Review

Methylation and Demethylation of *Ink4* Locus in Cancer Development

Da-jun Deng*, Qiang Li, Xiu-hong Wang

Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Cancer Etiology, Peking University Cancer Hospital & Institute, Beijing 100142, China

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ABSTRACT

The *Ink4* locus at chromosome 9p21 encodes $P15^{Ink4b}$, $P14^{Arf}$, $P16^{Ink4a}$, MTAP, ncRNA *ANRIL*/*p15AS* and *p16AS*, which play an important role in regulation of stem cell self-renewal. Loss of functions of these genes promotes cell proliferation through bypassing checkpoint between the G1 and S phase of the cell cycle. Transcriptional silence by methylation of CpG island around transcription starting site (TSS) is a frequent event in the early stage of carcinogenesis. Chronic inflammation is a strong initiator for methylation of CpG island of the *Ink4a* gene. Combination of transcriptional silencers such as Polycomb group (PcG) proteins with gene-specific ncRNA could result in histone modifications including trimethylation at H3K27 firstly, and then at H3K9 epigenetically. In the case of long-term silence of transcription, methylation of CpG sites initiates and spreads progressively within the full CpG islands of the target gene. The methylation status of *Ink4a* CpG island is very stable even if its host cells are fused with *Ink4a* active cells.

Key words: Methylation; *Ink4*; CpG island; *Ink4α* gene

Role of the *Ink4* Locus in Cancer Development

The *Ink4b-Arf-Ink4a* locus locates within the human chromosome 9p21 (Figure 1). Its antisense strand encodes a part of possible tumor suppressor MTAP, two long non-coding RNA (lncRNA) *ANRIL* (*p15AS*) and *p16AS* (GQ168815) characterized at our Lab recently. The *Ink4b-Arf-Ink4a* locus plays important roles in the cell senescence, control of the cell cycle, stem cell self-renewal, and apoptosis through $P15^{Ink4b}$-Cdk2, P14Arf-MDM2-P53-P21Cip1-Rb-Cdk2/4/6, and $P16^{Ink4a}$-Cdk4/6 pathways[1-3]. This locus is deleted frequently in a number of human cancers. Subjects with germline mutations of these genes are predisposed to malignant tumor such as melanoma[4]. Mice knockouting one of these genes are highly tumor prone. Thus, there are sufficient evidences to support that they are tumor suppressor genes[5].

Phylogenetically, *Ink4b* and *Arf* are older than *Ink4a* among vertebrates (Figure 2). Homologues of *Ink4a* exist only in mammalians such as human and mouse, but *Ink4b* and *Arf* also present in other vertebrates such as chicken, lizard, and stickleback. Interestingly, *Arf* in mouse plays more important roles than *Ink4a* in inhibition of cell proliferation. In contrast, *Ink4a* in human plays more roles than *Arf*[5,6]. Krimpenfort et al. reported that $P15^{Ink4b}$ protein levels were embryonic fibroblasts deficient for $P16^{Ink4a}$ under conditions of stress and concluded that $P15^{Ink4b}$ could fulfill a critical backup function for $P16^{Ink4a}$[7]. Moreover, Leong et al. reported that *p53* deficiency could lead to compensatory upregulation of *Ink4a* in mouse tissues[8]. Thus, $P16^{Ink4a}$-Rb-Cdk4/6 pathway might also serve as backup for dysfunction of $P14^{Arf}$-$P21^{Cip1}$-P53 pathway. It is likely that each gene at the *Ink4* locus serves as backup pathways for other genes within the same locus. This might account for that knockout of *Ink4a* in mouse is not lethal and concomitant deletion of two genes results in higher tumor prone. In fact, the frequent deletion of 9p21 in tumor cells are often involved more than one gene within the *Ink4* locus.

