Raffaella Villa

Center For Genomic Regulation (CRG), c/Dr Aiguader 88,
08003 Barcelona, Spain, Phone:+34-93-3160135
email: raffaella.villa@crg.es

Universitat Pompeu Fabra
Facultad de Ciencias de la Salud y de la Vida

Role of epigenetic modifications in Acute Promyelocytic Leukemia

Raffaella Villa

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Genómica (CRG) de Barcelona

Dr. Luciano Di Croce

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SUMMARY

Acute promyelocytic leukemia (APL) is defined as a malignant, marrow-based neoplasm of hematopoietic cells, characterized by the accumulation of immature granulocytes at the promyelocytic stage of differentiation and often featuring fusion proteins generated by chromosomal translocations. The fusion protein PML-RARa, originated from a chromosomal translocation that involves the gene PML on chromosome 15 and the retinoic acid receptor alpha (RARa) on chromosome 17, is the molecular cause for 98% of all APL cases. The ability of PML-RARa to block hematopoietic differentiation and to induce leukemia is based on aberrant gene repression. Mechanistically, this is achieved through recruitment of histone deacetylase and DNA methyltransferase enzymes to several target promoters.

The aim of this PhD thesis was to investigate how epigenetic mechanisms participate in PML-RARa-mediated gene silencing and contribute to its pathological potential. In particular, we characterized the role of DNA methylation by studying a family of proteins able to recognize and specifically bind to methylated CpG dinucleotides (methyl-CpG-binding proteins). Furthermore, we extended the study to an additional epigenetic layer, histone methylation.

In the first part, we show that MBD1 is a critical interpreter of PML-RARa-induced promoter hypermethylation. In fact, we demonstrate that MBD1, initially recruited by PML-RARa, through an HDAC3-mediated mechanism, to its target promoters, is then able to spreads along the methylated locus, thus playing a pivotal role in transcriptional repression and leukemia progression. In the second part, we provide evidence for the involvement of Polycomb-mediated H3K27 methylation in APL leukemogenesis. Indeed, we show that PML-RARa recruits the Polycomb repressive complex 2 (PRC2) to tumor suppressor loci leading to H3K27 methylation and subsequent gene silencing. Furthermore, depletion of PRC2 activity in a human APL derived cell line (NB4), leads to the reactivation of several PML-RARa target genes, thus facilitating cellular differentiation. At last, we investigate how the crosstalk among the different epigenetic layers contributes to the molecular pathology of

leukemia, demonstrating that both DNA and histone methylation are required to maintain PML-RARa aberrant gene silencing, and that, importantly, the impairment of one of the two epigenetic mechanisms affects the other as well. Overall, this work represents an important advance in our understanding of the early molecular mechanisms involved in the progression of APL, and thus, opens new possibilities for therapeutic treatments.

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INTRODUCTION

1. Chromatin structure

In eukaryotic cells DNA and associated proteins are organized into a three dimensional structure called "chromatin". The fundamental unit of chromatin is the nucleosome that is composed of an octamer of core histones (two molecules of each H3, H4, H2A, H2B,) around which 147 base pairs of DNA are wrapped (Fig. 1). In addition the histone H1 binds to the *linker DNA* region that connects each nucleosome thereby favoring the stabilization of the 30nm chromatin fiber (Fig. 1). The 30 nm fiber in turn is folded into ternary structures to achieve the maximum degree of compaction charcacteristical of metaphase chromosomes (Felsenfeld and Groudine, 2003).

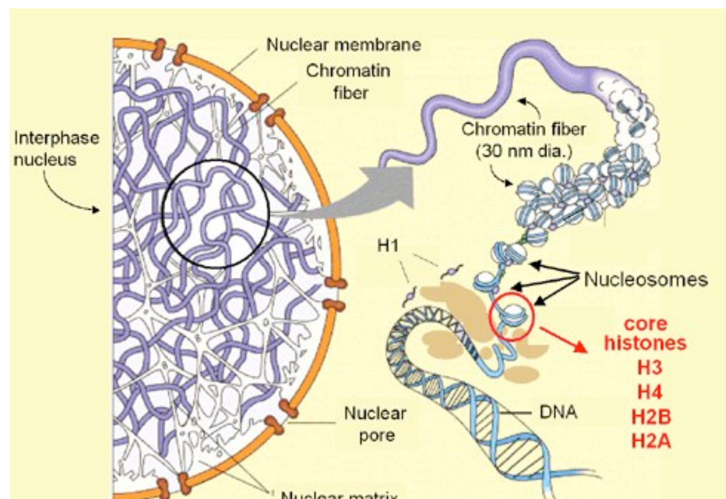

Figure 1. Chromatin structure. Schematic representation of chromatin structure. (Allis web page)

The structure of chromatin is dynamic and correlates with gene expression. In fact, the "open" vs. "closed" chromatin conformations determine access of regulatory factors to certain DNA sequences, which is essential for the regulation of transcription. Chromatin structure is mainly modulated by two mechanisms: sliding of nucleosomes catalyzed by chromatin remodeling complexes and covalent modifications of DNA and/or histones. Functionally, chromatin can be divided into euchromatin and heterochromatin. Euchromatin

contains genes that are actively transcribed and is decondensed during interphase. Conversely, heterochromatin is transcriptionally inactive and highly condensed. Heterochromatin can be further subdivided into constitutive (centromeric and telomeric repeats) and facultative (regions subjected to a developmentally regulated transcriptional silencing), (see below).

1.1. Epigenetics

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*" (Wolffe and Matzke, 1999). In the last years it became increasingly clear that to fully understand the genetic regulation of cells and their fate it is important to take into account not only the information contained in the primary DNA sequence, but also the information stored and generated by chromatin modifications. Epigenetic modifications are constituted, principally, by DNA methylation and histone covalent marks. Also non-covalent mechanisms, such as chromatin remodeling and topological replacement of histone variants have been considered to participate in epigenetic mechanisms. In fact, ATP-dependent chromatin remodeling complexes can modify chromatin structure by altering histone-DNA interactions and histone variants are exchanged in specific chromatin contexts (i.e. H2AX in DNA repair, CENP-A during mitosis, H3.3 in euchromatic active genes) (Gregory and Shiekhattar, 2004). More recently, also RNA (in particular noncoding RNA) has been implicated in epigenetic mechanisms. Indeed, repeat-associated siRNA, *Xist* RNA and small RNAs in *S. Pombe* have been shown to induce mitotically heritable gene silencing (Bernstein and Allis, 2005). Data of the last few years suggested that a crosstalk between different epigenetic mechanisms exists. The best example is the silencing of X-chromosomal gene-loci where *Xist* RNA, histone modifications and DNA methylation are all playing an important role. Furthermore numerous interactions between enzymes that mediate epigenetic processes have been recently identified. For example DNA methyltransferases (DNMTs) can interact with histone deacetylases (HDACs)

and/or histone methyltransferases (HMTs) (Bachman et al., 2003; Fuks et al., 2000; Fuks et al., 2001).

2. DNA methylation

DNA methylation is the best characterized covalent modification of chromatin and constitutes a stable and heritable epigenetic mark. In eukaryotes, DNA methylation involves mostly cytosine bases in the context of a CpG dinucleotide and is associated with a repressive state of chromatin (Bird and Wolffe, 1999). This dinucleotide tends to cluster in regions called CpG islands (Bird, 2002) which are found in promoters and in the 5' end of many genes (nearly 60% of all genes) and are unmethylated in normal cells. Hypermethylation of CpG-islands in certain promoters has been connected to tumorigenesis (Jones and Baylin, 2002) (see below). The components of the DNA methylation system in mammals can be classified into *de novo* and maintenance methyltransferases depending on the substrates (Klose and Bird, 2006). DNMT3A and DNMT3B preferentially methylate unmethylated CpGs (*de novo*), while DNMT1 introduces a methyl mark onto the newly synthesized strand during DNA replication (maintenance). DNMT1 was the first enzyme of the family to be identified in mammals (Bestor et al., 1988) and it was found associated with replication foci during S phase (Liu et al., 1998). Another evidence that this enzyme is responsible for maintaining and propagating epigenetic marks through cell division came from the use of embryonic stem cells (ES cells) derived from Dnmt1 knockout mice. Although these cells maintain just 30% of normal methylation, they are capable of methylating inserted retroviral DNA, demonstrating the existence of other enzymes able to methylate DNA (Lei et al., 1996; Okano et al., 1998). DNMT3A and DNMT3B were identified in 1998 due to homologies with the DNMT1 catalytic domain (Okano et al., 1998). The two family members seem to be essential for mouse development and knockout mice deficient either for Dnmt3a or Dnmt3b are not viable (Okano et al., 1999). They display differential tissue localization, but

their functions are partially redundant as ES cells mutated either in Dnmt3a or in Dnmt3b, still maintain the ability to *de novo* methylate DNA (Okano et al., 1999; Okano et al., 1998). During development, however, they show non-overlapping functions and DNMT3B is particularly required for the *de novo* methylation of pericentromeric repetitive DNA sequences. Mutations in DNMT3B, in fact, are correlated with a disease known as immunodeficiency, centromeric instability and facial anomalies syndrome (ICF). There are two other enzymes belonging to the DNMT family: DNMT2 and DNMT3L. DNMT2 has DNA methyltransferase activity *in vitro* but no effects on DNA methylation *in vivo* (Herman and Baylin, 2003; Okano et al., 1998). DNMT3L is related to the other DNMTs, but does not contain catalytic activity. It was recently demonstrated that in cells derived from the germline, DNMT3L, recognizes the unmethylated lysine 4 of histone 3 and recruits *de novo* methyltransferases, providing a new functional link between different epigenetic mechanisms (Ooi et al., 2007). Other mechanisms that can target the *de novo* methyltransferases to their substrates are the specific recognition of DNA sequences (Ge et al., 2004) or the interaction of the enzymes with transcriptional repressors (Brenner et al., 2005; Di Croce et al., 2002). Most programmed *de novo* methylation occurs at early stages of development and during gametogenesis (Reik et al., 2001). In fact, during these important developmental steps, it seems to be necessary to erase the epigenetic program and re-methylate genes. In germ line cells the methylation reprogramming is crucial for the resetting of imprints, for the removal of acquired epigenetic modifications and to avoid deamination of 5-methylcytosine (see below). In the early mouse embryo, methylation reprogramming could be linked to the first-lineage decisions during mammalian development. How the organism achieves global DNA demethylation still remains elusive since no demethylase enzymes have been identified so far. In the last twenty years, aberrant *de novo* methylation has also been linked to tumorigenesis (see below).

In mammals, DNA methylation has been implicated in several cellular functions including X-chromosome inactivation, genomic imprinting, tissue-

specific gene expression, regulation of chromatin structure and silencing of endoparasitic sequences (Bird, 2002; Gaudet et al., 2003). Indeed, alterations in the pattern of genomic methylation is correlated with several diseases (Egger et al., 2004). Mechanistically, a methylated cytosine can counteract the association of DNA-binding factors with their respective target sequences (Watt and Molloy, 1988) or, alternatively, can constitute an anchor site for a family of proteins able to bind methylated CpGs (methyl-CpG-binding proteins), that mediate transcriptional repression through interactions with co-repressors (Hendrich and Bird, 1998; Jones et al., 1998; Ng et al., 2000; Sarraf and Stancheva, 2004). Furthermore, it was recently shown that DNMTs can interact with chromatin modifying enzymes, demonstrating that they can also have a non-enzymatic role in transcriptional silencing (Bachman et al., 2003; Fuks et al., 2000; Fuks et al., 2001; Geiman et al., 2004).

