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Epigenetic gene silencing in acute promyelocytic leukemia

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Abstract

The recent explosion in our knowledge of how chromatin organization modulates gene transcription has highlighted the importance of epigenetic mechanisms in the initiation and progression of human cancer. These epigenetic changes—in particular, aberrant promoter hypermethylation that is associated with inappropriate gene silencing—affect virtually every step in tumor progression. Intriguingly, methylation patterns are severely altered in tumors, with an overall hypomethylation of the genome and hypermethylation of islands of CpGs clusters within specific DNA regions. Though overexpression of DNA methyltransferases (DNMTs) has been proposed to be a mechanism for aberrant genome methylation, it does not explain the specific regional hypermethylation in cancer cells. We have analyzed the role of chromatin modifying activities in cell transformation using acute promyelocytic leukemia as a model system. This disease is caused by expression of the PML-RARα fusion protein, thus offering the opportunity of studying the mechanisms of leukemogenesis through molecular investigation of the activity of the directly transforming protein. Recent evidence suggests that PML-RARα as well as other leukemia-associated fusion proteins induce changes in the chromatin structure. Specifically, aberrant recruitment of different chromatin modifying enzymes to specific promoters induces DNA hypermethylation and heterochromatin formation, which consequentially leads to the transcriptional silencing of that genes. Importantly, these epigenetic modifications were found to contribute to the leukemogenic potential of PML-RARα. These observations suggest that epigenetic alterations could actively contribute to the development of APL and other hyperproliferative diseases.

Keywords: Leukemia; PML-RARα; DNA methylation; Epigenetics; Chromatin structure; Methylated DNA binding proteins

1. Introduction

Epigenetics is defined as "*the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence*" [1]. Although the presence of epigenetic alteration in cancer was already observed more than 20 years ago, the underlying molecular mechanisms remained elusive and have just started to be understood.

The major epigenetic changes that take place during the development of cancer are the aberrant DNA methylation of genes that suppress tumorigenesis, and histone modifications of nucleosomes [2]. The nucleosome, the basic unit of chromatin, consists of an octamer composed of two copies each of the H2A, H2B, H3 and H4 core histone proteins, with 147 bp of superhelical DNA wrapped around in 1.65 left-handed turns [3]. Epigenetic alterations are all thought to involve modifications of chromatin through modifications of nucleosomes. Some of the most intriguing questions in this field is whether those alterations really drive tumorigenesis or are rather simply an epiphenomena of the cancer state; whether promoter hypermethylation is the cause or simply a consequence of gene silencing [4].

As discussed in this article, some important information have been obtained from the study of genes silenced by the

Abbreviations: APL, acute promyelocytic leukemias; DNMT, DNA methyltransferase; HDAC, histone deacetylase; MBDs, methyl-binding proteins; NCoR, nuclear corepressor; PML gene, promyelocytic leukemia; RAR, retinoic acid receptor; TRD, transcriptional repression domain; 5-Aza-dC, 5-aza-2′-deoxycytidine

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oncogenic protein PML-RARα, providing us with the first clues about the role of aberrant DNA methylation in cancer development.

2. Mammalian DNA methylation system

In mammals, approximately 1% of DNA bases are modified by the addition of a methyl group to the cytosines. This modification, which occurs within CpG dinucleotides, is an important regulatory mechanism of gene expression. CpGs are distributed unevenly and, overall, are underrepresented in the genome. Clusters of hypomethylated CpGs (termed CpG islands) are usually found in the promoter regions of housekeeping genes as well as in genes regulated in a tissue-specific or developmental manner with a frequency of about 60% of genes that are transcribed by RNA polymerase II [5,6].

Cancer cells present a global hypomethylation of the genome, relative to normal cells, which is accompanied by hypermethylation of CpG islands within specific DNA regions, including gene promoters and coding sequences [7,8]. Promoter hypermethylation is a frequent mechanism of inactivating tumor suppressor genes, and methylated CpGs within coding regions are hotspots for the acquisition of somatic mutations [9]. Both events may play a crucial role in the development as well as in the maintenance of a transformed phenotype [10].