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*Corresponding author.
E-mail: dengdajun@bjmu.edu.cn

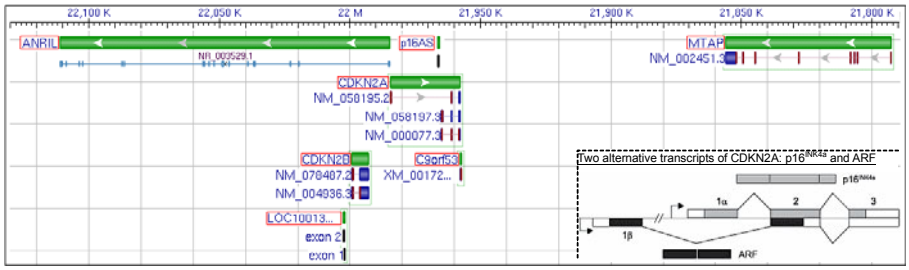

Figure 1. Models of genes [green arrowed box, including *LOC100130239*, *Ink4b* (*CDKN2B*), *Ink4a* (*CDKN2A*)/*Arf*, *C9orf53* on the sense strand, *ANRIL* (*p15AS*), *p16AS*, and *MTAP* on the reverse strand], their transcripts (gray arrowed line) and exons (brown open read frame or blue untranslated region) around chromosome 9p21 (top scaled ruler) (based on graphics of NC_000009.10 on the NCBI web site). Two alternative splicing patterns of the *Ink4a* transcript were illustrated on the right bottom corner.

Methylation of CpG Islands within the *Ink4* Locus during Carcinogenesis

There are a number of CpG islands around TSSs and other regions of all the three genes within the *Ink4* locus. Methylation of each CpG island around TSS results in transcriptional silence of the corresponding gene. Each of these genes is inactivated independently[9]. Methylation-silence of *Ink4a* is not accompanied with methylation-silence of *Arf*, although they share the same exon 2 using different reading frames (Figure 2). Frequency of *Ink4a* methylation is significantly higher than those of *Ink4b* and *Arf*[10]. Loss of expression of these genes promotes progression of cells at the G1 phase to the S phase of the cell cycle through the TGFβ-P15^{Ink4b}-Cdk2, P14^{Arf}-MDM2-P53-P21^{Cip1}-Cdk2/4/6, and P16^{Ink4a}-Rb-Cdk4/6 pathways, respectively.

Inactivation of the genes at the *Ink4* locus is a frequent event in a number of human cancers. Mice deficient for both P16^{Ink4a} and P19^{Arf} are viable but highly prone to tumors, succumbing to lymphomas and fibrosarcomas early in life[11]. Whether concomitant methylation of CpG islands around TSS of these genes promotes development of cancer is unknown.

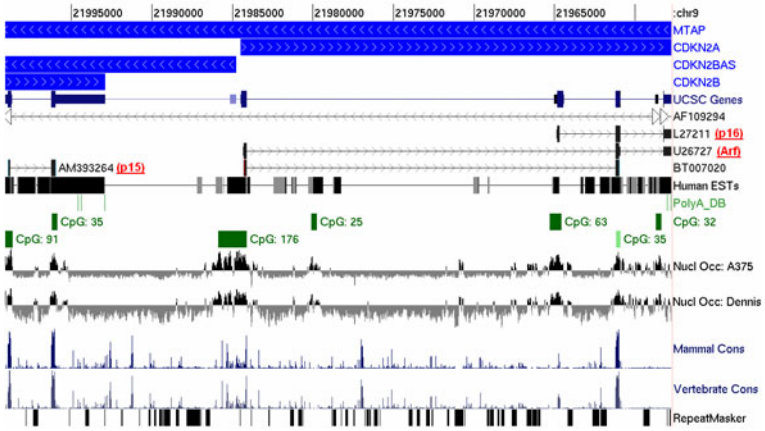

Figure 2. Illustration of gene location, transcripts, CpG islands, nucleosome occupation, and phylogenetic conservation of the *Ink4* locus at 9p3 (based on web http://genome.ucsc.edu)