2.1. DNA methylation and cancer

It has been known for some time that abnormalities of genomic methylation have implications in tumorigenesis. Three mechanisms have been demsonstrated so far: deamination of methylated CpGs, global DNA-hypomethylation and inactivation of tumor suppressor genes due to aberrant hyper-methylation.

C-T transition

Through a spontaneous deamination-reaction, a cytosine can be transformed into a uracil. Since uracil is not a DNA base, the repair machinery can recognize and eliminate it. The situation is different for spontaneous deamination of 5-methylacytosine. In this case the base is converted into a thymine and therefore it becomes more difficult to repair it. One well-studied example is the gene encoding the tumor suppressor p53: this gene is mutated in 50% of solid tumors and 28% of the mutations result from methyl-CpG deamination (Magewu and Jones, 1994).

Hypomethylation

Transformed cells display 20-60% lower cytosine-methylation-levels than normal cells (Lapeyre and Becker, 1979). One of the mechanisms that links genomic hypomethylation to cancer progression is aberrant mitotic recombination leading to chromosomal instability. Furthermore, parasitic sequences that are usually silenced by DNA methylation can become active upon demethylation: loss of methylation was observed in long interspersed nuclear elements (LINEs) and in Alu-elements (Yoder et al., 1997). In addition, genes that normally are silenced can be derepressed upon global DNA demethylation. One example is the oncogene K-ras that is hypomethylated in lung and colon cancer (Feinberg and Vogelstein, 1983).

Silencing of tumor-suppressor genes

DNA hypermethylation is the best characterized epigenetic alteration in cancer. It affects CpG islands at promoter of genes that are unmethylated in normal cells. Examples include hypermethylation of the $p16^{INK4a}$ promoter in several tumors, that keeps cancer cells from entering senescence (Merlo et al., 1995), the hypermethylation of the $p14^{ARF}$-locus leading to MDM2-mediated p53 degradation (Esteller et al., 2000) and hypermethylation of the DNA-repair gene hMLH1 in colorectal cancer (Kane et al., 1997).

2.2. Methyl-CpG-binding proteins

This family of proteins is composed of 5 members: MeCP2, MBD1, MBD2, MBD3 and MBD4, all containing the methyl-CpG-binding domain (MBD) and all capable of silencing gene expression (Fig. 2). More recently, Kaiso was also included into the family of methyl-CpG-binding proteins (Daniel and Reynolds, 1999). Despite the absence of a MBD domain, Kaiso can bind symmetrically methylated DNA through zinc-finger domains and repress transcription trough the POZ/BTB domain (Prokhortchouk et al., 2001) (Fig. 2).

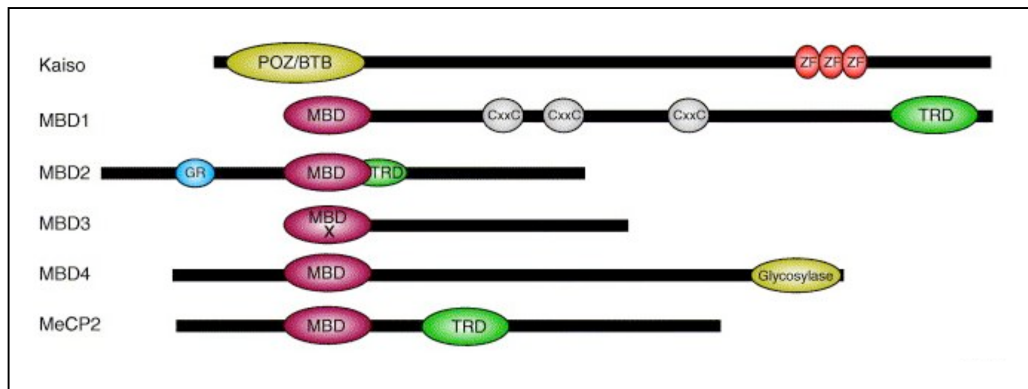

Figure 2. Schematic representation of methyl-CpG-binding proteins. The different functional domains are schematically indicated: POZ/BTB domain, the zinc-finger domain (ZF), methyl binding domain (MBD), cysteine rich domain (CXXC), transcriptional repression domain (TRD), glycine-arginine motif (GR) and glycosilase domain (Glycosilase) (Klose and Bird, 2006).

The MeCP1 complex and MeCP2 were the first proteins of the family to be identified more than a decade ago (Lewis et al., 1992; Meehan et al., 1989). MeCP2 is a heterochromatic protein that contains the MBD domain and a transcriptional repression domain (TRD) (Nan et al., 1997; Nan et al., 1996) (Fig. 2). Through the TRD MeCP2, is able to interact with mSin3A and thus recruits histone deacetylases (HDACs) to repress transcription (Jones et al., 1998; Nan et al., 1998). More recently, it was also demonstrated that MeCP2 interacts with a histone methyltransferase (HMT) activity specific for lysine 9 at histone 3 (Fuks et al., 2003), thereby supporting an interesting correlation between chromatin organization and DNA methylation in transcriptional silencing. MeCP2 is a X-linked gene predominantly expressed in differentiated neurons (Shahbazian et al., 2002). Mutations in MeCP2 are associated with a neuronal disorder (the Rett syndrome) that affects primarily women, with an incidence of 1/10000 to 1/15000 live births (Hagberg, 1985). How mutations in MeCP2 cause the RETT syndrome is still not completely clear. Microarray studies using a mouse model for Rett syndrome indicate that MeCP2 regulates a subset of genes that become upregulated in the absence of a functional MeCP2 (Nuber et al., 2005; Tudor et al., 2002). However, the possibility of novel MeCP2 functions unrelated to transcriptional regulation remains open.

Further bioinformatic investigations for MBD-containing proteins led to the identification of all the other family members (Hendrich and Bird, 1998). X-ray cristallography analysis of the MBD-domain revealed a α/β sandwich structure with the conserved residues Arg22, Tyr27, Arg30, Asp32, Tyr34, Arg44, Ser45 and Lys46 allowing the binding to methylated DNA (Hendrich and Bird, 1998; Ohki et al., 2001) (Fig. 3). Furthermore, the identification of a common intron in the MBD domain of the five proteins pointed towards an evolutionary correlation (Hendrich and Bird, 1998).

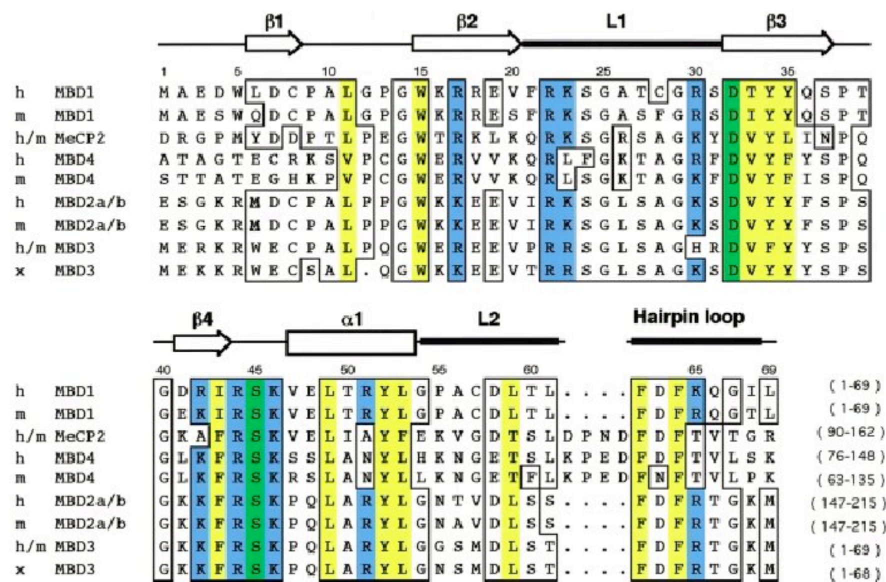

Figure 3. Sequence alignment of the MBD domain. MBD domain of MBD1, MeCP2, MBD4, MBD2a/b from human (h) and mouse (m) and of MBD3 from human (h), mouse (m) and xenopus (x). The secondary structure of MBD1 is indicated at the top. Conserved residues are boxed and colored blue if basic, yellow if hydrophobicand green if acidic or polar (Ohki et al., 1999).

Apart from MBD4 that is a thymine glycosilase that specifically repairs DNA lesions resulting from methyl-CpG deamination (Hendrich et al., 1999; Millar et al., 2002) (see above), the other members of the family achieve gene silencing by interaction with co-repressor complexes (Feng and Zhang, 2001; Jones et al., 1998; Klose and Bird, 2004; Nan et al., 1998; Ng et al., 2000; Sarraf and Stancheva, 2004; Wade et al., 1999; Yoon et al., 2003; Zhang et al., 1999). For example, MBD2 and MBD3 interact with the multisubunit Mi2/NuRD chromatin remodeling complex (Wade et al., 1998; Zhang et al.,

1999). Interestingly, the fact that Mbd3-/- mice die during early embryogenesis, while Mbd2-/- animals are viable, demonstrates that the two proteins are not functionally redundant (Hendrich et al., 2001). In addition, MBD3 is the only family member that does not bind methylated DNA due to changes in crucial amino acids in the MBD domain (Hendrich et al., 2001).

2.2.1. MBD1

MBD1 was the first protein to be identified due to the MBD-homology with MeCP2. MBD1 is unique among the family members in that it contains two or three cysteine rich domains (CXXC) also found in DNMT1 and in the histone methyltransferase mixed-lineage leukemia (MLL) (Fig. 2). There are five isoforms generated by alternative splicing involving the C-terminal region and the CXXC domains, that differ in their nuclear localization and in their functional role (Fujita et al., 1999). Similar to MeCP2, MBD1 also contains a TRD responsible for, at least, part of its repressive activity in a trichostatin A (TSA, a HDACs inhibitor) sensitive manner (Ng et al., 2000). The TRD domain contains 33% of hydrophobic amino acids and point mutations in one of these amino acids cause a loss in the ability to repress transcription (Ng et al., 2000). More recently, MBD1 was also shown to interact with HMT enzymes such as SUV39 (Fujita et al., 2003) and SETDB1 (Sarraf and Stancheva, 2004) and with the Polycomb group of proteins (PcGs) (Sakamoto et al., 2007) providing a new link between histone and DNA methylation. In particular, Sarraf and Stancheva showed that during S phase MBD1 and SETDB1 form a complex with the chromatin assembly factor CAFp150 postulating a role during DNA replication. Furthermore, MBD1 depletion was shown to affect H3K9 methylation as well and to cause reactivation of certain target genes. To better characterize the function of MBD1 *in vivo*, Zhao and coworkers generated Mbd1-deficient mice (mbd1-/-) (Zhao et al., 2003). Despite apparently normal development, further investigations showed that the animals suffered from neurological disorders and genomic instability. Furthermore neuronal stem cells lacking mbd1 show a decrease in neural

differentiation. It still remains to be verified whether the phenotype of mbd1-/- mice reflects the role of MBD1 in chromatin modifications.

