1. INTRODUCTION

The answer of biological systems to any external stimulus is now a well known physiological mechanism, starting from procariotic cells to superior organisms. However, the molecular mechanisms by which a biological system integrates external stimuli are much more complicated then expected. It is clear now that the genome dynamically integrates signals coming from the extracellular environment. Every cell follows a specific genetic program that establishes which genes have to be activated and transcribed and which have to be silenced. Such a genetic program is able to dynamically regulate itself in response to the environmental stimuli. This dynamic contest is due to specific **molecular marks** that cells use to generate signals of information that can be recognized and read by committed protein complexes. Such molecular marks target covalently not only the genome (the DNA sequence), but also other protein components such as histones. Taken together, DNA sequence and protein components represent the chromatin which undergoes several dynamic covalent modifications triggering or inhibiting specific biological processes as modulation of gene transcription. Such dynamic changes of the genetic program can be inherited during cell division and represent the **epigenome**.

Thus, the **epigenetic modifications** are the means by which cells manage the genetic program interpreting the external stimuli.

1.1 DNA methylation and its biological role

Symmetric methylation of cytosine in CpG dinucleotides is one of the widespread modifications in animal genomes. To date, this modification has been found both in invertebrates (*Drosophila melanogaster)* (Hung *et al.,* 1999) and in chordates (from *Ciona*

intestinalis to mammals) (Simmen *et al.,* 1999). It has been associated with an inactive chromatin state (heterochromatin) and, therefore, as negative regulator of transcription. It is now well known that DNA methylation plays an important role in epigenetic regulation of gene expression that is not directly dependent on primary structure of DNA, but is maintained by several protein or non-protein factors such as histone tail modifications and chromosome territory. It is generally accepted that DNA methylation is a unidirectional process. If any sequence acquires CpG methylation then this modification becomes stable and will be inherited after cell division. So, both daughter DNA molecules will have same pattern of methylation.

DNA methylation affects gene expression directly or indirectly. Some transcriptional factors (i.e. Sp1) can interact only with non-methylated DNA sequences, whereas methylation of cytosine abolishes interaction (Clark *et al.,* 1997). This in turn leads to less effective transcription of certain genes. On the other hand, there is a different mechanism of action of CpG methylation. MBD (methyl-DNA-binding domain) proteins (Hendrich and Bird 1998) specifically recognize and bind methylated DNA sequences and attract large protein complexes that can change chromatin conformation from “opened” to “closed”.

DNA methylation occupies up to 70% of the CpG dinucleotides in the genome, and represents likely the major epigenetic modification in mammals (Robertson and Wolffe, 2000). The organization of the mammalian genome is such that there is a high density of CpG in the upstream promoter regions in most of the approximately 30,000 genes, as well as within gene introns and exons (Bird, 2002). This organizational feature and the capacity for DNA methylation to silence transcription in mammals lead to the early speculation that

gene regulation might be highly sensitive to the methylation status of these so-called CpG islands (Holliday and Pugh, 1975). However, this proved not to be the case, as these potential targets for methylation were found to be unmethylated, at least for most genes under normal circumstances (Antequera and Bird, 1993; reviewed in Meehan and Stancheva, 2001). Notable exceptions to this general rule include imprinted genes and those of the inactive X-chromosome. Further genomic characterization identified other classes of sequence families with significant levels of CpG, leading to alternative proposals for the significance of this stable and heritable modification. The mammalian genome contains an extremely high burden of sequences that have arisen due to integration of retrotransposons. Uncontrolled expression of these sequences from their viral promoters would result in transcriptional chaos, were it not for the susceptibility of these promoters to be repressed by DNA methylation (Yoder *et al.,* 1997). This regulatory role for DNA methylation has been embodied in the genome defense hypothesis, and remains one of the significant functions of DNA methylation (Walsh *et al.,* 1998).

One of the most comprehensively studied roles of DNA methylation is the marking of parental alleles by genomic imprinting. Imprinted genes are expressed in a non-Mendelian fashion, in which parent-of-origin specifies the active allele (Reik and Walter, 2001). These genes are essential for fetal growth and development, and have been shown to also influence postnatal growth trajectories and diverse biological processes, for example, affecting thermogenesis in offspring, maternal care and suckling behavior, and adult behavior and cognition (Li *et al.,* 1999; Plagge *et al.,* 2004). The marking of the active and inactive alleles is achieved through differential DNA

methylation in critical regulatory regions. These differentially methylated regions (DMRs) are essential for expression or repression. It is interesting to note that a disproportionately high number of imprinted genes are found to be methylated on the maternal allele (Reik and Walter, 2001).

During development in mammals, there are at least two periods of genome-wide DNA methylation reprogramming (Reik and Walter, 2001). The two periods best characterized include a time during primordial germ cell differentiation and one during preimplantation development. The extent of this reprogramming and whether, in fact, it is required for normal development in all mammalian species remain unknown (Bestor, 2000). In this regard, the details of DNA methylation reprogramming in humans are only just beginning to be appreciated. The degree to which this important regulatory mechanism operates in early development and germ cell differentiation in humans will influence our understanding of the potential impact of DNA methylation in human health and disease.

A growing body of evidence indicates that DNA methylation, together with chromatin modifications, are competent to specify transcriptional states and perhaps more importantly, mutually reinforce transcriptionally repressive states (Tamaru and Selker, 2001; Fuks *et al.,* 2003; Tamaru et al., 2003). Also, epigenetic regulation of gene expression includes processes as embryonic development, transcription, chromatin structure, X chromosome inactivation, genomic imprinting and chromosome stability. Consistent with these important roles, a growing number of human diseases have been found to be associated with aberrant DNA methylation.