2.1. DNA methyltransferases

Methylation patterns are transmitted to the next generation during cell division. DNA methyltransferases (DNMTs) transfer the methyl group that is provided by *S*-adenosylmethionine to the 5′-carbon of the cytosine ring to form methyl cytosine [11,12]. There are three known biologically active DNMTs in mammalian cells: DNMT1, DNMT3a, and DNMT3b (Fig. 1). Each of these proteins is essential for embryonic development; deficiency in any of the corresponding genes in mice causes embryonic or early postnatal death [13–15]. Furthermore, murine cells lacking DNMTs display genomic instability [16], implying that methylation has wide-ranging effects on global genome function and integrity.

DNMT1 is the most abundant methyltransferase in somatic cells, and it localizes to replication foci [17] and interacts with the proliferating cell nuclear antigen (PCNA) [18]. It is often referred to as the ‘‘maintenance’’ methyltransferase because it is believed to be the enzyme responsible for copying methylation patterns after DNA

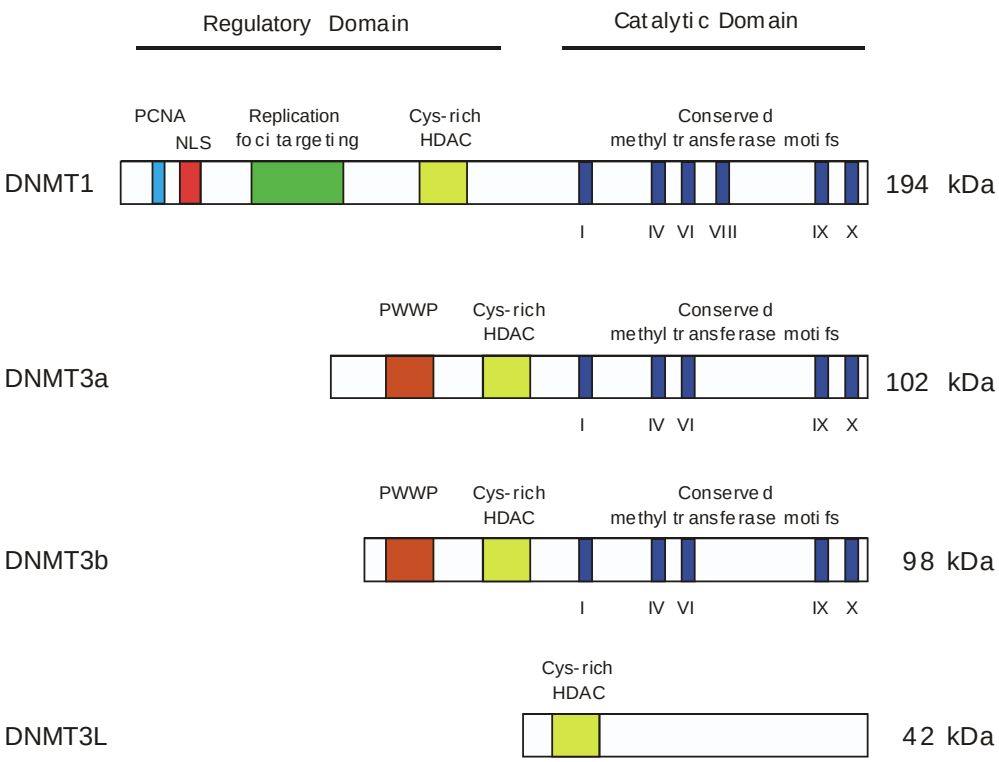

Fig. 1. Mammalian DNMT family members. Schematic structure of catalytically active DNMTs and DNMT-like protein. The catalytic domains of DNMT1, DNMT3a and DNMT3b are conserved, but there is little similarity between their N-terminal regulatory domains. The domain that interacts with proliferating cell nuclear antigen (PCNA) is depicted as a cyan box; the nuclear localization signal (NLS) as a red box; the domain responsible for the targeting to the replication foci, as a green box; plant homeodomain motif/cystidine-rich domain, and the histone deacetylase (HDAC) interaction domain, as yellow boxes; and the conserved proline and tryptophan domains (PWWP), as brown boxes. DNMT3L lacks the conserved catalytic domain but is otherwise closely related to the C-terminal DNMT3 domain.

replication. Indeed, DNMT1 has a 10–40-fold preference for hemimethylated (i.e. methylated on one strand of DNA) versus unmethylated DNA. The catalytic domain at the C-terminus of DNMT1 is a shared feature of the family with high homology among all three DNA methylating enzymes. The N-terminal domain is unique and is considered to be the regulatory domain, and it interacts with several other proteins [11].