3. Histone modifications

Together with DNA methylation, covalent histone modifications are determinants of epigenetic maintenance and control. They have been associated with transcriptional regulation, with some modifications seemingly correlating with a repressive state of chromatin (heterochromatin), while others seem to indicate transcriptionally active chromatin (euchromatin). These modifications can have two mechanistic functions. Chromatin accessibility for DNA-binding proteins, is altered directly by changing electrostatic charge or nucleosomal interactions. On the other hand, epigenetic marks can function as anchoring sites for effector proteins that can regulate transcription. There are at least eight distinct types of modifications of histones: acetylation, lysine methylation, arginine methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination and proline isomerization (Kouzarides, 2007; Lehnertz et al., 2003) (Fig. 4). Most modifications affect the N-terminal and C-terminal histone tails and few of them the globular domains. Lysine is the best characterized residue due to the fact that it can be acetylated, methylated, ubiquitylated and sumoylated.

Chromatin Modifications	Residues Modified	Functions Regulated
Acetylation	**K**-ac	Transcription, Repair, Replication, Condensation
Methylation (lysines)	**K**-me1 **K**-me2 **K**-me3	Transcription, Repair
Methylation (arginines)	**R**-me1 **R**-me2a **R**-me2s	Transcription
Phosphorylation	**S**-ph **T**-ph	Transcription, Repair, Condensation
Ubiquitylation	**K**-ub	Transcription, Repair
Sumoylation	**K**-su	Transcription
ADP ribosylation	**E**-ar	Transcription
Deimination	**R > Cit**	Transcription
Proline Isomerization	**P-cis > P-trans**	Transcription

Figure 4. Covalent modifications of histones. Each modification is shown with the residues that are modified and the associated functions (Kouzarides, 2007).

As mentioned above, different combinations of histone modifications mark different types of chromatin environment (euchromatin *vs* heterochromatin) and can generate synergistic or antagonistic interactions. For example, phosphorylation at serine 10 of histone 3 (H3S10P) favors acetylation at lysine 9 and 14 and inhibits methylation at lysine 9 on the same histone (Zhang and Reinberg, 2001). In summary, the repeated occurrence of certain marks in defined chromatin environments led to the postulation of the so-called "histone code" hypothesis, which states that certain histone modifications can indicate functional chromatin domains providing a putative new layer of genetic regulation (Jenuwein and Allis, 2001). All histone post-translational modifications are removable, e.g. HDACs removes acetyl groups (see below), ubiquitin proteases remove mono-ubiquitin, Ser/Thr phosphatases remove phosphate groups. Recently also two classes of demethylase enzymes have been discovered: the LSD1 class and the jumonji class (see below).

Euchromatin *vs* heterochromatin

Originally, two forms of chromatin have been distinguished cytologically because of their different staining: heterochromatin stains darkly, indicating a tighter packing of chromatin, while euchromatin staining is lighter. Euchromatin contains actively transcribed genes and is characterized by hyperacetylation of histones (Roth et al., 2001). Also H3K4 methylation is associated with active genes: H3K4me1 and H3K4me2 are usually enriched along the coding sequence while H3K4me3 is particularly enriched at the transcriptional start site (TSS) (Barski et al., 2007). Furthermore, methylation at lysine 36 and lysine 79 of histone 3 and monoubiquitination of histone H2B have been recently described as being enriched along transcribed genes and associated with the elongation process of transcription (Shilatifard, 2006). In general, genes in heterochromatic regions are silenced and contain hypoacetylated histones. Constitutive heterochromatin represents a gene poor region and is mostly associated with pericentromeric and centromeric chromatin. The best characterized mark of this chromatin environment is H3K9me3 mediated by the SUV39 enzyme providing an anchoring site for the

heterochromatin-like protein 1 (HP1). This histone modification, in turn, induces DNA methylation leading to a stable and irreversible repression (Lehnertz et al., 2003). Conversely, facultative heterochromatin features genes that become silenced during developmentally regulated processes. An example is the mammalian X-chromosome inactivation or the regulation of Hox genes expression during cellular differentiation. Both processes are regulated by the PcGs that specifically mediate methylation at lysine 27 of histone 3 (see below). Another mark of facultative heterochromatin is G9a-mediated H3K9me2 that was identified during the inactivation of the chicken β-globin locus and during the silencing of Oct3/4 in the differentiation of mouse ES cells (Litt et al., 2001; Roopra et al., 2004; Tachibana et al., 2002).

3.1. Histone acetyltransferases (HATs) and Histone deacetylases (HDACs)

The acetylation of histone tails is always associated with a transcriptional active state of chromatin and is regulated by two families of enzymes with antagonistic activities: HATs that add an acetyl group and HDACs that remove it. It was recently demonstrated that the acetyl group neutralizes the basic charge of the lysine and leads to chromatin unfolding (Shogren-Knaak et al., 2006). In addition, acetylated lysine residues constitute a docking site for bromodomain proteins (Dhalluin et al., 1999). In general, both the HAT and HDAC families of proteins show limitated substrate specificity implying an indirect recruitment through other factors. Additionally, recent data point towards a role for these two enzyme families also in the regulation of acetylation of non-histone proteins. Due to their important function in gene regulation (HATs correlate with gene activation, while HDACs correlate with gene silencing), is not surprising that alterations in their activity have been linked to several human diseases. For example, the histone acetyltransferase p300/CBP was shown to display tumor suppressor features and to be involved in one of the chromosomal translocations responsible for acute myeloid

leukemia (AML) (Chaffanet et al., 2000; Giles et al., 1998; Muraoka et al., 1996; Timmermann et al., 2001). Furthermore, HDAC functions have been associated with several tumors and now represent important targets for cancer therapy (Bolden et al., 2006; Kelly and Gilliland, 2002; Kelly et al., 2002; Marks and Jiang, 2005). HATs can be divided into six main groups, GNAT, MYST, CBP/p300, nuclear receptor co-activators such as SRC-1, ACTR, and TIF, TATA-binding protein-associated factor TAFII250 and its homologues, and subunits of RNA polymerase III general factor TFIIIC (Sterner and Berger, 2000). They all share a conserved core domain, responsible for the binding of the acetyl-CoA factor, that mediates the catalytic activity, but they differ in the N-terminal and C-terminal domains that facilitate substrate-specific activities (Hodawadekar and Marmorstein, 2007). HDACs are a class of enzymes identified due to their ability to remove the acetyl group from an acetyled lysine on histone tails. In the past few years, HDACs have been intensely investigated due to their involvement in cancer progression, leading to the development of inhibitors with promising effects in preclinical models of cancer. This group of enzymes can be further subdivided into three classes depending on their homology with yeast HDAC proteins. Recently bioinformatic analysis identified a new class of HDACs (IV) including proteins similar to human HDAC11 (Gregoretti et al., 2004). HDACs of class I, II and IV share structural homology and the catalytic mechanism that requires a Zn metal ion, while HDACs of class III do not show sequence homology with class I and II HDACs and require NAD^+ as a cofactor.

Class I

HDAC1, HDAC2, HDAC3 and HDAC8 belong to class I and are related to RPD3 from yeast (Hu et al., 2000; Johnson and Turner, 1999). They all have a conserved catalytic domain, but they differ in the C-terminal region (Khochbin and Wolffe, 1997). HDAC1 and HDAC2 are part of multiprotein complexes, such as Sin3/HDAC (Hassig and Schreiber, 1997), NuRD/Mi2 (Xue et al., 1998) and CoREST (Humphrey et al., 2001; You et al., 2001) and their functional differences are still not very clear. However, the observation that

Hdac1-/- mice show embryonic lethality, clearly demonstrates that HDAC2 cannot functionally replace HDAC1 functions (Lagger et al., 2002). Conversely, HDAC3 is functionally distinct from the other members of the class in that its activity is related to the nuclear receptors co-repressor complexes. In fact, HDAC3 was co-purified with the silencing mediator for retinoid and thyroid receptors (SMRT) and with the nuclear receptor co-repressor (NCoR) complexes (Urnov et al., 2000; Wen et al., 2000). Furthermore, it is the only HDAC of class I that can shuttle between the nucleus and the cytoplasm (Takami and Nakayama, 2000). However, it is still not clear whether cytoplasmic HDAC3 is enzymatically active. HDAC3 is implicated in the regulation of several cellular processes such as differentiation, proliferation and apoptosis (Jepsen and Rosenfeld, 2002). Indeed, aberrant expression and localization of this enzyme has been recently connected with carcinogenesis. For example, overexpression of HDAC3 was found in lung cancer cells while, conversely, HDAC3 repression is associated with colon cancer cell lines (Bartling et al., 2005; Wilson et al., 2006). In addition, astrocytic glial tumors display pronounced cytoplasmatic localization of HDAC3 (Liby et al., 2006). All these features indicate HDAC3 as a suitable target for the development of new drugs for cancer therapy.

Class II

This class includes HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10 that were identified due to their homology with the HDA1 protein of yeast (Fischle et al., 1999; Grozinger et al., 1999; Verdel and Khochbin, 1999). The catalytic domain of these proteins is in the C-terminal region. Furthermore HDAC6 is the only protein among HDACs to have two catalytic domains. It is possible that class II HDACs participate with class I members during nuclear-receptor-mediated gene silencing (Huang et al., 2000; Kao et al., 2000), but they are subjected to an additional level of regulation, as their activity seems also to be controlled by nucleocytoplasmic shuttling (Miska et al., 1999; Verdel et al., 2000; Wang et al., 2000).

Class III

Human homologues of the Sir2 NAD^+ dependent deacetylase of yeast (SIRT1-7) belong to this class. Class III HDACs are involved in different functions compared to class I and II and they are still not well characterized. SIRT1 is the most studied and is responsible for the deactylation of H4 and p53 (Michishita et al., 2004; Vaquero et al., 2004). SIRT2, 3 and 4 are localized in the mitochondria and an interesting hypothesis is that they can constitute a connection between metabolic and aging processes (Michishita et al., 2004). Like the yeast Sir protein, SIRT6 and SIRT7 are associated with heterochromatin regions and nucleoli respectively, but fail to deacetylate H4 in vitro (Liszt et al., 2005).

3.2. Histone methyltransferases (HMTs)

All HMTs contain a SET domain that is responsible for their catalytic activity with the exception of DOT1 (the enzyme responsible for H3K79 methylation) (Kouzarides, 2000). Conversely to HATs, HMTs are quite specific enzymes that usually modify one single lysine on a specific histone. Furthermore, methylation is associated either with activation or repression of transcription. Indeed, this modification can have different biological outcomes depending on the residue and on the timing on which it occurs on the same residue (mono-, di- and tri-methylation). For example, trimethylation of lysine 4 at histone 3 (H3K4me3) is associated with activation of transcription, while H3K9me3, usually indicates pericentromeric heterochromatin (see above). Another example is represented by H4K20 that marks active genes when it is monomethylated (Barski et al., 2007) while it marks heterochromatin when is trimethylated (Schotta et al., 2004). Recent studies performed in ES cells further complicate the situation. In fact, promoters of genes important for differentiation were shown to be enriched, in their repressed state, in both H3K4me3 and H3K27me3, constituting the so-called “bivalent domains” (Bernstein et al., 2006). Unlike histone acetylation, histone methylation does not change nucleosome charge, but usually it can be recognized by effector

proteins displaying chromo-like domains belonging to the “royal family”. For example, BPTF, a component of the NURF chromatin-remodelling complex, recognizes H3K4me3, while HP1 recognizes H3K9me3. For years, histone methylation has been considered a permanent post-translational modification, but the recent discovery of histone demethylases (see below) has changed this view. Despite this, histone methylation still plays a central role in the maintenance and in the transmission of the genetic state of the cell. The best examples are the involvement of H3K9 methylation (and HP1 binding) in heterochromatin maintenance, and the role of H3K27 methylation (and PcGs binding) in gene silencing during development (see below).