When one allele is inactivated by LOH or germline mutations, it is common that the remaining functional allele of some tumor suppressor genes such as *Ink4a*, *MLH1*, *LKB1*, *FZD9*, and *E-cadherin* will be silenced in tumor tissues by methylation of CpG island as "second hit"[12-16]. However, the methylation hit of the functional allele is not a universal phenomenon. For example, the prevalent second hits for inactivation of *BRCA1, BRCA2*, and *APC* is somatic mutations or loss of heterozygous (LOH), but not methylation of CpG island[17]. Though it is often to see inactivation of *Ink4b*, *Arf*, *Ink4a* by methylation

without somatic mutation in various cancer tissues, the abnormal methylation accompanied with LOH of 9p21 locus can be observed in primary cancer tissues[18,19]. The possible reason for the methylation hit following the genetic hit might be a consequence of the failure of adaptation. For the haploinsufficient genes such as *Ink4b* and *Arf*, inactivation of one functional allele might lead to compensatory upregulation of both the inactivated (if accessible for transcription factors) and wild-type alleles themselves and/or compensatory activation of backup pathways, as described above, to overcome the obstacles of metabolism or cell cycle. In the case of failure of sufficient adaptation, the compensatory mechanisms would be shifted from upregulation of the involved genes to alternative backup pathways to avoid the long-term ineffective compensation. Consistent silence of transcription of a gene is a strong signal to initiate methylation of CpG island and chromatin remodeling.

Methylation-silence of gene within the *Ink4* locus is an early frequent event in multistep processes of carcinogenesis. It exists not only in malignant tissues, but also in precancerous lesions and tissues with inflammation. It was reported that *Ink4b* methylation was observed in 38% of the cases (20/53) with myelodysplastic Syndrome (MDS) and that 83% of patients (20/24) with greater than 10% bone marrow blasts had *Ink4b* methylation and nine of these 20 patients progressed to acute myeloid leukemia (AML)[20]. In fact, DNA hypomethylation agent 5-aza-cytidine (5-Aza-C, azacitidine) is the first epigenetic drug to receive FDA-approved labeling for the treatment of MDS.

In a large-scale population-based study at high risk area for stomach cancer in China, we found that frequency of *Ink4a* methylation in *H. pylori* associated superficial and chronic atrophic gastritis (CAG) was up to 68% (13/19) and 82% (108/132), respectively[21]. Interestingly, the methylation positive rates in CAG with intestinal metaplasia (IM), indefinite for dysplasia, and low-grade noninvasive neoplasia were reduced to 57% (81/142), 67% (145/215) and 46% (35/77), respectively (Figure 3). Further analysis showed that the prevalence of *Ink4a* methylation among all kinds of gastric lesions was *H. pylori* density-dependent. These results suggest that *H. pylori* infection may be a strong initiator for the methylation in gastric mucosa. Both progression and cancerization rates of CAG, with or without IM and dysplasia, in *H. pylori* serum-antibody-positive patients were significantly higher than those in the serum-negative patients among this population[22]. In a nested case-control study in the area, we observed that all of five cases with *Ink4a*-methylated low-grade gastric dysplasia progressed to gastric carcinoma within 5 years[23]. The role of *Ink4a* methylation in *H. pylori*-related gastric carcinogenesis is under investigation.

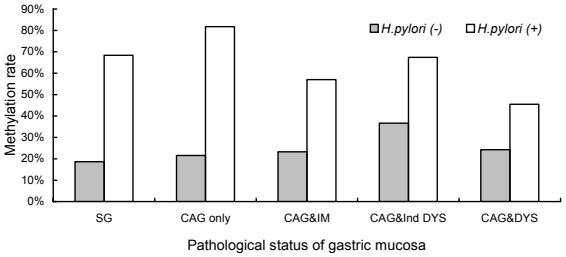

Figure 3. Prevalence of *Ink4a* methylation (by methylation specific-PCR) in human gastric mucosa biopsies with various lesions from Chinese patients with and without *H. pylori* infection (by ELISA assay). SG, superficial gastritis; CAG, chronic atrophic gastritis; IM, intestinal metaplasia; Ind DYS, indefinite for dysplasia; DYS, dysplasia (noninvasive neoplasia)

Similar methylation changes could also be observed in gastric carcinomas with Epstein-Barr virus infection and hepatocellular carcinomas with hepatitis B and C virus infection[24,25]. Thus, aberrant methylation of CpG islands in tissues with inflammation might be one kind of defense response of host cells to invading microorganisms, which could return to normal methylation status after eradication of the microorganisms generally through demethylation or cell regeneration. Others and we reported that *Ink4a* methylation was also an early event during carcinogenesis of experimental animals by chemicals previously[26,27]. Whether *Ink4a* methylation is a carcinogen-specific response or a universal adaptive phenomenon during inflammation is worth to be studied further.