1.2 The DNA-methyltransferases

Introduction of the methyl group into the symmetrical dinucleotide 5-CpG-3 results in its positioning into the major groove of the DNA without interference with the base-pairing of nucleotides. Methylation of cytosine residues imposes a greater level of risk to the stability of the genome as deamination of methyl-cytosine results in the transition of meC-T, a nucleotide base change less easily repaired and recognized than the deamination of cytosine to uracil (Hermann *et al.,* 2004). The lack of high fidelity repair capacity for the meC-T transition explains the observation that CG sites are a major mutational hotspot, accounting for up to 30% of point mutations in the germline, and are decidedly underrepresented in mammalian genomes (Bird *et al.,* 1985). This situation suggests that the maintenance of this modification and its functions have been the result of considerable evolutionary pressure, and that the continued maintenance of cytosine methylation must confer a significant advantage given the substantial costs. The complexity of DNA methylation in the genome suggests that there must be a number of activities responsible for its establishment and maintenance, able to operate in both specialized and generalized functions (Chen *et al.,* 2003).

DNA methyltransferases (MTases) represent a collection of three family groups numbered in order of their discovery (Bestor, 2000). These enzymes serve the two distinctive processes of DNA methylation, the establishment of DNA methylation state by de novo methylation and, thereafter, the maintenance of those states by templating this information to daughter strands arising from replication (Lei *et al.,* 1996; Okano *et al.,* 1999). Despite some sequence similarities, the divisions of labor amongst this group can,

in part, be inferred by their functional organization. Broadly, their organization can be resolved into two functional domains; the N-terminal domain comprising regulatory functions and the C-terminal catalytic domain (Fig.1) (Bestor, 2000; Robertson, 2002).

Dnmt1 was the first of the group of MTases identified, and is the largest of these activities with an extensive N-terminal regulatory domain and a smaller C-terminal domain (Bestor *et al.,* 1988). This large regulatory domain contains a wide variety of functional motifs, including a nuclear localization signal, a proliferating cell nuclear antigen (PCNA) (Chuang *et al.,* 1997) interacting domain, and a replicating foci targeting region (Leonhardt *et al.,* 1992). Dnmt1 does not function in the cell in an isolated manner, and is capable of interacting with many proteins via the N-terminus. This interaction is likely to be facilitated by the polybromo domain (PBHD), a hallmark of protein-protein interaction (Bestor and Verdine, 1994). Furthermore, this domain has been implicated in a transport role for Dnmt1 to the replication foci (Liu *et al.,* 1998). Dnmt1 methylates DNA specifically at CG sites, with a strict preference for hemimethylated over unmethylated substrates in vitro (Pradhan *et al.,* 1997, 1999). It is this preference for hemimethylated substrates that forms the basis for its function as a maintenance methylase. In this respect, it is not surprising that Dnmt1 expression is tightly coordinated with DNA replication. In addition to its function as a MTase, Dnmt1 has been shown to be associated with a wide variety of chromatin modifying activities, including histone methyltransferases, methyl CpG binding proteins, and heterochromatin binding protein HP1 (Hermann *et al.,* 2004). Collectively, these associations share in common the properties of transcriptional repressors leading to the understanding that Dnmt1 and, hence, DNA methylation stably reinforces chromatin

silencing (Bird, 2002). Also this enzyme is required for the survival of the organism as its knock out is lethal in embryos (reviewed in Jaenisch and Bird, 2003).

The **Dnmt2** gene is the most highly conserved of the MTases in eukaryotes, found in both organisms that show methylation and those that have no detectable DNA methylation. Although it is ubiquitously expressed at low levels in most human and mouse tissues as well as mouse embryonic stem cells, mice homozygous for a Dnmt2 null mutation are viable, and display normal levels of methylation at endogenous sequences (Okano *et al.,* 1998b). Introduction of an inducible transgene containing Dnmt2 indicated that a genuine methylase activity could be demonstrated, albeit on CpT and CpA targets (Kunert *et al.,* 2003). A weak but reproducible in vivo methyltransferase activity was recently demonstrated for the recombinant protein in mammalian cell lines (Liu *et al.,* 2003).

Dnmt3 family. The highly related enzymes, Dnmt3a and Dnmt3b, are encoded by different genes but share the preference for methylation of unmethylated CG dinucleotides. This substrate preference identifies them as de novo DNA methylases (Okano *et al.,* 1998a). Dnmt3a and Dnmt3b are thought to differ mechanistically due to inherent differences in the catalytic domains, suggesting that Dnmt3a is distributive while Dnmt3b is processive (Gowher and Jeltsch, 2002). These intrinsic differences allow for an effective division of labor between these related de novo methylases. The processive Dnmt3b is more suited to methylation of CG-rich regions of the genome, such as the CG-rich pericentromeric repeats (Hermann *et al.,* 2004). The distributive nature of Dnmt3a requires that it adds back methylation to dinucleotides on a target by target basis, and is thus implicated in de novo methylation at single genetic

loci (Hata *et al.,* 2002). A specific function for Dnmt3a was not detectable with the knockout model but studies using the various Dnmt-knockout ES cells in addition to transgenic *Drosophila melanogaster* expressing Dnmt3a revealed that this enzyme may be specialized to methylate non-CpG sequences like CpA, and CpT although the function of non-CpG methylation in ES cells is unknown (Ramsahoye *et al.,* 2000). Dnmt3a is the major form in adult tissues, where it colocalizes with heterochromatin. In contrast, the isozyme Dnmt3a2 is the major form during embryogenesis, and has been shown to localize with euchromatin (Chen *et al.,* 2002). Both Dnmt3a and Dnmt3b are required for the survival of an adult organism: lack of Dnmt3b is embryonic lethal and lack of Dnmt3a lethal in mice of few weeks (Okano *et al.,* 1999, reviewed in Robertson KD, 2005).