3.3. Histone demethylases

Histone demethylases catalyze the removal of methyl groups from histone lysines and arginines. Peptidylarginine deaminase 4 (PAD4/PAD14) is the only arginine demethylase identified so far. This enzyme is able to convert monomethyl arginine at H3 and H4 to citrulline (Cuthbert et al., 2004; Wang et al., 2004b). However, since PAD4/PAD14 can act also on unmethylated arginines and there is no known enzyme that converts citrulline back to arginine, the situation is not clear.
Since the half-life of histones and of their methylated lysines is identical, lysine methylation has been considered for years to be an irreversible mark (Byvoet et al., 1972; Duerre and Lee, 1974). However, recent progress in the field led to the identification of two enzyme-families capable of catalyzing this reaction, the LSD1-family and the jumonji-family. LSD1 was first characterized as a histone demethylase specific for H3K4me1/2 (Shi et al., 2004). Further studies also reported a role for LSD1 as a H3K9me1/2 demethylase when associated with the androgen receptor (Metzger et al., 2005). LSD1 is a flavin-containing amine oxidase that, by reducing the cofactor FAD, catalyzes the cleavege of the α-carbon bond of the substrate to generate an imine intermediate (Fig. 5). The imine is then hydrolyzed yielding an aldehyde and an amine (Fig. 5). Due

to the requirement of protonated histones for the reaction, LSD1 cannot demethylate trimethyl histones.

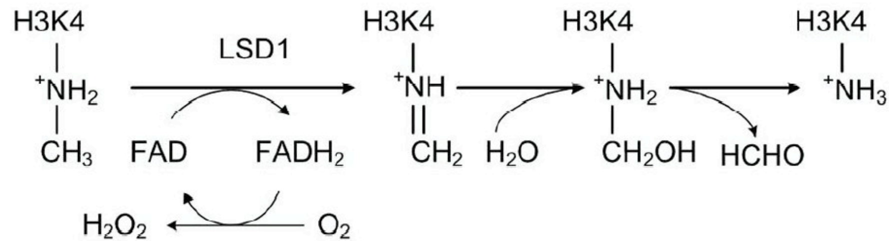

Figure 5. Mechanism of histone demethylase LSD1. LSD1 catalyzes an oxidation reaction transferring two hydrogen atoms from methylated H3K4 to FAD and forming an imine intermediate. The imine intermediate is then spontaneously hydrolyzed to produce an amine and formaldehyde (Tian and Fang, 2007).

LSD1 has been found in multiprotein complexes associated with HDAC activity (Hakimi et al., 2002; Hakimi et al., 2003; Humphrey et al., 2001; Shi et al., 2003) and was recently connected to cancer progression (Kahl et al., 2006). The jumonji family of genes contains more than 100 proteins and was first identified due to its important role in development (Takeuchi, 1997; Takeuchi et al., 1995) (Fig. 6).

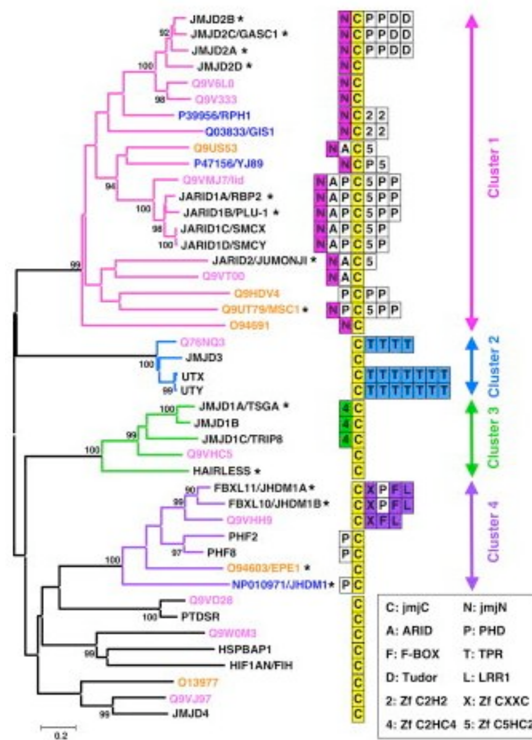

Figure 6. Unrooted neighbor-joining tree for the jumonji family of proteins. The tree was constructed based on jmjC domain sequences by using Poisson correction distance for amino acid sequences and the MEGA package. Protein names are represented in black for human, magenta for *Drosophila*, blue for budding yeast and orange for fission yeast. Specific domains for each protein are shown as colored boxes (Takeuchi et al., 2006).

More recently the JmjC domain, present in all members of this family, was shown to have histone demethylase activity (Tsukada et al., 2006). It acts through hydroxylation reactions using Fe (II) and α-ketoglutarate as cofactors (Fig. 7).

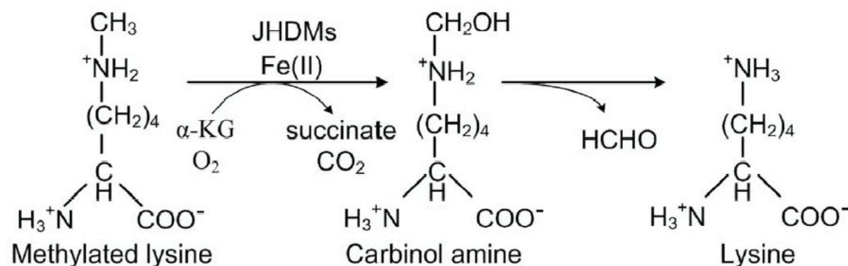

Figure 7. Mechanism of histone demethylase jumonji-family. Monomethylated lysine is hydroxylate by the jmjC-domain proteins, using Fe and α-ketoglutarate as cofactors, into carbinol amine and succinate. The carbinol amine is then spontaneously transformed into lysine and formaldehyde (Tian and Fang, 2007).

The family-members, so far characterized, display different substrate specificity. For example, JHDM1 only demethylates H3K36me1/2 (Tsukada et al., 2006), JHDM2A is associated with the androgen receptor and demethylates H3K9me1/2 (Tsukada et al., 2006), the JMJD2 subfamily is specific for trimethyl lysine demetylation (Cloos et al., 2006; Fodor et al., 2006; Klose et al., 2006), the JARID1 subfamily specifically demethylates H3K4me3/2 (Christensen et al., 2007; Lee et al., 2007a), while the UTX subfamily specifically demethylates H3K27me3/2 (Agger et al., 2007; Lee et al., 2007b). The discovery of these new families of enzymes constitutes a fundamental step in the epigenetic field, changing the view of histone methylation as a stable mark. However, their complete biological functions during development (i.e. how they are triggered and controlled) are not fully understood.

4. The Polycomb group of proteins (PcGs)

PcG proteins together with Trithorax group of proteins (TrxGs) were first identified in *Drosophila* as regulators of genes important for embryonic development (Ringrose and Paro, 2004). Mutational studies, performed nearly 60 years ago, revealed a role for PcGs in spatial gene regulation, silencing genes in body segments where their products don’t belong. Gene inactivation mediated by PcGs requires at least the cooperation of two complexes: the Polycomb repressive complex 1 (PRC1) and the Polycomb repressive complex 2/3/4 (PRC2/3/4) (Fig. 8). The PRC1 is composed of several proteins, such as BMI1, HPH1-3, HPC proteins and RING1-2 (Ringrose and Paro, 2004; Zhang and Reinberg, 2001) (Fig. 8). The PRC2/3/4 comprises EZH2, SUZ12, different isoforms of EED and the histone-binding proteins RbAp48/46 (Zhang and Reinberg, 2001) (Fig. 8).

PROTEIN NAME		PROTEIN DOMAIN	FUNCTION
Drosophila	Human		
PRC1			
Polycomb, PC	CBX2/HPC1 CBX4/HPC2 CBX8/HPC3	Chromodomain	Methyl-lysine binding
RING	RING1/RNF1/RING1A RNF2/RING1B	RING-finger domain	Ubiquitin ligase
Posterior sex combs, PSC	BMI1 ZNF134 RNF110/ZFP144/PCGF2	RING-finger domain	
Polyhomeotic, PH	EDR1/HPH1 EDR2/HPH2 EDR3/HPH3	Zinc-finger domain	
Pleiohomeotic, PHO	YY1	Zinc-finger domain	DNA-binding
PHO-like			DNA-binding
Polycomb like, PCL	SCML1	PHD-finger domain	
Sex combs on midleg, SCM	PHF1	Zinc-finger domain	
PRC2			
Enhancer of zeste, E(Z)	EZH1 EZH2	SET domain	Histone methyltransferase
Suppressor of zeste, SU(Z)12	SUZ12	Zinc finger domain	
Extra sex combs, ESC	EED	WD40 repeats	

Figure 8. The Polycomb group of proteins (PcGs). Nomenclature of PcGs in *Drosophila* and human. For each protein are shown the characteristic domains and the associated functions (adapted from Sparmann and van Lohuizen, 2006).

The EZH2 protein, catalyzing the specific methylation at lysine 27 of histone 3, is responsible for the initiation of repression (Fig. 9). Interestingly, it was recently demonstrated that for the PRC2 to be enzymatically active, the presence of SUZ12 is strictly required (Cao and Zhang, 2004; Pasini et al., 2004). H3K27me3 is then recognized by the chromodomain-containing proteins HPCs, that maintain genes repressed, by recruiting PRC1. Gene silencing is achieved through chromatin compaction (Francis et al., 2004), direct inhibition of the transcriptional machinery (Dellino et al., 2004) and/or ubiquitination of lysine 119 at histone H2A (Cao et al., 2005; Wang et al., 2004a) (Fig. 9). More recently, EZH2 was also shown to interact with DNMTs, pointing towards an additional mechanism of silencing and an interesting link between these two epigenetic pathways (Vire et al., 2006) (Fig. 9). While in *Drosophila* the PRC2 targeting is regulated by specific consensus sequences, the so-called Polycomb repressive elements (PREs), homologous sequences in mammals have not been identified so far and the mechanism of PcGs recruitment thus remains elusive.