Ink4a methylation may be a potential biomarker to predict malignant potential of precancerous lesions at several organs. In addition to our observation that the cases with *Ink4a*-methylated gastric dysplasia progressed to adenocarcinoma[23], it was also reported that *Ink4a* methylation in sputum was a significant predictor for the development of lung cancer in another nested case-control study in USA[28]. In a multi center double-blinded nested case-control study, a significant positive correlation was also observed between malignant progression of Barrett's esophagus and methylation of *Ink4a*, *HPP1*, and *RUNX3*[29]. In a small prospective study in Britain, it was reported that oral epithelial dysplasia with *Ink4a* methylation progressed to oral squamous cell carcinoma at a higher risk than those without the methylation (8/14 vs. 2/24)[30]. In a larger follow-up clinical cohort in China (NCT00835341), we found that *Ink4a*

methylation promoted malignant transformation of mild or moderate oral epithelial dysplasia significantly[31]. The sensitivity and specificity of *Ink4a* methylation for oral cancer prediction were up to 76.9 % and 78.3 % for patients at age over 60 years, respectively. If these results could be confirmed further in multicentre clinical trials, *Ink4a* methylation might became another very useful biomarker for diagnosis of cancer, after the discovery of α-fetal protein (AFP) and prostate specific antigen (PSA).

Generally, loss of $P16^{Ink4a}$ protein is correlated with *Ink4a* methylation in monoclonal cultured cell lines and cancer cell clonally expanded malignant tissues very well. However, such relationship could not be observed often in nonmalignant tissues, in which only a limited number of cells are transformed neoplastically. In contrast, *Ink4a* methylation in a few cells might sometimes accompany with upregulation of the protein in other cell subpopulations within the same lesions such as HPV-associated cervical dysplasia (CIN), tissues with inflammation, and epithelial dysplasia. The "contradictory" phenomenon could be observed for various genes such as *Cox2* in gastritis lesions. $P16^{Ink4a}$ is essential for cell senescence and anoikis[32], which are frequent events in inflammation lesions because of increased oxidative stress there. It was reported that knockdown of $P16^{Ink4a}$ by siRNA was accompanied by the upregulation of P53, $P21^{cip1}$ and RB in the transfected cells and augmented DNA damage-induced apoptosis in cervical cancer cells induced by ultraviolet-irradiation and cisplatin treatments[33]. Whether *Ink4a* methylation leads to compensatory upregulation of p53 is unknown. It is likely that expression change of *Ink4a*, whether upregulated or silenced, might be one kind of the adaptive response of host cells to DNA damage. On one hand, the upregulation inhibits the progression of cells at G1 phase to S phase of the cell cycle for repair of DNA damage at un-lethal level or promotes apoptosis of the lethally damaged cells. On another hand, in the case of failure of both apoptosis and DNA repair, the *Ink4a*-silenced cells might be prone to immortalization, especially when it is inactivated by DNA methylation stably.

Mechanism of Methylation Initiation of the *Ink4* Locus

Expression of the genes within the *Ink4* locus is regulated by a number of transcription factors, including activators Ras, Myc, TGFβ, ETS2, and silences pRB, PcG protein EZH2, BMI1, CBX2, CBX7, etc[34]. CDC6 and MLL1 could bind with the Regulatory domain (RD) at upstream of the locus and downregulate the expressions of all of three genes[35,36]. These proteins consist of the *Ink4* locus-specific PcG repressive complexes (PRC) and play a crucial role in the epigenetic silencing of these genes[37].