The third significant member of the Dnmt3 family of enzymes is Dnmt3L. This highly degenerate protein shows clear homology to Dnmt3a and Dnmt3b, but despite conserved folding like MTases, Dnmt3L lacks any catalytic activity but is expressed together with Dnmt3a and Dnmt3b during gametogenesis and embryogenesis (Bourc'his *et al.,* 2001; Hata *et al.,* 2002). Dnmt3a and Dnmt3L are essential for establishment of imprinted regions in oocytes (Hata *et al.,* 2002). The exact mechanism of this process is not fully worked out, but there is the suggestion that the sequence specific function of Dnmt3a may require an activator to enforce the accuracy of this targeting. In this regard, Dnmt3L may function as an activator protein in the methylation of single copy genes.

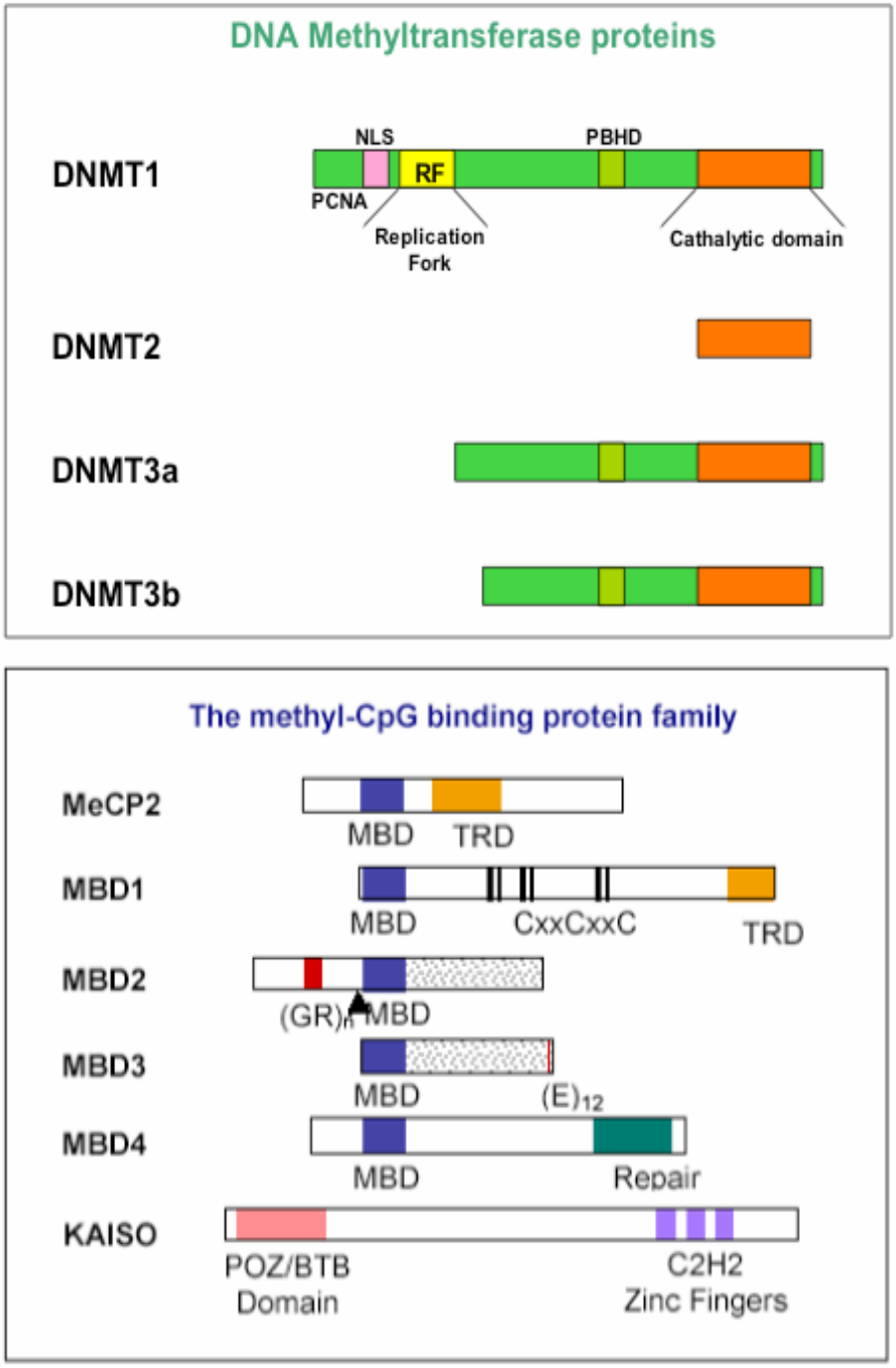

Fig.1 Schematic representation of proteins of the mammalian DNA methylation system.

1.3 The methyl-binding proteins: linking DNA methylation and chromatin structure

A. Bird identified the first methyl-DNA-binding activity called MeCP1 (methyl-CpG-binding protein 1) (Meehan *et al.,* 1989). This activity included two complexes (400 and 800 kD) consisting of different components. It was shown that MeCP1 plays an important role in regulation of expression of reporter genes if these genes had methylated promoter regions. Then a protein called MeCP2 was identified (Lewis *et al.,* 1992). It had a number of biochemical and functional characteristics different from MeCP1. For example, its molecular weight was about 50 kD and only one symmetrically methylated CpG was sufficient for specific interaction of MeCP2 with DNA sequence (MeCP1 needed 12 symmetrically methylated CpGs). It was shown that an 85-amino-acid domain in the N-terminal part of protein was responsible for interaction with methylated DNA. This domain was called MBD (methyl-DNA-binding domain). An analogous domain was found for MBD1 protein (formerly PCM1).