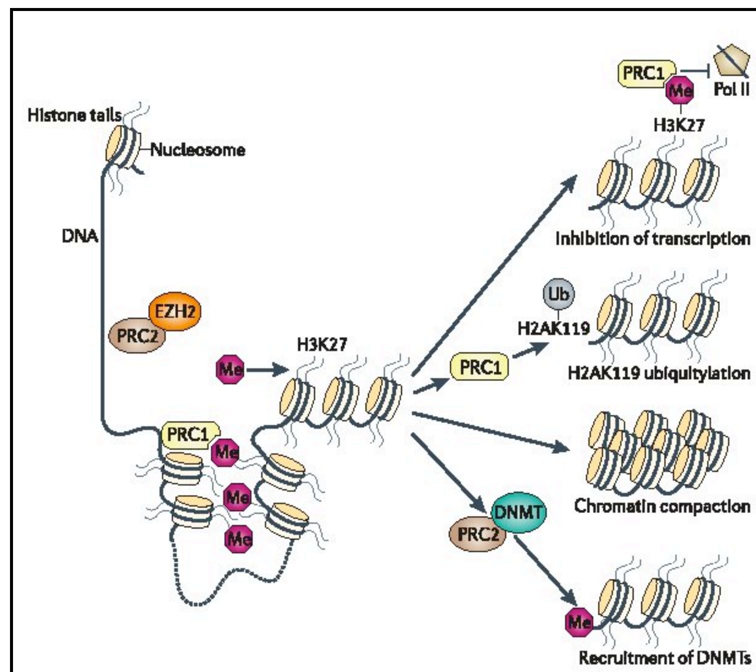

Figure 9. Mechanisms of Polycomb-mediated gene silencing. Polycomb-mediated silencing is initiated by the PRC2 that, methylating H3K27, creates docking sites for the PRC1. This causes a compaction of chromatin. PRC1 silencing could also involve direct inhibition of RNA polymerase II (Pol II) and ubiquitination (Ub) of H2AK119. Another possible mechanism of silencing includes the direct recruitment of DNA methyltransferases (DNMTs) (Sparmann and van Lohuizen, 2006).

PcGs are conserved during evolution and their central role in mammals was confirmed by the severe developmental and proliferative defects displayed by mice lacking Eed, Ezh2, Suz12 or Ring1A (del Mar Lorente et al., 2000; Erhardt et al., 2003; Faust et al., 1998; Faust et al., 1995; O'Carroll et al., 2001; Pasini et al., 2004). In fact, they are involved in several developmental processes such as regulation of Hox gene expression, silencing of the paternal X-chromosome in mouse female cells from extra-embryonic tissues (Heard, 2005), maintenance of imprinting (Umlauf et al., 2004) and they also seem to play a general role in cell proliferation and plasticity (Lund and van Lohuizen, 2004; Valk-Lingbeek et al., 2004). Silencing of Hox genes is the best characterized function of PcG proteins and it is conserved in *Drosophila*, vertebrates and plants. In the last years it was demonstrated that H3K27me3 is essential for Hox genes repression and that for this function integral PcGs are required (Muller et al., 2002). Several studies have shown that epigenetic

modifications are characteristical for X-chromosome inactivation. Indeed, DNA methylation, presence of the histone variant macroH2A and hypoacetylation of H4 are features of inactive X (Xi) (Brockdorff, 2002). The first evidence that the PcGs were also involved in this process came from data showing that X inactivation was not maintained in extrambryonic tissues of mice homozygous for a mutation in the Eed protein (Wang et al., 2001). In fact, H3K27me3 was found enriched on the Xi in both embryonic and extraembryonic cells. In addition, it was demonstrated that this epigenetic mark occurs early during the initiation stage of X-chromosome inactivation and immediately follows *Xist* coating of the Xi (Plath et al., 2003; Silva et al., 2003). PcG mediated silencing was also recently linked to the regulation of the expression of a subset of autosomal imprinted loci (Mager et al., 2003). Indeed, in mice, a subset of paternally silenced genes was shown to be re-expressed in Eed-null embryos. Beyond the role of PcGs in keeping chromatin in a repressive state during development, recent studies point towards a role also in the maintenance of stem cell pluripotency. Stem cells have the ability to self-renew and/or to differentiate into several cell types. O’Carrol and collaborators demonstrated that Ezh2 is essential for the derivation of pluripotent embryonic stem cells in mice (O'Carroll et al., 2001). In addition Ezh2 and H3K27me3 are enriched in stem cells and regulate the expression of transcription factors (Oct3/4) important for their pluripotency (Erhardt et al., 2003).

4.1. PcGs and cancer

The identification of genes regulated by PcGs that are involved in cell growth and division, opens the possibility of a link between PcGs and cancer progression (Ringrose et al., 2003). The first molecular connection came from work that showed that BMI1 regulates cell proliferation by repressing the Ink4a/Arf locus (Jacobs and van Lohuizen, 1999), an important tumor-suppressor locus. Furthermore, it was recently demonstrated that the Rb-E2F pathway regulates the expression of EZH2 and EED and that this is of central importance for the proliferation of transformed and non-transformed cells

(Bracken et al., 2003). In addition PcGs were found overexpressed in several cancers. For example, BMI1 was identified as a proto-oncogene, collaborating with MYC in generating B and T cell lymphomas (Haupt et al., 1991; van Lohuizen et al., 1991). EZH2 is found overexpressed in lymphoma and in prostate and breast cancer and its expression increases during cancer progression (i.e. with the formation of metastatic lesions) (Sparmann and van Lohuizen, 2006). Furthermore, SUZ12 upregulation is associated with colon and breast cancer (Kirmizis et al., 2003). The precise role of PcGs in neoplastic development remains to be clarified. Since it was recently shown that loci representing Polycomb target genes were DNA methylated in tumors, one hypothesis could be that the PRC2 mediates tumor-suppressors silencing by recruitment of DNMTs (Ohm et al., 2007; Schlesinger et al., 2007; Vire et al., 2006; Widschwendter et al., 2007) (Fig. 10A). Another hypothesis argues for a role of PcGs in regulating stem cell pluripotency. Recent work in pluripotent stem cells demonstrated that PcGs maintain genes important for cellular differentiation silenced (Boyer et al., 2006; Bracken et al., 2006; Lee et al., 2006). Thus, overexpression of PcGs in tumorigenesis could convey self-renewal properties to the cells, favoring neoplastic growth and tumor progression (Sparmann and van Lohuizen, 2006) (Fig. 10B). Further investigations into the molecular involvement of PcG proteins in neoplastic progression (for example how it is recruited to different target genes) will open new possibilities for therapeutic intervention in molecular oncology.

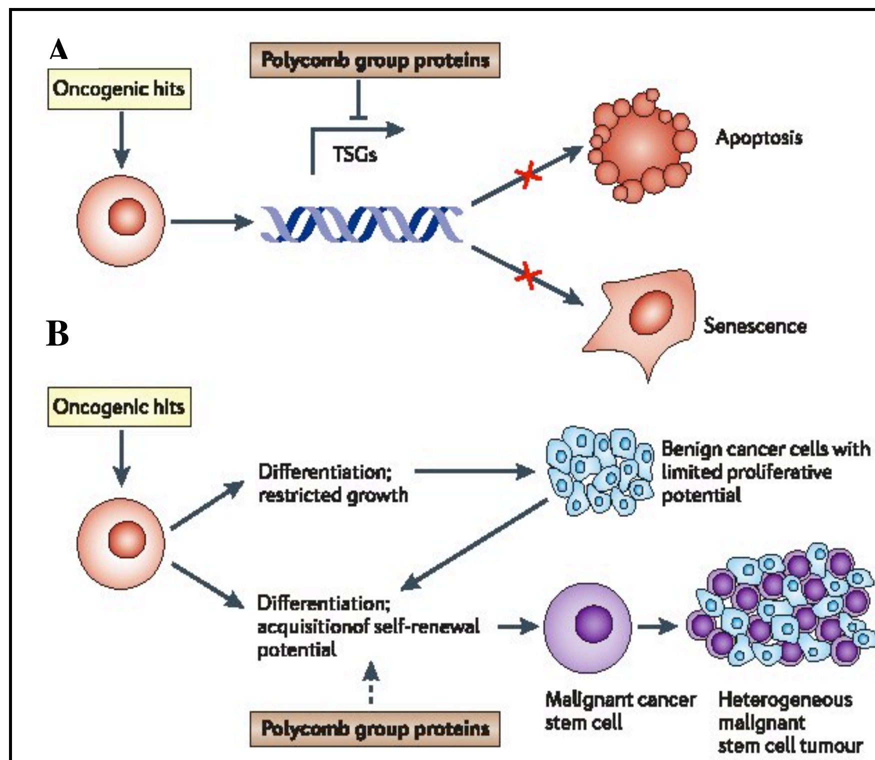

Figure 10. Functions of PcGs during tumor development. (A) PcGs transcriptionally repress tumor-suppressor genes (TSGs) that normally control cell proliferation through apoptosis and senescence. (B) Overexpression of PcGs in tumors could confer self-renewal properties to the cells, favoring neoplastic growth and tumor progression (Sparmann and van Lohuizen, 2006).

5. Acute promyelocytic leukemia (APL)

Leukemias are cancers of blood or bone marrow cells, characterized by alterations in the processes of synthesis and maturation of leukocytes. Typically, the immature cells proliferate abnormally substituting normal cells and spreading to other organs of the body. Leukemias can be classified into two major groups depending on the cell type that undergoes the neoplastic transformation: lymphocytic leukemia (affecting lymphoid cells) and myeloid leukemia (affecting myeloid cells). Furthermore, from a clinical point of view, we can distinguish an acute form, with rapid growth of immature blood cells and fast progression, from a chronic form that can progress for a long time period, since blood cells can partially mature.

Acute myeloid leukemia (AML) is characterized by the presence of chromosomal translocations that generate fusion proteins (Caligiuri et al.,

1997; Strout and Caligiuri, 1997). Usually one of the genes involved is a transcription factor important for hematopoietic differentiation while the other gene confers the oncogenic characteristics (Lin and Evans, 2000; Minucci et al., 2000). 5-10% of AML cases are represented by acute promyelocytic leukemia (APL). APL is characterized by an abnormal accumulation of immature granulocytes called promyelocytes that display chromosomal rearrangements that always involve the retinoic acid receptor alpha (RARa) on chromosome 17 and one of five different fusion-partners (PML, PLZF, NUMA, NPM or STAT5b). The translocation that involves the PML gene on chromosome 15 is found in 98% of APL cases. Also the reciprocal fusion proteins (RARa-X) are expressed in leukemic cells, but their role is still not clear (Melnick and Licht, 1999). Only RARa-PLZF was clearly demonstrated to participate in leukemogenesis in transgenic mice (Pandolfi, 2001).
The RARa-ligand, retinoic acid (RA), and the compound arsenic trioxide are currently used in clinical practice. However, many patients show RA resistance after two relapses or more (Gallagher, 2002). For this reason and due to the recent research progresses, new drugs such as HDAC and DNMT inhibitors alone or in combination with RA are now under preclinical investigation (Altucci et al., 2005; Fazi et al., 2005; Insinga et al., 2005).

5.1. RARa

The retinoid signal is transduced by two families of nuclear receptors, the retinoic acid receptors (RARs) and the retinoic X receptors (RXRs), which work as heterodimers (Kastner et al., 1997; Mark et al., 1999). They have a modular structure composed of six conserved regions, named A-F (Fig. 11). Regions C and E contain the conserved DNA binding domain (DBD) and ligand binding domain (LBD) respectively (Fig. 11). LBDs are formed by 12 alpha-helices and a beta-turn which are folded in a three-layered, antiparallel helical sandwich, the helix 12 (in the AF-2 activation domain) is outside the LBD core (Fig. 12). The N-terminal A/B region is a ligand-independent transactivation domain while the D region serves as a hinge between the DBD

and the LBD. The F region is absent in RXRs and its function in RARs is still not clear.