Chromatin-associated PcG proteins are repressors of development- and differentiation- related genes including the *Ink4* locus. They are also epigenetic gene silencers implicated in carcinogenesis [38]. Overexpression of PcG proteins is associated with poor prognosis of cancer. PcG proteins are classified into PRC1, PRC2, and RhoRC mainly. PhoRC can directly bind to DNA [39]. PRC2 is an initiation repressive complex that binds in a localized manner at PcG response elements (PREs) of target genes. In fact, SET domain at PcG protein EZH1 and EZH2 in PRC2 exhibits histone methyltransferase (HMTase) activity that specifically trimethylates H3K27 in nucleosomes containing PREs. Polycomblike PRC2 (Pcl-PRC2) is another H3K27- specific HMTase that mono-, di- and trimethylates H3K27 and generates high levels of H3K27me3 in nucleosomes without PRE within PcG-target genes that are needed to maintain a PcG-repressed chromatin state[40]. Another PRC2 member EED plays a role in propagation of repressive histone markers through specifically binding to histone tails containing H3K27me3 with its C-terminal domain[41].

EZH2 is one of the core members of PRC2. It was reported that EZH2 within PRC2 could directly control DNA methylation by recruiting DNA methyltransferases (DNMTs) to EZH2-target genes[42]. Generally, transcription repression by PRC2-H3K27me3 pathway is considered as an initiation process during epigenetic silencing of PRC-target gene (Figure 4). However, PRC1 binding, H3K9 trimethylation, and DNA methylation are recognized as epigenetic markers during PRC-target gene silencing. Whether PRC1 binding and H3K9 trimethylation are mediated in the process of PRC2 recruiting DNMTs is unknown.

PRC1 is a maintenance repressor complex. After additional binding with PRC1, the PRC2-repressed genes will be inactivated epigenetically. SUV39H-catalyzed trimethylation of H3K9 is a landmark of histone modifications within heterochromatin and epigenetically silenced domains in euchromatin. It is well known that the N-terminal chromodomain (CD) of some PcG components such as CBX7 could bind with both H3K9me3 and H3K27me3 *in vitro*. However, it was unknown whether PRC1 binding to the PRC2-target genes promotes formation of H3K9me3. Our recent work demonstrated that expression level of CBX7 among 20 human cancer cell lines was correlated with methylation status of

Ink4a positively. Further studies showed that upregulation of CBX7 expression recruits high level of SUV39H2 to the locus of gastric cancer MGC803 cells that trimethylates H3K9 at the promoter of *Ink4a* subsequently, and resulted in downregulation of the genes within the *Ink4* locus finally. The expression of the *Ink4* locus could be rescued by siRNA-interference of *Suv39h* expression[43]. These results suggest that CBX7, the core member of PRC1, could directly induce trimethylation of H3K9 in nucleosome near transcription starting site of *Ink4a* through recruitment of SUV39H to its target sequences (Figure 4).