The DNMT3a and DNMT3b are the de novo methylating enzymes [19]. These proteins use both unmethylated and hemimethylated DNA as their template. It is not known what controls the specificity of these enzymes, but one could imagine that they could contribute to aberrant gene silencing by methylation if targeted to the wrong CpG sequence. Noteworthy, mutations in the carboxy-terminal catalytic domain of the human DNMT3b are associated with ICF (immunodeficiency, centromeric instability, and facial anomalies) syndrome [20]. More recently, an alternative form of DNMT3 has been identified, termed DNMT3L, which acts as cofactor for both DNMT3a and DNMT3b [21,22].

2.2. Methyl-CpG binding proteins

The first methyl-CpG binding protein, known as MeCP1, was identified in nuclear extracts through its ability to bind a methylated DNA probe containing multiple methylated CpGs [23]. Later, a second protein, MeCP2, was detected due to its ability to bind a single methylated CpG [24]. MeCP2 can be divided into two structural domains: the methyl-CpG binding domain (MBD), which recognizes a symmetrically methylated-CpG dinucleotide through contacts in the major groove of the double helix, and the so-called transcriptional repression domain (TRD), which interacts with several regulatory proteins [25]. Elucidating the properties of MeCP2 has been central to understanding the mechanisms of DNA methylation-dependent silencing. The recent discovery that its gene is mutated in individuals with Rett syndrome has placed even more interest in studying MeCP2 [26].

The search for other MBD-like domains revealed another four proteins, named MBD1, MBD2, MBD3 and MBD4 (Fig. 2), which now create the MBD family [27].

MBD2 and MBD3 form a complex with the multisubunit Mi-2/NuRD complex, which contains an ATP-dependent chromatin-remodelling protein, Mi-2, as well as other components, such as RbAp46/p48, MTA-1, and HDAC1/2. The MBD2/3–NuRD complex is not only involved in repressing methylated genes but it can also remodel methylated chromatin. The MBD2/3–NuRD complex as well as another consisting of MeCP2–Sin3a–HDAC provide a mechanistic link between DNA hypermethylation and histone deacetylation in transcriptional repression [28,29].

In addition to its function within the NuRD complex, MBD2 has been proposed to possess intrinsic DNA demethylating activity [30]. Although this putative enzymatic activity of MBD2 is still matter of debate, recent results suggest that mammalian cells might indeed possess

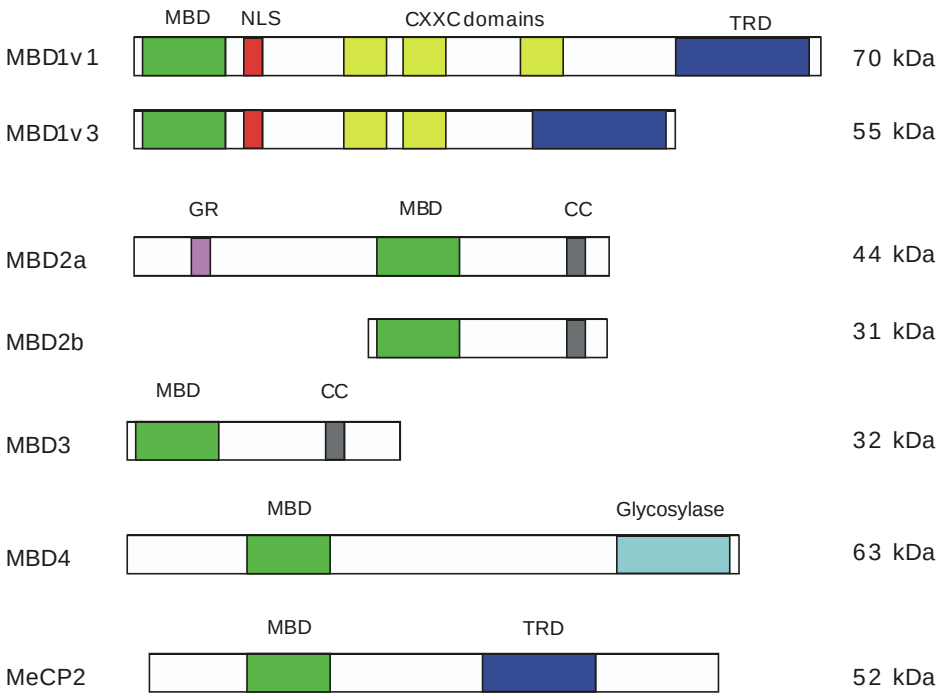

Fig. 2. Mammalian methyl-CpG binding protein family. The highly conserved methyl-CpG binding domain (MBD) is depicted as a green box; the transcriptional repression domain (TRD), as a blue box; the nuclear localization signal (NLS), as red boxes; the CXXC motifs of MBD1, as yellow boxes; and the glycine–arginine repeats (GR), as a purple box. MBD4 possesses a thymine glycosylase activity. The coiled coil (CC) domain, depicted as gray boxes, may contribute to the protein–protein interaction.

