoake TL, Sim A, Parker A, Oscier DG, et al. (2007) Inactivation of HOXA genes by hypermethylation in myeloid and lymphoid malignancy is frequent and associated with poor prognosis. Clin Cancer Res 13: 5048–5055.

14. Strathdee G, Sim A, Saurat R, Holyoake TL, Brown R (2007) HOXA5 is targeted by cell-type-specific CpG island methylation in normal cells and during the development of acute myeloid leukaemia. Carcinogenesis 28: 299–309.

15. Yuan X, Geng Y, Yue J, Jiang B, Yuan J, et al. (2008) Cooperation between EZH2, NSD1-mediated histone H2A ubiquitination and Dnmt1 in HOX gene silencing. Nucleic Acids Res 36: 3590–3599.

16. Klose RJ, Bird AP (2006) Genomic DNA methylation: the mark and its mediators. Trends Biochem Sci 31: 89–97.

17. Belting HG, Shashikant CS, Ruddle FH (1998) Multiple phases of expression during transdifferentiation in Drosophila. Curr Biol 9: 688–693.

18. De La Fuente R, Baumann C, Fan T, Schmidtmann A, Dobrinski I, et al. (2006) Lsh, a SNF2 family, is required for genome-wide methylation. Genes Dev 15: 432–444.

19. Ooi SK, O’Donnell AH, Bestor TH (2009) Mammalian cytosine methylation at a glance. J Cell Sci 122: 2717–2718.

20. Denns K, Fan T, Geiman TM, Yau Q, Muegge K (2003) Lsh, a member of the SNF2 family, is required for genome-wide methylation. Genes Dev 15: 1980–1989.

21. Jeddeloh JA, Stokes TL, Richards EJ (1999) Maintenance of genomic DNA methylation during cell cycle progression. EMBO J 18: 2457–2468.

22. Zeitlinger J, Stark A, Kellis M, Hong JW, Schones DE, et al. (2007) High-resolution profiling of histone modifications in the human genome. Cell 129: 835–847.

23. Zhu H, Geiman TM, Xi S, Jiang Q, Schmidtmann A, et al. (2006) Lsh is an epigenetic guardian of heterochromatin at repeat elements. Nucleic Acids Res 32: 5019–5028.

24. Muegge K (2005) Lsh, a guardian of heterochromatin at repeat elements. Biochim Biophys Acta 1743: 540–554.

25. Sun LQ, Lee DW, Zhang Q, Xiao W, Raabe EH, et al. (2004) Growth retardation and premature aging phenotypes in mice with disruption of the SNF2-like gene, PASH. Genes Dev 18: 1035–1046.

26. Fan T, Schmidtman A, Xi S, Briones V, Zhu H, et al. (2008) DNA hypomethylation caused by Lsh deletion promotes erythroderma development. Epigenetics 3: 134–142.

27. Myant K, Stancheva I (2008) LSH cooperates with DNA methyltransferases to repress transcription. Mol Biol Cell 20: 215–226.

28. Xi S, Zhu H, Xu X, Schindlum A, Geiman TM, et al. (2007) Lsh controls Hox gene silencing during development. Proc Natl Acad Sci U S A 104: 14366–14371.

29. Zhu H, Geiman TM, Xi S, Jiang Q, Schindlum A, et al. (2006) Lsh is involved in de novo DNA methylation of Hox genes. EMBO J 25: 335–345.

30. De La Fuente R, Baumann C, Fan T, Schmidtman A, Dobrinski I, et al. (2006) Lsh is required for mitotic chromosome synopsis and retrotransposon silencing in female germ cells. Nat Cell Biol 8: 1448–1454.

31. Huang J, Fan T, Yan Q, Zhu H, Fox S, et al. (2004) Lsh, an epigenetic guardian of repetitive elements. Nucleic Acids Res 32: 5019–5028.

32. Han T, Huang J, Zhe Z, Hua H, Bao J, et al. (2008) Nucleosome deposition and transcription elongation by native chromatin at specific gene loci by native chromatin immunoprecipitation of nucleosomes isolated using hydroxyapatite chromatography. Nat Protoc 3: 396–409.

33. Ozolinkas F, Song JS, Liu XS, Fisher DE (2007) High-throughput mapping of the chromatin structure of human promoters. Nat Biotechnol 25: 244–248.

34. Schones DE, Cui K, Corden-Bright S, Robb RF, Barski A, et al. (2008) Dynamic regulation of nucleosome positioning in the human genome. Cell 132: 887–898.

35. Yuan GC, Liu YJ, Dion MF, Slack MD, Wu LF, et al. (2005) Genome-scale identification of nucleosome positions in S. cerevisiae. Science 309: 626–630.

36. Guelenler MG, Levine SS, Boyer IA, Jarnisch R, Young RA (2007). A chromatin landmark and transcription initiation at most promoters in mammalian cells. Cell 130: 77–88.

37. Lsh Mediated Pol II Stalling