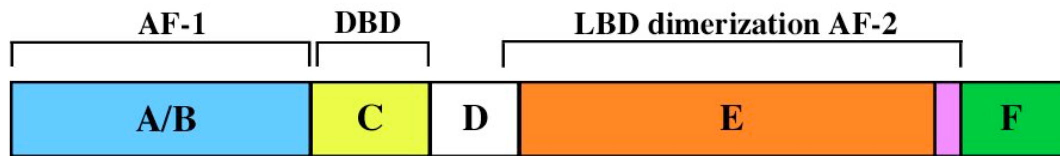

Figure 11. Schematic representation of the retinoic acid receptor alpha (RARa). The conserved regions are indicated with different colors (A/B blue, C yellow, D white, E orange, F green). The functional domains are schematically indicated: activation domain 1 and 2 (AF-1 and AF-2), DNA binding domain (DBD), ligand binding domain (LBD) (adapted from Bastien and Rochette-Egly, 2004).

The DBD mediates the interaction of retinoid acid receptors with specific DNA regions, known as retinoic acid responsive elements (RAREs). RAREs are usually composed of two direct repeats of a core hexameric motif, PuG(G/T)TCA (Leid et al., 1992; Mangelsdorf and Evans, 1995). The classical RARE is the DR5 (5-bp-spaced direct repeats), however RAR/RXR can also bind DR1 (1-bp-spaced direct repeats) and DR2 (2-bp-spaced direct repeats). The presence of RARE in key genes for hematopoietic differentiation (as for example, p21, RARb, STATs, vitamin D receptors and several Hox genes) points towards a central role of retinoid receptors in this homeostatic process. In the absence of RA, retinoid receptors behave as transcriptional repressors through the recruitment of the co-repressor complexes NCoR and SMRT which contain HDAC activity (Glass and Rosenfeld, 2000) The binding of RA causes a structural change in LBD (H12 becomes tightly packed against H3 and H4) (Fig. 12), leading to the release of the co-repressor complexes and the recruitment of co-activators containing HAT activity.

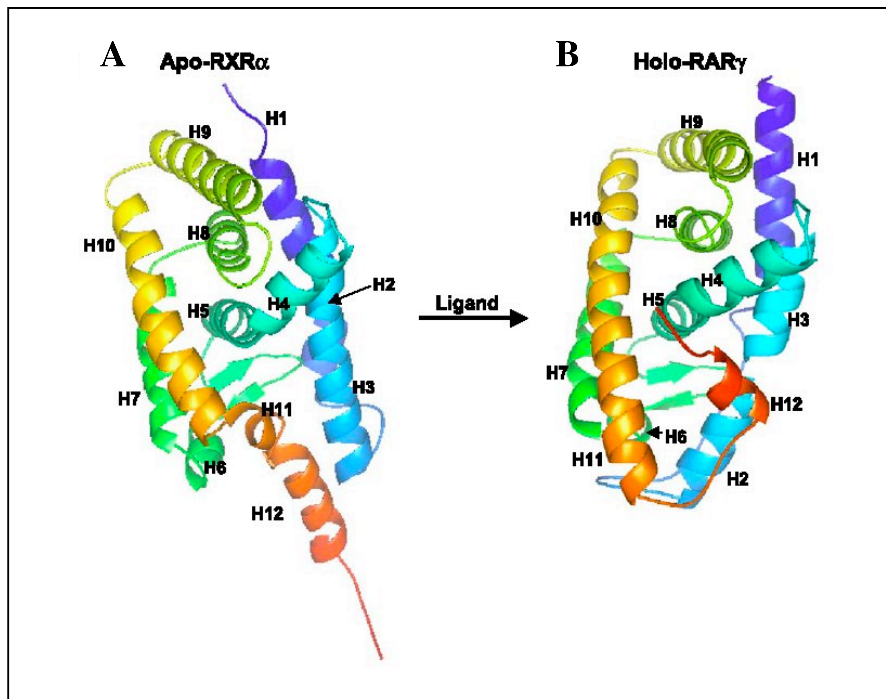

Figure 12. RA-induced conformational change in the LBD of RARa and RARγ . The LBD is formed by 12 conserved alpha-helices (H1-12) and a beta-turn (light green). In absence of the ligand, the H12 points away from the LBD core (apo-RARa), while in presence of the ligand H12 becomes tightly packed against H3 and H4 (holo-RARγ) (Bastien and Rochette-Egly, 2004).

Acute promyelocytic leukemia is an example of how alterations in the RA-signal- transduction pathway can lead to cancer. However, recent studies using RARa transformed in a potent repressor (either expressing HDAC1-RARa or using RARa mutated in the LBD in transgenic mice), show that deregulation of the retinoid signaling *per se* is not enough to induce leukemia (Matsushita et al., 2006). The specific properties of the other proteins involved in the chromosomal translocation are also required for transformation.

5.2. Promyelocytic leukemia (PML)

In the majority of APL cases the fusion partner of RARa is the promyelocytic protein PML. PML is a RING-fingerlike protein that localizes in nuclear structures of unknown function (0,2-1um in size) called PML-nuclear bodies (NB). Cells normally contain 10-30 NB while in APL blasts, PML-RARa causes the disruption of NB and the location of PML into microspeckled

structures. Recent work pointed towards the importance of the functional disruption of PML-NB in the development of APL (Pandolfi, 2001). PML contains a RING domain in the N-terminal region, followed by the RBCC domain (two Zinc finger domains, B boxes, and a α-helical coiled-coil motif), a nuclear localization signal (NLS) and a serine/proline rich domain of unknown function. The RBCC domain mediates protein-protein interactions and is responsible for PML oligomerization.

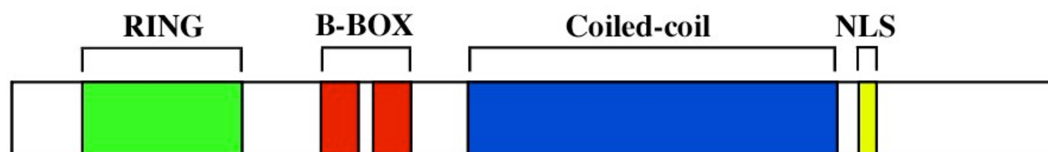

Figure 13. Schematic representation of the promyelocytic leukemia (PML). The functional domains are schematically indicated: Ring finger domain (RING), B boxes (B-BOX), coiled-coil motif (Coiled-coil), nuclear localization signal (NLS).

There are seven PML isoforms generated by alternative splicing that differ in the C-terminal region and thus in their subcellular localization (two of them do not contain NLS) (Jensen et al., 2001). Intriguingly, the PML region shared by all the isoforms is the one contained in the PML-moiety of the PML-RARa fusion protein. PML is not just associated with the NB, but is directly responsible for their formation. Cells derived from pml-knock-out mice, do not display NB structures and show an aberrant nuclear localization of the proteins usually compartimentalized in the NB (Ishov et al., 1999). More than 30 proteins, in fact, have been found to colocalize with the PML-NB and a few of them directly interact with PML, such as for example, p53, pRb, DAXX, CBP. Recent *in vivo* and *in vitro* studies point towards a role of PML in the induction of growth suppression, apoptosis and senescence, all involved in tumor suppression (Mu et al., 1994; Pearson and Pelicci, 2001; Quignon et al., 1998; Salomoni and Pandolfi, 2002) (Fig. 14).

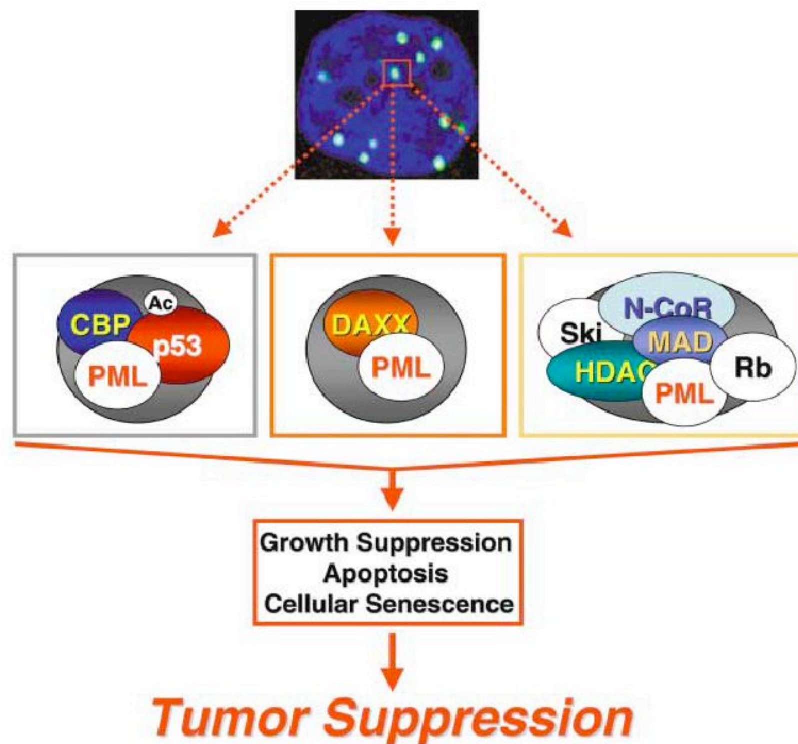

Figure 14. The PML-NB regulate tumor suppression functions. PML regulates p53 activation for apoptosis and cellular senescence (left panel). The co-repressor DAXX associates with PML-NB and induces apoptosis (middle panel). MAD and Rb transcription are controlled by the accumulation of repressive complexes in the PML-NB (right panel) (Pandolfi, 2002).

Therefore, the disruption of PML may be critical for APL leukemogenesis. Indeed pml-/- mice are highly susceptible to develop tumors if subjected to drug-induced carcinogenesis (Salomoni and Pandolfi, 2002). In addition, mice obtained crossing PML-RARa transgenic animals with pml-/- animals have a higher leukemia incidence and an accelerated onset of the disease (Rego and Pandolfi, 2001). PML disruption could also contribute to leukemogenesis by promoting the accumulation of other aberrant genetic events. In fact, proteins responsible for the maintenance of genomic stability, such as BLM, nibrin/p95 and MER11, are also contained in the PML-NB (Zhong et al., 1999). Interestingly, it was recently demonstrated that a posttranslational modification of PML (sumoylation at lysine 160) is essential for PML-RARa mediated leukemic transformation. In fact, transgenic mice with PML-RARa mutated in

lysine 160 show a myeloproliferative syndrome, which never progresses into leukemia (Zhu et al., 2005).