In 1998, Hendrich and Bird published a paper about identification and characterization of a family of methyl-DNA-binding proteins (Hendrich and Bird 1998). Three new proteins MBD2, MBD3, and MBD4 were described in addition to previously characterized MBD1 and MeCP2. Expression of all MBD proteins was found in almost all somatic tissues. Moreover, methyl-specific interaction with DNA and co-localization with constitutive heterochromatin were shown for MBD1, MBD2, and MBD4 (Fig.1).

MeCP2 is a protein with molecular weight of about 50 kD. It has two functional domains - MBD and TRD (transcription repression domain). TRD is necessary for interaction of MeCP2 with the mSin3A/HDAC nucleosome remodeling complex and plays crucial role in repression of transcription of target genes. The N-terminal domain (MBD) is responsible for interaction with methylated DNA and, as previously stated, only one symmetrically methylated CpG is sufficient for specific interaction of MeCP2 with DNA sequence. However, the flanking sequences may influence the binding as shown by Cheng *et al.,* 2003. MeCP2 can also interact with other transcriptional factors and co-repressors (like SMRT) (Klose and Bird, 2004). The mRNA coding for MeCP2 is present in all adult somatic tissues tested. Recently, Zhou *et al.,* showed that calcium influx membrane-depolarization dependent during synaptic activity triggers phsphorylation on serine 423 of MeCP2 by CaMKII. This posttranslational modification attenuates its repressor activity promoting BDNF (brain-developed neurotrophic factor) transcription (Zhou *et al.,* Neuron 2006). MeCP2 is the most intensively studied MBD protein because defects (i.e. point mutations) in MeCP2 gene are the cause of Rett syndrome (RTT). RTT is an X-linked dominant disease; it is a progressive neurologic developmental disorder and one of the most common causes of mental retardation in females. Patients appear to develop normally until 6 to 18 months of age, then gradually loss of speech and purposeful hand movements and develop microcephaly, seizures, autism, ataxia, intermittent hyperventilation, and stereotypic hand movements. After initial regression, the condition stabilizes and patients usually survive into adulthood. Also, conditional brain-specific deletion of this gene leads to generation of symptoms analogous to symptoms of Rett syndrome

(Johnston *et al.,* 2003). Some of these symptoms are mental retardation, heavy breathing, stereotyping, increased tone, macroorchidism, memory dysfunctions such as manic depressive psychosis and impairments in adaptative behaviour.

MBD1 is the largest member of the MBD family. It consists of ~640 amino acid residues and has molecular weight ~75 kD. MBD1 interacts with DNA in a methylation-dependent manner in EMSA experiments. But it can repress either methylated or non-methylated transitory transfected constructs. Moreover, MBD1, unlike MBD2 and MBD4, can co-localize with regions of constitutive heterochromatin even in cell with aberrant DNA methylation maintenance system (i.e., without DNMT1) (Hendrich and Bird 1998). In early works, only two functional domains of MBD1 were described-MBD and a domain consisting of three CxxC-motifs (homologous to motifs found in DNA-methyltransferase DNMT1), but another domain was described quite soon. This domain (TRD, by analogy to transcriptional repression domain of MeCP2) is involved in transcriptional repression of reporter constructs as well as one of the CxxC-motifs (Ng *et al.,* 2000). It was shown at the same time that MBD1 can utilize different functional domains for interaction with different proteins. For example, TRD is important for interaction with MCAF (MBD1-containing chromatin-associated factor) (Fujita *et al.,* 2003a). Complexes Suv39h1-HP1*alpha* (Fujita *et al.,* 2003b) and p150-CAF-HP1 (Reese *et al.,* 2003) interact with the MBD domain. MBD1 can also interact with SETDB1 histone-methyltransferase. Protein p150-CAF was characterized as a partner of MBD1 in a yeast two-hybrid screen. Complex CAF (chromatin associated factor) takes part in nucleosome assembly after DNA replication and maintenance of active/inactive chromatin state. One of three subunits of this complex, p150,

interacts with HP1 protein and is involved in maintenance of inactive heterochromatin. Moreover, p150-CAF can interact with PCNA (proliferating cell nuclear antigen) during DNA replication. PCNA, in turn, interacts with DNMT1. p150-CAF attracts MBD1-SETDB1 complex that leads to methylation of lysine 9 of histone H3 and to formation of inactive chromatin.

MBD2 and MBD3 are related proteins. There is a hypothesis that genes coding these factors diverged from a common precursor. The genes have common exon-intron structure and amino acid sequences of MBD2 and MBD3 are identical by 70% (Hendrich *et al.,* 1999a). But despite these similarities, MBD2 and MBD3 have different functions. MBD3 is a structure subunit of one of the major chromatin-remodeling complexes-NuRD (nucleosome remodeling and histone deacetylase complex) (Zhang *et al.,* 1999), whereas MBD2 is only one of the DNA-binding subunits of MeCP1 (Ng *et al.,* 1999). Knockout of MBD3 leads to embryonic lethality in mice immediately after implantation of the embryo (Hendrich *et al.,* 2001). Deletion of MBD2 does not have such serious consequences. Moreover, there are no abnormalities either in imprinting/X-inactivation or repression of mobile elements in MBD2-/- mice. There is only one phenotypic effect of knockout of MBD2-abnormal maternal behavior (Hendrich *et al.,* 2001).

MBD4 is a protein with molecular weight ~60 kD having two functional domains-MBD and a glycosylase domain. MBD4 co-localizes with regions of constitutive heterochromatin and can bind methylated DNA *in vitro*, but despite other MBD-proteins does not participate in regulation of gene expression. This protein belongs to the mismatch repair system. Methylated cytosines are so-called “hot spot” of mutagenesis they can be converted to thymines after

spontaneous deamination. MBD4 is involved in processes of recognition and correction of such mutations (Hendrich *et al.,* 1999b). Deletion of MBD4 leads to accumulation of mutations and higher frequency of carcinogenesis (Miller *et al.,* 2002).