a mechanism to demethylate DNA. Thus DNA methylation could be considered as a reversible reaction that regulates transcription similar to acetylation and phosphorylation of histone tails [31].

MBD4 is unique among MBD proteins since it does not only contain a MBD but also glycosylase domain [32], thus suggesting a possible role in DNA repair. Although MBD4 is capable of binding to methyl-CpG sites, it has a higher affinity for mCpG/TpG mismatches, which arise from spontaneous deamination of 5-methylcytosine to thymine. Both in vitro and in vivo studies have conclusively confirmed that not only MBD4 has DNA *N*-glycosylase activity at G-T mismatches but it also suppresses CpG mutability and tumorigenesis in animal models [33].

An exception of MBD proteins listed above is Kaiso, member of the BTB/POZ (Broad complex, Tramtrak, Bric a brac/Pox virus, and zinc finger) family of transcription factors. Kaiso was identified in a yeast two-hybrid screen as a human transcription factor that interacts with p120 catenin [34]. Despite the absence of MBD motif, Kaiso binds to symmetrically methylated CpGs and represses transcription via direct interaction with the nuclear corepressor (NCoR) complex [35].

2.3. Histone tails modifications and DNA methylation

The correlation between CpG methylation and transcriptional silencing in vertebrates has been recognized for the past 20 years. Nevertheless, definitive biochemical evidence connecting the two has only recently been obtained [36,37]. It has been recently shown that the binding of MBD proteins to methylated CpG is also responsible for the recruitment of silencing complexes [6]. MeCP2, for instance, builds complexes with corepressors that include histone deacetylases (HDACs). This is supported by the observation that the HDAC inhibitor trichostatin A (TSA) partially relieves transcriptional inactivation in hypermethylated genes. The interactions of DNMTs, MBDs and HDACs provide a synergistic connection of DNA methylation and histone deacetylation in transcriptional repression [6,38].

Recently, methylation on lysine 9 and 27 of histone H3 tail has been demonstrated to be implicated in transcriptional control and chromatin organization [9,39]. The enzymes responsible for those modifications—histone methyltransferases (HMTs)—have now been identified and characterized [40]. A link between DNA methylation, histone deacetylation and histone methylation in regulating appropriate transcriptional repression is emerging. For example, it has been reported that MeCP2 associates with H3-K9-specific HMT activity [41]. These observations collectively link histone H3-K9 and DNA methylation to silencing of specific genes, and support the idea that histone modifications and DNA methylation cooperate to establish long-term states of transcriptional repression [42,43].

3. The "methylation paradox" of cancer cells

Cancer cells present a global hypomethylation of the genome, relative to normal cells, which is accompanied by hypermethylation of CpG islands within specific DNA regions, including gene promoters and coding sequences [7]. Tumor suppressor genes are often found transcriptionally inactive due to hypermethylation of their promoters in cancer cells. Moreover, methylated CpGs within coding regions are hotspots for somatic mutations (Fig. 3). The biological significance of DNA hypomethylation in cancer is less clear. Early experiments suggest that inhibition of DNMTs activity has an oncogenic effect both in vitro and in vivo [44,45]. More recently, Jaenisch and coworkers demonstrated that DNA hypomethylation cause genome instability and high frequency of lymphomas in mice harboring hypomorphic DNMT1 mutations [46]. Surprisingly, previous results from the same group indicate just the opposite, namely DNMT1 hypomorphic mutation reduces the frequency of intestinal neoplasia when crossed to Apc^{Min} mice [47]. These data together indicate that a disruption in the balance of DNA methylation is associated with cancer risk, and the overall epigenetic equilibrium—rather than hypomethylation or hypermethylation per se—must be preserved in the cells.

Thus, both events can contribute to initiation and/or the progression of cancer. The molecular basis underlying altered DNA methylation patterns in cancer cells is still unknown.