5.3. PML-RARa

The PML-RARa fusion protein features the RBCC domain of PML and the DBD and LBD of RARa. These two characteristics convert PML-RARa into a constitutive repressor of RARa target genes. Due to the RBCC domain, in fact, PML-RARa can form oligomers and interacts more efficiently with the SMRT-NCoR-HDAC complex, becoming insensitive to physiological concentrations of RA (10^{-9} to 10^{-8} M) (Grignani et al., 1998; Lin et al., 1998). This aberrant gene silencing leads to the differentiation block observed in hematopoietic precursors and the development of leukemia. Just pharmacological concentrations of RA (10^{-6} M) can partially revert the fusion protein-induced gene silencing. Furthermore, PML-RARa oligomerization allows the binding of the fusion protein to non-consensus RARE, enlarging the number of target genes in comparison to wild type RARs (Kamashev et al., 2004).

PML-RARa-mediated silencing not only involves HDACs, but also other epigenetic mechanisms such as DNA methylation (Fig. 15A). Indeed, Di Croce and coworkers recently showed that the fusion protein interacts and recruits DNMT1 and DNMT3A to the RARb2 promoter (one of the well-characterized PML-RARa target genes), thus inducing aberrant DNA methylation (Di Croce et al., 2002) (Fig. 15B). Interestingly PML-RARa-induced DNA methylation was shown to be stable throughout the cell cycle even in the absence of the fusion protein, supporting a role for DNA methylation in the maintenance of the transformed phenotype. Use of RA and 5-Aza-2’-deoxycytidine (5-Aza-dC, a DNMTs inhibitor) in combination reactivated RARb2 expression in both APL-derived cell lines as well as in APL blasts from patients.

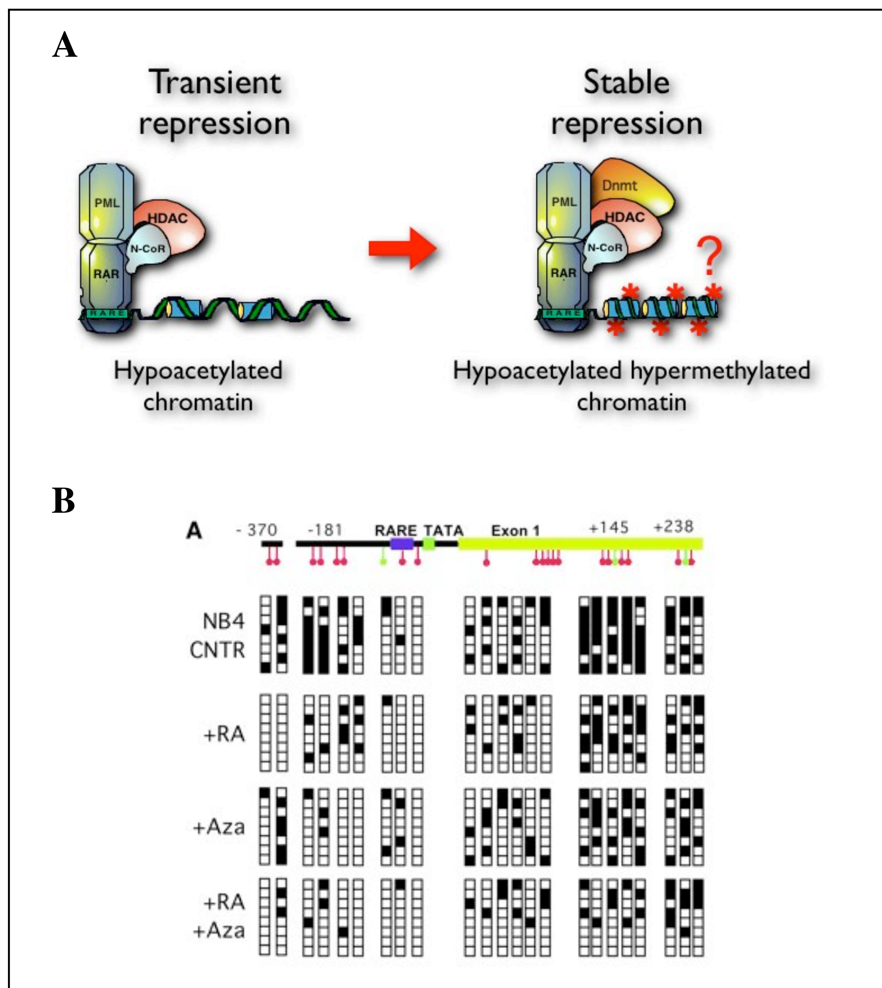

Figure 14. PML-RARa-mediated gene silencing. **(A)** Model that shows PML-RARa-mediated repression. Initially, the fusion protein recruits the SMRT-NCoR-HDAC corepresor complex inducing chromatin deacetylation at target promoters. In a second step, the recruitment of DNA methyltransferases (DNMTs) produces also DNA methylation (red asterisks). Methylated CpGs might provide docking site for methyl-CpG-binding proteins (red question mark). (B) PML-RARa-induced DNA methylation. The scheme represents DNA methylation at the RARβ2 promoter obtained with bisulfite genomic sequencing of an APL derived cell line not treated (NB4 CNTR) or treated with retinoic acid (RA) and/or 5-Aza-2’-deoxycytidine (Aza). White squares represent unmethylated CpGs while black squares represent methylated CpGs (adapted from Di Croce et al. 2002)

OBJECTIVES

Research in our group is focused on the investigation of epigenetic mechanisms involved in cancer using APL as a model system. APL is characterized by the presence of the chimeric fusion-protein PML-RARa that, blocks hematopoietic differentiation at the promyelocytic stage through aberrant gene silencing. The mechanisms so far identified for PML-RARa-mediated gene repression involve deacetylation of histones and DNA methylation (Di Croce et al., 2002; Grignani et al., 1998; Lin et al., 1998). The aim of this thesis project was to further characterize the physiological implications of the presence of methylated CpGs at PML-RARa target genes and to explore the possibility that, together with DNA methylation, other epigenetic mechanisms are involved in PML-RARa-mediated gene repression during leukemogenesis.

Specifically, the main objectives of my PhD project were:

- The investigation of the methyl-CpG-binding protein MBD1 and its role in PML-RARa-mediated aberrant DNA methylation.
- The characterization of other epigenetic mechanisms involved in APL, such as Polycomb-mediated H3K27 methylation and its possible cross-talk with DNA methylation.

RESULTS

Villa R, Morey L, Raker VA, Buschbeck M, Gutierrez A, De Santis F, Corsaro M, Varas F, Bossi D, Minucci S, Pelicci PG, Di Croce L.
The methyl-CpG binding protein MBD1 is required for PML-RARalpha function.
Proc Natl Acad Sci U S A. 2006 Jan 31;103(5):1400-5. Epub 2006 Jan 23.

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DISCUSSION

In the past, cancer was considered as a disease caused by genetic abnormalities. Growing evidences are changing this view, postulating that not only alterations of the genome but also of the cellular epigenome contribute to aberrant gene expression during tumor formation. In fact, it is well established that DNA methylation is altered in several tumors resulting in a global hypomethylation of the genome and hypermethylation of CpG islands (see introduction). Furthermore, DNMTs and methyl-CpG-binding proteins were shown to be overexpressed in several human cancers (Herman and Baylin, 2003; Lopez-Serra et al., 2006). In addition, other chromatin modifications play a role in the aberrant gene silencing characteristic of transformed cells. For example, features of several cancers are H4K16 deacetylation and H4K20me3 (Fraga et al., 2005), overexpression of PcGs (see introduction) and mutations in the chromatin remodeling complex SWI/SNF (Roberts and Orkin, 2004). The principal difference between these two alterations is that while it is not likely that several genetic mutations affect a single-cell pathway, multiple epigenetic events may occur at higher frequency and may function as networks in the same pathway influencing each other (Jones and Baylin, 2007).

Among all types of cancer, leukemias provide a good model to study alterations of the cellular epigenome. Indeed, these diseases are characterized by the presence of chimeric fusion proteins that are often responsible for chromatin alterations at target gene loci. One example is the mixed-lineage leukemia (MLL) protein that has been found fused to different partners in acute lymphoblastic and myeloid leukemia and is a chromatin modifying enzyme itself (Daser and Rabbitts, 2004). In fact, the MLL protein is the mammalian orthologue of the trithorax complex in *Drosophila* and regulates H3K4 methylation. This mark is particularly present at promoters of actively expressed Hox genes in progenitor cells and concomitantly decreases when these genes are silenced during cellular maturation (Milne et al., 2002; Nakamura et al., 2002). It is believed, that MLL fusion proteins skew the balance between proper gene-activation and -repression. For example, the fusion products of MLL and the HATs p300 t(11;22) or CBP t(11;16) lead to

aberrant acetylation of MLL targets (Daser and Rabbitts, 2004), while the fusion product of MLL and ENL t(11;19) associates with SWI-SNF to activate transcription of HoxA7, a gene required for transformation (Nie et al., 2003). Two other examples of how aberrant regulation of epigenetic mechanisms can lead to leukemia are represented by the fusion proteins AML1-ETO t(8;21) and PML-RARa t(15;17). The acute myeloid leukemia 1 (AML1) protein in normal cells binds both the co-repressors Groucho/transducin-like enhancer and Sin3 and the activator CBP, and thus is capable of gene-activation or -repression, depending on the target. In contrast, when AML1 is fused to ETO (the homologue of *Drosophila* nervy), it represses transcription of AML1 targets, due to the ability of ETO to interact with the nuclear co-repressor complexes NCoR-SMRT-HDAC (Hug and Lazar, 2004).

PML-RARa is the fusion protein responsible for the accumulation of hematopoietic cells at the promyelocitc stage in the majority of APL cases. This is principally caused by an aberrant gene silencing, achieved through the recruitment of the nuclear co-repressor complex NCoR-SMRT-HDAC and of DNMTs, that leads to hypoacetylation and hypermethylation of target genes, respectively (Di Croce et al., 2002; Grignani et al., 1998; Lin et al., 1998).

The main focus of this PhD thesis has been the study of epigenetic mechanisms involved in APL. In particular, we aimed to further expand the investigation on the role of DNA methylation in PML-RARa-mediated repression and to evaluate the involvement of other epigenetic modifications, such as histone methylation.

The first part of this thesis was dedicated to the study of the methyl-CpG-binding family of proteins (see introduction) in APL. The studies on this family of proteins provided one of the first evidence for a connection between different epigenetic modifications during tumor formation. In fact, methyl-CpG-binding proteins form a complex with histone deacetylases and nucleosome remodeling machineries and were found associated with hypermethylated tumor suppressor genes in human cancer cells (Lopez-Serra et al., 2006), proving a link between DNA methylation and histone modifications

in aberrant gene silencing. Since this family of proteins represents the interpreters of DNA methylation and DNA methylation has a prominent role in tumor suppressor gene silencing during tumorigenesis, it's not surprising that a methyl-CpG-binding protein plays an important role in cancer.

The first article presented in this PhD thesis shows that MBD1 (a methyl-CpG binding protein expressed in our leukemic system) is recruited by PML-RARa to several target genes, thus cooperating in cellular transformation. Indeed, the use of a MBD1 protein mutated in both the MBD and the TRD domain could in part rescue the differentiation block imposed by PML-RARa. Furthermore we demonstrated that this interaction occurs through an HDAC3-mediated mechanism and is not limited to the promoter region, but spreads over the locus. The association of MBD1 at PML-RARa target genes, following DNA methylation, is required for maximal and stable transcriptional repression. Indeed, depletion of HDAC3 using specific shRNAs led to reactivation of PML-RARa target genes. The new perspective proposed by the results presented in this article, is that a methyl-CpG-binding protein is not just attracted by methylated CpGs, but, during cancer progression, it can also be targeted by an oncoprotein prior to significant CpG-methylation. Indeed, this initial recruitment could be necessary to increase the local concentration of MBD1, thus facilitating the binding to methylated CpGs and the spreading along the locus. Subsequently, MBD1 bound along those chromosomal regions, further interacts with HDAC3 and DNMTs (Fuks et al., 2001) enhancing the recruitment of co-repressors and promoting chromatin compaction (Fig. 15).