Kaiso is a unique methyl-DNA-binding protein. It does not have classical methyl-DNA-binding domain and interacts with DNA via a zinc finger domain consisting of three zinc fingers of C2H2 type. Another functional domain of Kaiso is the N-terminal 120 amino acid BTB/POZ-domain (Prokhortchouk *et al.,* 2001). This domain is usually located in the N-terminal part of BTB-proteins and serves for homo- or heterodimerization during protein-protein interactions. Most BTB-proteins are transcriptional repressors. Kaiso was initially identified by a yeast two-hybrid screen as a partner of p120-catenin which is an important predominantly cytoplasmic protein interacting with and stabilizing E-cadherin (Davis *et al.,* 2003). It was also shown that Kaiso is a component of double MeCP1 complex. It is a part of a rapidly migrating band called Kaiso-generated band (KGB) (Prokhortchouk *et al.,* 2001). The molecular weight of KGB is ~700 kD. There is a possibility that Kaiso-containing complex serves as an effector in signal-transduction pathway from cell membrane to nucleus and is responsible for repression of target genes in response to extracellular signals.

However, the activity of methyl-binding proteins likely depends on their association with different molecular partners. One example of a multiple function methyl-binding protein is MBD2. MBD2 exerts different activities depending on its association with different partners. MBD2 can be a component of a large protein complex, MeCP1, which represses transcription from densely methylated genes. MeCP1 includes HDAC1, HDAC2, and RbAp46/48 proteins (Ng and

Bird 1999; Ng *et al.,* 1999). By this way, MBD2 targets deacetylase activity at methylated sites. MBD2 can also recruit on methylated DNA a different corepressor complex, Mi-2/NuRD, through its hetrodimerization with MBD3 (Hendrich *et al.,* 2001; Bowen *et al.,* 2004). Other authors have identified a protein, MIZF, that associates with MBD2 and significantly enhances HDAC proteins recruitment and activity (Sekimata *et al.,* 2001). In addition, MBD2 form a complex with DNA methyltransferase 1 (DNMT1) on hemimethylated DNA at replication foci and may help to establish or maintain the repressed state of chromatin (Tatemat *et al.,* 2000). While the above described functions result in transcriptional repression, recently it is emerging that when MBD2 associates with other partners, it may have opposite effects resulting in transcriptional activation of methylated genes. In a recent study, it was shown that the viral protein Tax can activate transcription from the methylated HTLV-1 long terminal repeat (LTR) through the interaction with MBD2 (Ego *et al.,* 2005). The transcription factor GATA-3 can displace MBD2 from a methylated promoter causing the transcriptional reactivation of GATA-3 responsive genes (Hutchins *et al.,* 2002).

We recently described a novel MBD2 associating protein, MBD*in*, showing the unique ability to reactivate MBD2-repressed genes still in methylated status (Lembo *et al.,* 2003). In the case of MBD*in*-mediated reactivation, as well as for GATA-3, transcriptional reactivation occurs prior to demethylation. However, MBD*in* is recruited by MBD2 on methylated DNA and acts by interfering with the ability of MBD2 to associate with the repressor complex rather than through displacement of MBD2 from methyl-CpG sites (Lembo *et al.,* 2003)**.** Recently it has been shown that MBD2 interacts with RNA helicase A, a component of CREB transcriptional co-activator

complex (Fujita et al., 2003). All these mechanisms lead to activation of methylated genes not through a possible active demethylase function of MBD2, but rather, through the association of MBD2 with a variety factors that can be recruited on methylated DNA and determine different interpretations of DNA methylation signals.

We have also recently described another unrelated MBD2 interactor, TACC3, displaying a similar activity on methylated genes. MBD2/TACC3 form a complex *in vivo* with the histone acetyltransferase pCAF. MBD2 could also associate with HDAC2, a component of MeCP1 repression complex. However, we found that complexes formed by MBD2 with TACC3/pCAF and with HDAC2 were mutually exclusive (Angrisano *et al.,* 2006). Moreover, HAT enzymatic assays demonstrated that HAT activity associates with MBD2 in vivo and that such association significantly increased when TACC3 was over-expressed (Angrisano *et al.,* 2006). Thus, TACC3 can be recruited by MBD2 on methylated promoters and is able to reactivate transcription possibly by favouring the formation of a HAT-containing MBD2 complex and, thus, switching the repression potential of MBD2 in activation (Angrisano *et al.,* 2006).

1.4 Chromatin, histone *code* hypothesis and *Code-readers*

The basic unit of chromatin is the nucleosome core particle, which contains 147 bp of DNA wrapped nearly twice around an octamer of the core histones. Each of the core histones has a related globular domain that mediates histone–histone interactions within the octamer, and that organizes the two wraps of nucleosomal DNA. The histone octamer is composed of a central heterotetramer of histones H3 and H4, flanked by two heterodimers of histones H2A and H2B. Each nucleosome is separated by 10–60 bp of 'linker' DNA, and the

resulting nucleosomal array constitutes a chromatin fiber of 10 nm in diameter. Also, each histone harbours an amino-terminal 20–35 residue segment that is rich in basic amino acids and extends from the surface of the nucleosome; histone H2A is unique in having an additional 37 amino acid carboxy-terminal domain that protrudes from the nucleosome. These histone 'tails' do not contribute significantly to the structure of individual nucleosomes nor to their stability, but they do play an essential role in controlling the folding of nucleosomal arrays into higher-order structures. This simple 'beads-on-a-string' arrangement is folded into more condensed, 30 nm thick fibers that are stabilized by binding of a linker histone to each nucleosome core. Such 30 nm fibers are then further condensed *in vivo* to form 100–400 nm thick interphase fibers or the more highly compacted metaphase chromosome structures (Fig.2). Indeed, *in vitro* removal of the histone tails results in nucleosomal arrays that cannot condense past the beads-on-a-string 10 nm fibre (reviewed in Mellor, 2006).