Overexpression of DNMTs has been proposed as a putative mechanism for aberrant genome methylation. However, whether the expression of DNMTs is indeed increased in tumors is still not clear [48]. DNMT overexpression, however, does not explain the specific regional hypermethylation observed in cancer cells, or the global hypomethylation of the genome. Additionally, despite the large body of evidence that changes occur in DNA methylation in cancer cells, the fundamental contribution of DNA methylation to tumorigenesis is still uncertain. Our recent results (see below) suggest a scenario in which oncogenic transcription factors induce epigenetic modifications, including DNA methylation, by recruitment of DNMTs to specific regulative regions, and in which these epigenetic alterations directly contribute to cancer progression.

4. The oncogenic transcription factor PML-RARα

Acute promyelocytic leukemias (APLs) are phenotypically characterized by the accumulation of clonal hematopoietic precursors blocked at the stage of promyelocytic cells [49]. Genetically, they are consistently associated with chromosomal translocations, resulting in the generation of chimeric genes and fusion proteins [50]. Ectopic expression of fusion proteins induces a differentiation

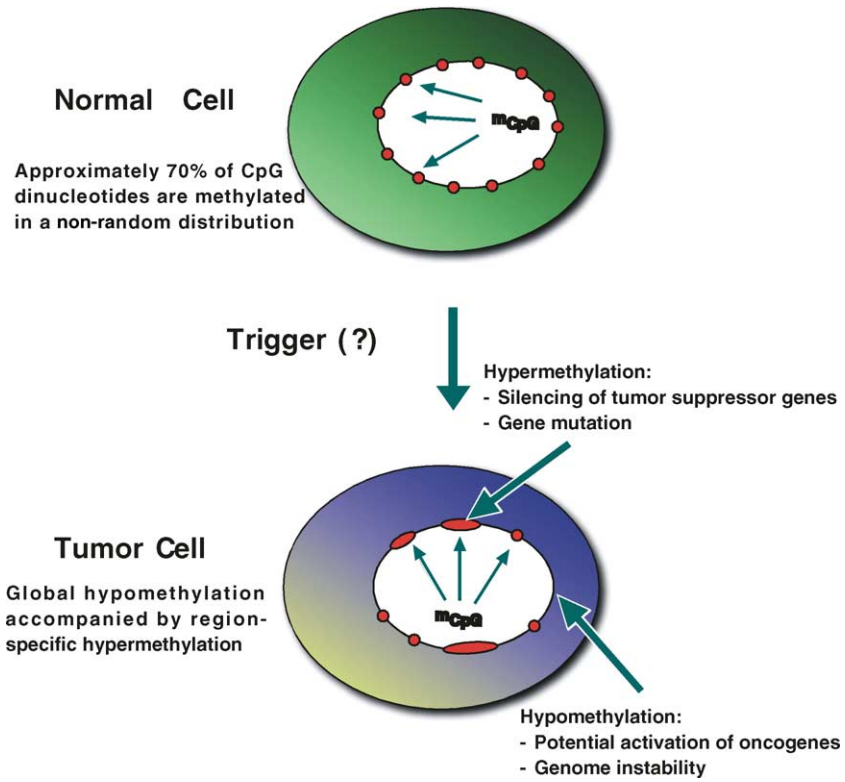

Fig. 3. The ''methylation paradox'' of cancer cells. Cancer cells present loss of methylation at genomic loci that are normally methylated (hypomethylation), concomitantly with a gain of methylated streches at regions that are usually unmethylated (hypermethylation). Hypomethylation may cause genome instability and expression of oncogenes, while hypermethylation may cause gene mutation and silencing of oncosuppressor genes. The event(s) that trigger this have not yet been identified.

block of hematopoietic precursors in vitro, and leukemia in animal models [51].

One of the two genes involved in each APL-associated translocation almost invariably encodes for a transcription factor that is physiologically involved in hematopoietic differentiation. According to the current model of leukemogenesis, a block in the differentiation process is a direct consequence of altered transcriptional properties of these chimeric transcription factors.

The PML-RARα fusion protein, found in the majority of human acute promyelocytic leukemias [52], arises from t(15;17) balanced reciprocal chromosomal translocation. It involves the PML gene and the gene for the retinoic acid receptor α [51].