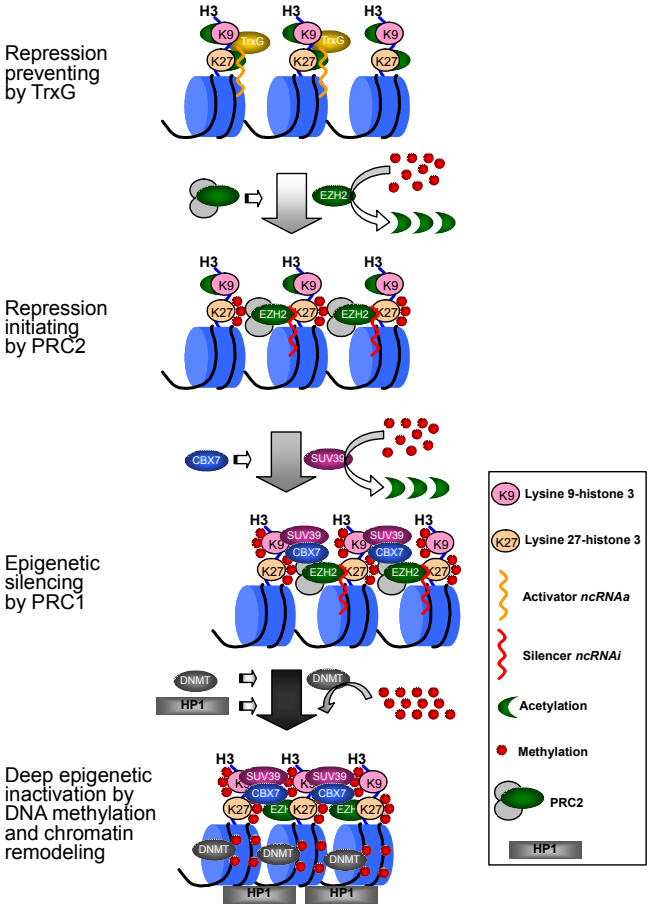

Figure 4. Model for epigenetic silencing of the mammalian *Ink4* locus

Like *Ink4a*, phylogenetically, CBX7 is a late developed gene that only presents in mammals (Figure 5). We observed that CD mutation of CBX7 only partially abolished function of wild type CBX7 on downregulation of the *Ink4* locus. There is a very conservative Pc box at the C-terminal of CBX proteins, which is a 15-amino acid motif necessary for transcriptional repression of target genes and for interaction with dRing, the catalytically active subunit of PRC1[44]. We found that the Pc box was also involved in the SUV39H recruitment to the *Ink4a* promoter. It was reported that CBX7 could bind to DNMT1 and induce the downregulation of *E-cadherin*[45]. However, we did not observe methylation of CpG islands within the *Ink4* locus in the CBX7-upregulated cells even after 80 passages. These results suggest that other silencers are necessary for deep epigenetic silence of the *Ink4* locus by DNA methylation.

It was reported that single-stranded and double-stranded RNAs (ssRNA and dsRNA) might be involved in combination of these CBX7 with DNA[46,47]. ssRNA *p15AS*, a fragment of *ANRIL*, was involved in the epigenetic inactivation of *Ink4b* through Dicer-independent way[48]. It was even supposed that the act of transcription itself, rather than ncRNA transcript, might mediate the regulation of transcription of target gene[49]. Recently we characterized an antisense transcript within the *Ink4a* promoter (412bp; *p16AS*; Wang and Deng, GenBank

accession number GQ168815). Trans-cription level of *p16AS* in cell lines and cancer tissues with *Ink4a* expression were significantly higher than that in cell lines with *Ink4a* inactivation by methylation. Unlike dsRNAs targeted against the first exon that downregulated *Ink4a* expression, we observed that dsRNAs matching with *p16AS* within the *p16* promoter region upregulated *Ink4a* transcription (Wang and Deng, unpublished data). Yap *et al*. reported similar results[50]. They found that binding to the antisense transcript of the first exon and intron of *Arf* disrupts the ability of CBX7 to repress *Ink4b-Arf-Ink4a* locus. Thus, it is likely that the endogenous *p16AS* might be involved the maintaining of *Ink4a* transcription. We proposed that the binding of silencer ssRNA (*ncRNAi*) and activator ssRNA (*ncRNAa*) might play a role in recruitment of PcG silencers and activators such as trithorax group proteins (TrxG) to target-genes, respectively (Figure 4).

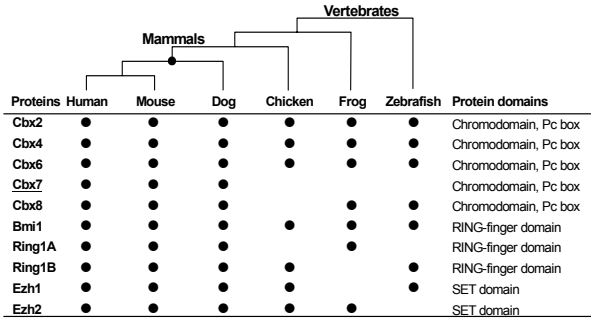

Figure 5. Phylogenetic representation of selected vertebrate organisms and their PcG homologs[44].

Traditionally, TrxG-containing trithorax acetylation complex (TAC1) is recognized as the activator to maintain the expression of *Homeobox* genes (*Hox*) through acetylation of core histone and trimethylation of H3K4[51]. Hsp90 might be an essential cooperator for the maintenance of active chromatin at sites of gene expression by TrxG[52]. Recently, it was found that some members of TrxG such as SWI/SNF and MLL1 might also play an important role in the maintenance of activation at the *Ink4* locus[36,53,54]. It is likely that TrxG and PcG proteins cooperate with each other for regulation balance of both *Hox* and *Ink4* genes during embryo development and carcinogenesis. Knockout of *Mll* gene only resulted in defective maintenance of *Hoxa7* expression in $Mll^{-/-}$ embryos of mouse[55]. But the *Hox* gene was still accessible for transcription factors. Thus, downregulation of *TrxG* expression is likely not necessary for the initiation of methylation of CpG islands.