Such an organized DNA/Protein structure, which is called chromatin, carries not only genetic information encoded in the DNA component but also epigenetic information carried by histone proteins in the form of reversible covalent modifications. The function of different chromatin structures depends on a large number of post-translational covalent modifications occurring on specific chromatin components, with different modifications yielding distinct functional consequences. Most of these modifications occur at the unstructured histone "tails" that are predicted to protrude between the gyres of nucleosomal DNA that encircle the histone core.

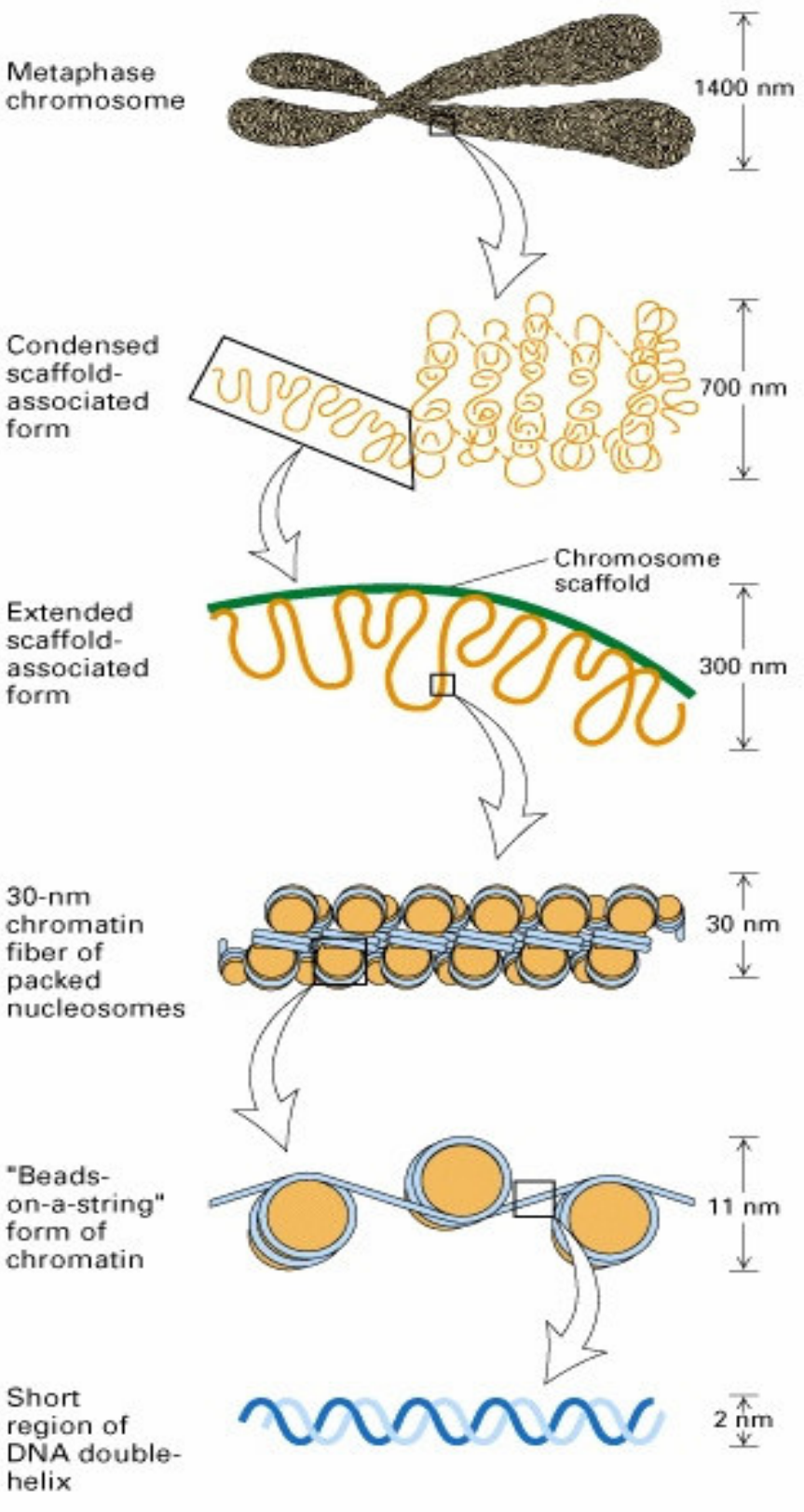

Fig. 2: structure of chromatin and fiber/chromosome assembly

In fact, histones are subject to an enormous number of post-translational modifications, including acetylation and methylation of lysines (K) and arginines (R), phosphorylation of serines (S) and threonines (T), ubiquitylation and sumoylation of lysines, as well as ribosylation.

Adding to the complexity is the fact that each lysine residue can accept one, two or even three methyl groups, and an arginine can be either mono- or di-methylated. The majority of these post-translational marks occur on the amino-terminal and carboxy-terminal histone tail domains, although more and more examples of modifications within the central domains of the histones have been identified. Given the number of new modification sites that are identified each year, it seems likely that nearly every histone residue that is accessible to solvent may be a target for post-translational modification. These modifications may regulate access to the DNA and thus influence nuclear processes, such as transcription. Accumulating evidence suggests that these modifications are part of a **histone code** and that they act as highly selective binding platforms for the association of specific regulatory proteins. Such proteins have the ability ***to read the code*** and modify, directly or indirectly, the chromatin structure.