4.1. Interaction with HDACs

Aberrant recruitment of the nuclear corepressor (NCoR) and the HDAC complex is crucial to the activation of the leukemogenic potential of some of the transcription factors involved in the fusion proteins, thus providing the molecular basis for a common pathogenetic mechanism in APLs. Histone acetylation levels influence chromatin structure in a manner tightly linked to transcriptional activity: high levels of histone acetylation are observed at the promoters of transcribed genes, whereas hypoacetylation has been correlated to silenced genes. It is expected, therefore, that modification of the chromatin structure at the target promoters of the fusion proteins represents one important mechanism of leukemogenesis.

PML-RARα, like the wild type form of retinoic acid (RA) receptor (RAR), represses transcription of target genes through binding to specific DNA sequences (so called RA responsive elements, or RARE) and subsequently recruiting of corepressors such as the NCoR–HDAC complex [53,54]. In case of the wild-type RARα, RA triggers dissociation of the NCoR–HDAC complex and recruitment of several coactivators endowed with histone acetylase activity, thus leading to transcriptional activation (and differentiation of normal hematopoietic precursors). In contrast, the fusion protein PML-RARα is rendered insensitive to physiological concentrations of RA that would usually trigger transcriptional activation. Due to its oligomerization state [55], PML-RARα forms stable complexes with NCoR–HDAC corepressors and functions as a constitutive and potent transcriptional repressor of RARE-containing promoters.

4.2. Interaction with DNMTs

Our group has shown that the transforming protein PML-RARα alters transcription not only through HDAC, but also via interactions with DNMTs [56]. In hematopoietic cells, PML-RARα coordinates the action of both