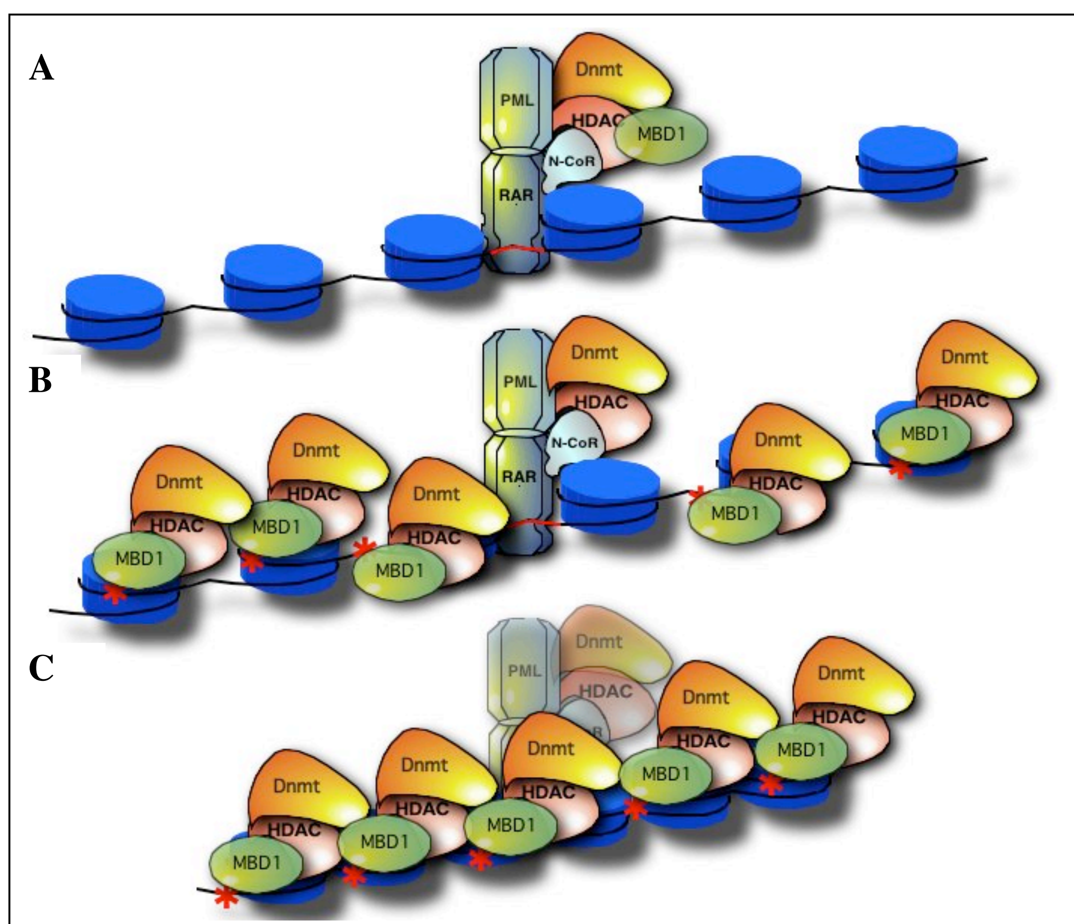

Figure 15. Model for MBD1 spreading along the RARb locus. (A) Initially, PML-RARa recruits MBD1 through HDAC3 to the RARE region. **(B)** Subsequently the local concentration of MBD1 facilitates its binding to methylated CpGs (red asterisks) all over the locus. **(C)** Further recruitment of HDAC3 and DNMTs causes a chromatin compaction of the locus.

The second part of the thesis focused on a complementary epigenetic mechanism, histone methylation. Recently, the analysis of aberrant gene silencing in cancer cells suggested several connections between DNA and Polycomb-mediated H3K27 methylation. First, hypermethylated genes in human colorectal cancer have been shown to be also enriched in H3K27me3 (McGarvey et al., 2006). Subsequently, Vire and collaborators demonstrated that EZH2 recruits DNMTs to PRC2-target genes in a cancer cell line, thus controlling DNA methylation (Vire et al., 2006). In addition, at the beginning of 2007 three different groups showed that genes hypermethylated at CpGs in cancer cells are often premarked by H3K27 methylation early in development (Ohm et al., 2007; Schlesinger et al., 2007; Widschwendter et al., 2007). All

these findings pointed towards a role for the PRC2 in directing aberrant DNA methylation during carcinogenesis.

The second article of this PhD thesis not only provides evidence for the importance of both H3K27 and DNA methylation in the maintenance of aberrant gene silencing during leukemogenesis, but it also shows a possible mechanism for the targeting of these epigenetic complexes. In particular, we demonstrated that PML-RARa targets the Polycomb complex to tumor suppressor genes during leukemogenesis leading to methylation of histone 3 at lysine 27. In addition, we observed that abolishing the activity of the Polycomb complex by interfering against the subcomponent SUZ12, not only affected histone modifications, but also provoked DNA de-methylation of PML-RARa target genes, thus favoring gene reactivation and cellular differentiation. Furthermore to better characterize the potential cross-talk between DNA and H3K27 methylation at PML-RARa silenced promoters, we tested whether inhibition of DNA methylation, by 5-Aza-dC treatment, would affect the methylation status of H3. Strikingly, we observed that impairment of DNA methylation causes changes in H3K27 methylation as well, supporting the hypothesis of a connection between these two key epigenetic cellular memory systems in PML-RARa-expressing cells. Interestingly, just recently, the link between these two epigenetic mechanisms was extended to the regulation of gene silencing during normal development, providing evidence that such a connection is not just a feature of cancer cells (Xi et al., 2007).

RA is commonly used to treat APL patients. Here we demonstrated that pharmacological doses of RA both in APL-derived cells as well as in freshly isolated blasts from patients could restore the correct balance of epigenetic marks. Nevertheless, RA is not sufficient to completely cure this disease and patients often acquire resistance to the hormone and experience relapse. The discovery that alterations of epigenetic mechanisms are involved in cancer, led to the development of new therapeutic approaches to revert gene silencing. In fact, at the moment, inhibitors against DNMTs, HDACs and HMTs are investigated for their use in cancer therapy. In the future, combinatorial

treatment of RA and HDAC/HMT inhibitors and/or de-methylating agents might be tested for the removal of epigenetic marks in order to facilitate complete remission of APL in patients.

Our results identified PML-RARa as an oncoprotein that functions as a platform for epigenetic modifier enzymes. The RARa moiety is responsible for the recruitment of MBD1 through the HDAC3 protein contained in the nuclear co-repressor complex NCoR-SMRT, while the PML moiety mediates the interactions with DNMTs, SUV39H1 and the PRC2 (Carbone et al., 2006; Di Croce et al., 2002). In addition, the ability of PML-RARa to form oligomers, enhancing the binding to co-repressors, leads to the creation of a heterochromatin-like environment at tumor-suppressor loci and thus to aberrant silencing. Furthermore, DNMTs and MBD1 could also interact with the PRC2 reinforcing the network among epigenetic layers created by PML-RARa (Vire et al., 2006) and unpublished results) (Fig. 16). For the first time, we provided evidence for a targeting mechanism for both MBD1 and the PRC2 in cancer and we further demonstrated that PML-RARa-mediated recruitment of these chromatin-modifying complexes seems to be regulated in a continuous feed-back loop-fashion that is essential for driving APL-tumorigenesis. Current work in the laboratory is now focusing on clarifying the precise kinetics of these events.

In conclusion, the work presented in this PhD thesis, leads to the characterization of multiple epigenetic layers involved in APL and sheds new light on the transcriptional repression mediated by PML-RARa, thus opening new alternatives for therapeutic intervention. Furthermore it provides a model of how networks of epigenetic mechanisms control leukemia progression that could prove to be useful for future investigations into the molecular causes of other cancers.

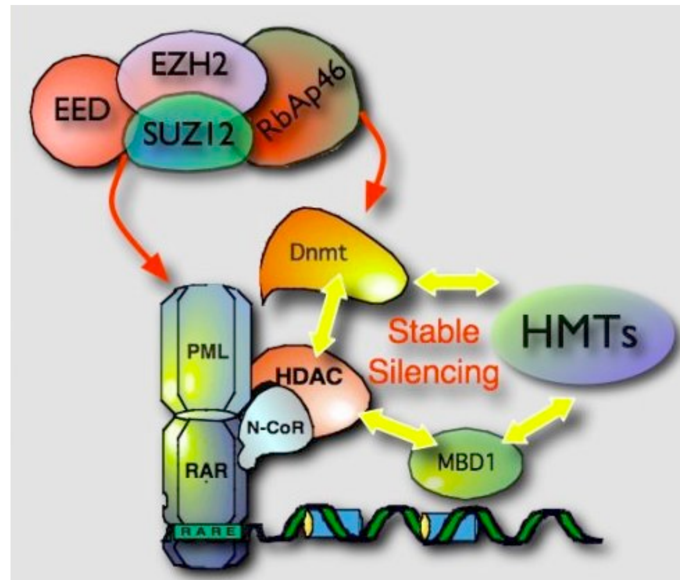

Figure 16. Network of interactions among epigenetic complexes in APL. PML-RARa recruits to its target genes the nuclear co-repressor complex NCoR-SMRT-HDAC3, DNMTs, MBD1 and the PRC2. These chromatin modifier enzymes could then establish cross-interactions with each-others, creating a regulatory network (controlled in a feed-back loop fashion), that leads to aberrant chromatin compaction and stable gene silencing.

CONCLUSIONS

The following conclusions can be drawn from the results presented in this PhD thesis:

- PML-RARa recruits MBD1 to the RARb promoter through HDAC3-mediated protein-protein interaction.
- MBD1 binding to methylated DNA spreads outside the RARb-promoter region in a time dependent fashion.
- MBD1 and HDAC3 cooperate with PML-RARa in silencing tumor-suppressor loci and in leukemia progression.
- Mutations in the MBD and in the TRD domains of MBD1 restore normal hematopoietic differentiation by preventing PML-RARa-mediated transcriptional repression.
- PML-RARa interacts and recruits the PRC2 to its target genes leading to H3K27 methylation and gene silencing.
- The impairment of Polycomb activity in an APL derived cell line (NB4) facilitates its differentiation, rendering PML-RARa target genes prone to activation.
- Polycomb-mediated histone methylation and DNA methylation are functionally interconnected and both required for the maintenance of PML-RARa repression: perturbation of one of the two epigenetic mechanisms affects the other one as well.
- RA treatment restores the proper balance of epigenetic marks both in NB4 and in APL primary blasts from patients.

SUPPLEMENTARY ARTICLES

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