In recent work, high-resolution chromatin immunoprecipitation has revealed distinct distributions and associations for the different modifications throughout the genome. For example, methylated lysine 9 (K9) or K27 on histone H3 are generally associated with genes whose transcription is repressed, whereas methylated K4, K36, and K79 are found in active chromatin. Also, the combination of H4 K8 acetylation, H3 K14 acetylation, and H3 S10 phosphorylation is often associated with transcription. Conversely, the lack of H3 and H4 acetylation correlates with transcriptional repression in higher

eukaryotes. Moreover, “active” marks show distinct distributions over transcribed genes. The trimethylated form of K4 (K4me3) is found at the 5′ region of active genes together with acetylated lysines. By contrast, K36me3 generally accumulates toward the 3′ region of active genes that is also associated with deacetylated lysines. Particular patterns of histone modifications also correlate with global chromatin dynamics, as diacetylation of histone H4 at K4 and K12 is associated with histone deposition at S phase, and phosphorylation of histone H2A (at S1 and T119) and H3 (at T3, S10 and S28) appear to be hallmarks of condensed mitotic chromatin (reviewed in Mellor, 2006 and in Peterson & Laniel, 2004). Such covalent modifications are performed by specific enzymatic activities which have been now organized into large histone acetyl transferase (HAT), histone deacetylase (HDAC), histone methyltransferase (HMT) and histone kinase families. Each member of a specific family shows a strict specificity for individual histone tails and for specific histone residues. For example, yeast and human Gcn5 and human PCAF preferentially acetylate lysine residues within the histone H3 amino-terminal tail, at K9 and K14. In contrast, the yeast and human NuA4 HAT complexes preferentially acetylate K4, K8, K12 and K16 of histone H4. Even more extreme specificity is seen with HMTs. For instance, the HMT Set7/9 is restricted to mono-methylation of histone H3 at K4, whereas the Dim-5 HMT is a tri-methylase specific for H3 K9. Thus, recruitment of different HATs or HMTs can result in distinct combinations of histone modifications. Cross-talk among different histone marks can also have a profound effect on enzyme activity. For instance, ubiquitylation of H2B K123 by the E2 ubiquitin conjugating enzyme Rad6 is required for subsequent di-methylation of H3 K4 by Set1p or H3 K79 by Dot1p. Prior histone marks can also

inhibit subsequent modifications. For instance, H3 S10 phosphorylation inhibits subsequent H3 K9 methylation, and of course H3 K9 methylation can also block acetylation of this same residue. An excellent example of even more complex cross-talk is exemplified during p53-dependent transcriptional activation *in vitro*. In this case methylation of H4 R3 by protein arginine methyltransferase 1 (PRMT1) stimulates CBP-p300 acetylation of H4 K5, K8, K12 and K16, which in turn promotes the methylation of H3 R2, R17 and R26 by another PRMT family member, CARM1. Thus, positive and negative crosstalk ultimately generates the complex patterns of gene or locus-specific histone marks associated with distinct chromatin states (reviewed in Mellor, 2006 and in Peterson & Laniel, 2004).

Once a pattern of histone modifications is established at a target locus, what happens? Many older models proposed that histone modifications might directly influence either the structure of individual nucleosomes or the folding dynamics of nucleosomal arrays. Indeed one common misconception is that histone modifications that alter the charge of a residue, such as lysine acetylation or serine phosphorylation, will disrupt histone–DNA interactions leading to 'open' or 'active' chromatin structures. There is not actually much evidence for such models. For example, the histone H3 tail contains 13 positively charged amino acids, and thus acetylation of one to four residues will only yield a 10–30% decrease in positive charge, levels that are unlikely to perturb ionic interactions with DNA Consistent with this view, *in vivo* laser crosslinking studies have shown that histone hyperacetylation does not release tails from DNA, and nucleosomes that harbor >12 acetates per octamer wrap DNA normally *in vitro* and have hydrodynamic properties that are nearly identical to unmodified nucleosomes. Although it is true that histone hyperacetylation does

disrupt the folding dynamics of nucleosomal arrays *in vitro*, even in this case 6–12 acetates per nucleosome are required. Although most site-specific patterns of histone modifications have yet to be generated and tested *in vitro*, the prevailing view is that these histone marks may not alter nucleosomal dynamics by themselves (reviewed in Mellor, 2006 and in Peterson & Laniel, 2004). An *in vivo* chromatin fibre is actually an extremely heterogeneous nucleoprotein filament, even at the nucleosome level. First and foremost, in addition to canonical nucleosomes, *in vivo* chromatin arrays also contain novel types of nucleosome that harbour one or more variant isoforms of the core histones. For instance, nucleosomes assembled at yeast and mammalian centromeres contain a histone H3 variant, Cse4/CENP-A, which is essential for centromere function or assembly. Another histone H3 variant, H3.3, replaces canonical histone H3 during transcription, generating a mark of the transcription event. Several variants of histone H2A have also been identified. In most cases, how histone variants alter nucleosome structure or change the folding properties of nucleosomal arrays is not known. It is also not clear how many of these variant nucleosomes are localized to specific DNA sequences. Notable exception, as previously stated, includes the deposition of H3.3 to chromatin of RNA polymerase II transcribed genes via a novel replication-independent assembly complex. Once a histone variant is targeted to a specific locus, there is the potential for creation of novel chromatin domains that have distinct regulatory properties. For instance, the amino-terminal tail of CENP-A lacks the phosphorylation and acetylation sites that are normally modified in histone H3 at transcriptionally active regions. Thus, CENP-A might produce islands of unmodified histone H3 that help to maintain centromeric chromatin in its condensed, inactive state. In contrast,

the histone H3.3 variant contains an amino-terminal tail that is virtually identical to that of histone H3, and thus it seems likely that many of the transcription-associated marks that have been attributed to histone H3 are likely also occurring on the histone H3.3 variant. Thus, incorporation of histone variants into chromatin fibres might enhance chromosome dynamics by creating domains of chromatin with novel properties (reviewed in Mellor, 2006 and in Peterson & Laniel, 2004).