SET-containing proteins such as members of TrxG and PcG proteins in PRC2 contain motifs near SET region that tightly bind to single-stranded DNA (ssDNA). Such binding withstands nucleosome assembly *in vitro*, interfering with the formation of regular nucleosomal arrays[56]. The binding of ssDNA might play a role in the initial recruitment of TrxG proteins to target genes in sustaining DNA stretches in a single-stranded configuration to allow for continuous transcription or silence (Figure 4).

Taken together, TrxG-containing transcription complex may play a role in maintaining expression and preventing repression of target genes including *Ink4a-Arf-Ink4b* through binding to target ssDNA or *ncRNAa* sequence and trimethylation of H3K4 and acetylation of core histones. Gene repression pressure within cells recruits EZH2-containing PRC2 to the transcription complex with the guide of *ncRNAi*, then trimethylates H3K27 that leads to the initiation of repression. The PRC2-related repression is reversed under condition of transcription pressure. However, in the case of long-term silence, PRC1 is recruited to the PRC2-target genes and trimethylation of H3K9 subsequently, which initiates DNA methylation and chromatin remodeling with the assistance of other silencers including DNMTs, HDACs, and hetero-chromatin binding protein HP1 (Figure 4).

Maintenance of the Methylation of CpG Islands under Transcription Pressure

In cancer cell lines, status of aberrant methylation of CpG islands is very stable. It was reported that re-methylation of *Ink4a* CpG island happened gradually in the 5'-aza-deoxycytidine (5-Aza-CdR)-treated T24 bladder carcinoma cells ten days after the removal of 5-Aza-CdR in the medium[57]. One of the possible pathways for the re-methylation may relate to trimethylation of H3K9, the inactive chromatin marker near TSS, which is not changed during the treatment. It was also reported that the knockout of both *DNMT1* and *DNMT3b* would result in re-activation of transcription and de-methylation of CpG island and loss of H3K9me3 of the *Ink4a* locus in HCT116 cell line[58]. However, methylation of H3K9 at the *Ink4a* promoter was recovered at passages 22, and then re-methylation of the CpG island at passages 87. These phenomenons imply that it may be the maintaining of silence pressure of target genes within a cell, but not the maintaining methylation of CpG islands by DNMT1, that could contribute to the epigenetic maintenance of methylation of CpG islands.

To confirm above hypothesis, we used a cell fusion model to investigate whether expression pressure of a gene in a cell line would induce de-methylation of CpG islands (Li and Deng, unpublished data). Gastric cancer cell line AGS with *Ink4a* methylation was fused with another gastric cancer cell line MGC803 without *Ink4a* methylation. We observed that $P16^{Ink4a}$ was expressed in each of the fused cells. Interestingly, proportion of the methylated *Ink4a* alleles in the sub-tetraploid fused cell subclones was decreased very slightly, although clones with de-methylation of CpGs could be observed. Moreover, for the *Bikunin* gene, which is methylated in MGC803 cells and unmethylated in AGS cells, the proportion of methylated *Bikunin* in the fused cells was also decreased little. *Bikunin* mRNA was detected in the fused cells. Bisulfite-clone sequencing of *Ink4a* and *Bikunin* showed that there were de-methylated CpG sites within some of the original methylated CpG islands. These results suggest that the expression pressure of target genes would promote de-methylation of the formerly methylated CpG islands weakly. Altogether, we suggest that transcription/silencing pressure itself might play weak roles in passing the methylation status of CpG islands during cell division. That most of methylated copies of *Ink4a* and *Bikunin* are persistent in the fused cell after passages 60 indicates that the maintaining methylation by DNMT1 and related repressor may play a more important role in the maintenance of methylation of CpG islands.

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