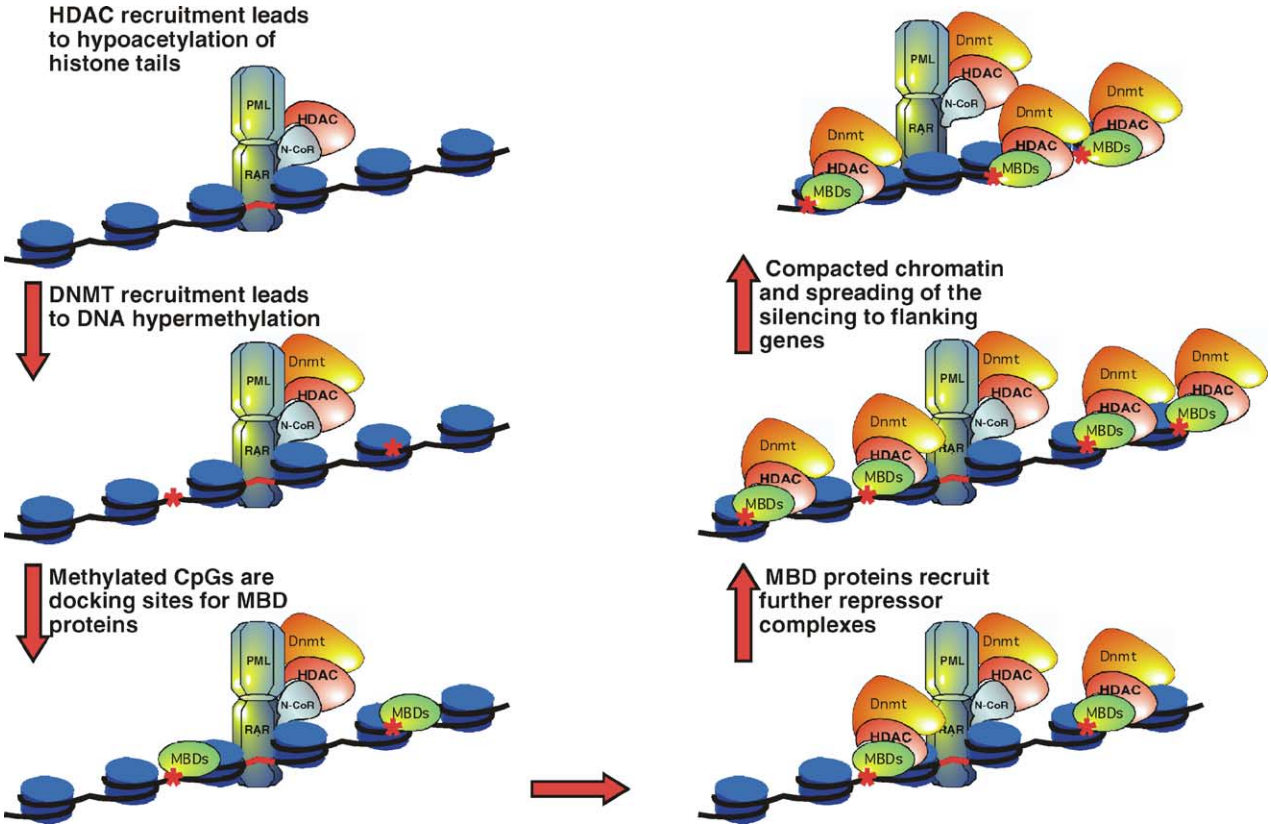

Fig. 4. PML-RARα mediated gene repression. Schematic representation of the step-wise silencing of PML-RARα target genes. The oncoprotein recognizes a well-defined DNA sequence (depicted in red, [53]) and recruits repressor enzymes, such as HDACs and DNMTs, the activity of which leads to hypoacetylation of histone tails, DNA methylation, and transcriptional silencing. Methylated CpGs are potential docking sites for MBD proteins, which can in turn recruit further repressor enzymes. The progression wave of the proposed hypothetical mechanism might "close" the chromatin structure and could even influence neighboring genes.

classes of enzymes, leading to intensive histone deacetylation and DNA hypermethylation at target promoters. Specifically, induction of PML-RARα resulted in increased CpG methylation in the 5′ region of the retinoic acid receptor RARß2, which is a direct target gene of PML-RARα and important for hematopoietic differentiation. Physical interactions between PML-RARα and both DNMT1 and DNMT3a were detected by coimmunoprecipitation experiments using an inducible PML-RARα expression cell line. The recruitment of DNMT1 and DNMT3a to the RARß2 promoter was critically dependent on the presence of PML-RARα. Moreover, DNMTs and PML-RARα were found to colocalize within intact nuclei. The interaction and colocalization of both proteins, as well as the concomitant methylation and repression of the RARβ gene, were also confirmed in cells isolated from APL patients. Once established, the PML-RARα-induced epigenetic modifications and the resulting gene repression are stable and maintained throughout cell divisions, even in the absence of the oncogene. These observations support not only an active role of de novo DNA methylation in the process of carcinogenesis, but also a role of established methylation patterns in the maintenance of the transformed phenotype. Furthermore, pharmacological doses of RA and the DNMT inhibitor 5-Aza-dC synergistically reduced methylation at the RARß2 locus and reactivated RARß2 expression in both a APL-derived cell line as well as in cells isolated from APL patients.

We have expanded the analysis of the PML-RARα association with DNMTs to further explore the relationship between DNA methylation, transcriptional repression and biological activity of the fusion protein. Among the critical players involved in induced gene repression, we have identified the MBD class of transcription cofactors to be important for PML-RARα actions. MBD proteins are found associated with PML-RARα target genes upon DNA methylation, and their presence is required for maximal and stable transcriptional repression. Experiments are in progress to establish whether this association is required for the leukemogenic potential of the fusion protein (Fig. 4).

5. Conclusions

An accumulating set of data points towards alterations of the cellular epigenome as a fundamental aspect of tumor development [57]. The paradigmatic PML-RARα model system shows that interactions among DNMTs, MBDs, and HDACs could cooperate to ensure transcriptional

repression of putative tumor suppressor genes or of genes involved in cell differentiation via direct targeting. A stochastic mechanism for gene repression with subsequent selection for cells having growth advantage has also been proposed [58]. In this scenario, histone methylation initiates, and DNA methylation maintains the transcriptional silencing of tumor suppressor genes.

Modifications of the chromatin structure, such as loss of DNaseI hypersensitive sites, also occur at these loci, which indicates that the nucleosome adopts a closed conformation, and thus adding a further layer of repression [59,60]. In this regard, it will be interesting to investigate a potential involvement of the polycomb repressor complexes in this process, and analyze in general whether heterochromatinization can occur in an otherwise transcriptionally active region of the chromatin. This hypothesis predicts that a consequence of the initial repression would be spreading of inactivation into flanking regions, unless boundary elements maintain and protect active domains from this process (Fig. 4) [61].

Since inhibition in vitro of DNMT and HDAC enzymes involved in PML-RARα-dependent gene repression was able to block cell growth and to revert the tumor phenotype, the improvement and further development of small compound inhibitors such as TSA and 5-Aza-dC represent a future hope for the therapies of APL and other hyperproliferative disorders [62].

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