1.5 Controlling enzyme and *code-readers* substrate specificity

How do the enzymes committed to modify chromatin select the specific target locus to be modified? The precise combination of locus-specific histone modifications is due to the combined effects of targeting histone modifying enzymes to specific loci, as well as to the inherent substrate specificity of the enzymes themselves. In the case of transcription, it is clear that targeting of histone modifications is achieved by direct interactions between histone modifying enzymes and DNA sequence-specific transcriptional regulators. For instance, the yeast HAT complex SAGA interacts with the transcriptional activation domains of a variety of yeast gene-specific activator proteins, and these interactions target HAT activity to specific promoter regions *in vivo*. Likewise, unliganded nuclear hormone receptors interact with HDAC complexes, such as NCoR and SMRT, which direct histone deacetylase activity to target genes and contribute to subsequent gene repression. In addition to targeting via gene-specific regulators, the yeast Set1 and Set2 HMTs are found associated with RNA polymerase II holoenzymes, directing histone

H3 K4 or K36 methylation, respectively, during transcriptional elongation (reviewed in Mellor, 2006 and in Peterson & Laniel, 2004).

Targeting histone modification enzymes is not unique to transcriptional control, as DNA repair and centromeric heterochromatin use distinct mechanisms to generate novel patterns of histone marks. In the case of DNA repair, the DNA lesion itself seems to play a central role in targeting histone modifications. For instance, the DNA damage checkpoint kinase ATM (Mec1p in yeast) is recruited to a DNA double strand break where it phosphorylates histone H2A (in yeast) or the histone H2A variant, H2AX (in mammals). Likewise, the human STAGA HAT complex contains DNA binding subunits that recognize DNA backbone-distorting base adducts, targeting histone H3 acetylation activity to sites of nucleotide excision repair (reviewed in Mellor, 2006 and in Peterson & Laniel, 2004).

A quite different strategy uses small noncoding RNAs to target histone H3 K9 methylation to chromatin surrounding mammalian and fission yeast centromeres. These centromeric regions are characterized by repetitive DNA sequences that are transcribed at low levels. The resulting double-stranded RNAs provide substrates for processing by the RNA interference (RNAi) machinery which produces small, 21–23 nucleotide RNAs. Recent studies have shown that an intact RNAi pathway is essential for targeting H3 K9 methylation to centromeric chromatin, and furthermore that these small RNAs actually associate with several chromatin components. The resulting novel ribonucleoprotein complex ultimately targets the Clr4p HMT to centromeric repeats, via either RNA–RNA (nascent centromeric transcripts) or RNA–DNA homologous pairing. Subsequent histone methylation leads to recruitment of proteins such

as Heterochromatin Protein 1 (HP1), which directs formation of highly condensed, heterochromatin structures required for centromere function (reviewed in Mellor, 2006 and in Peterson & Laniel, 2004).

Overlaid on top of these locus-specific marks are the genome-wide, bulk chromatin modifications that may control the day-to-day folding dynamics of chromosomes. For instance, newly synthesized histones that are deposited after passage of replication forks in S phase are enriched in acetylated isoforms of histones H3 and H4, and the formation of condensed chromosomes in mitosis is associated with phosphorylation of histones H3 and H2A (reviewed in Mellor, 2006 and in Peterson & Laniel, 2004).

In addition to these marks linked to the cell cycle, there appears to be a constant battle among HATs and HDACs on a global, nontargeted level that maintains a baseline equilibrium level of histone acetylation throughout the genome. Histone deacetylase inhibitors, such as trichostatin A or sodium butyrate, disrupt this equilibrium, leading to a general increase in bulk histone acetylation. Such genome-wide activities of histone modifying enzymes likely act in concert with the cell-cycle-linked changes in bulk chromatin to enhance the general dynamic nature of eukaryotic chromosomes (reviewed in Mellor, 2006 and in Peterson & Laniel, 2004).

Anyway, targeting the enzymes or the *code-reader* proteins to a specific locus is just the first step in the process. A higher level of specificity must be achieved at the level of the histone tails each of which bears specific modifications. Therefore, a key question is how these simple small chemical modifications, found on relatively large histone proteins, make such a big difference to nuclear processes, particularly gene regulation. Accumulating evidence suggests that evolutionarily conserved domains within code-reader proteins bind to

certain histone modifications with very high specificity, thereby distinguishing the same modification at different residues, for example trimethylation at K4, K9, and K27. Both the sequence environment surrounding the methylated lysine and the distinctive folds in otherwise conserved domains on the reader proteins appear to be major determinants of site discrimination at this level. However, how different states of modification at one residue, such as K4, K4me1, K4me2, or K4me3, are discriminated is far from clear. At the simplest level, different domains associate with different marks. For example, previous work has shown that the **bromodomain** shows a high affinity for acetylated lysine, whereas the **chromodomain** shows high affinity for methylated lysine such as the plant homeodomain (PHD) finger that is found in a variety of proteins and regulates gene expression (reviewed in Mellor, 2006 and in Peterson & Laniel, 2004). Other examples are the chromodomain containing proteins heterochromatin protein 1 (HP1) and polycomb that potentiate the formation of repressive chromatin environments via interactions with methylated K9 or K27, respectively. Even though lysines 9 and 27 are found in an identical local sequence environment (ARKS) swapping the chromodomains of HP1 and polycomb switches the specificity of the lysine that is recognized. This suggests that the chromodomains are involved in both binding target sites and discriminating between them (reviewed in Mellor, 2006). The basis of this discrimination is explained by the high-resolution structures of the polycomb and HP1 chromodomains in complex with H3 peptides. These structures indicate that the chromodomain of polycomb distinguishes K27 from K9 via an extended recognition groove that binds five additional residues preceding the ARKS motif (Fischle et al., 2003). Members of the chromodomain protein (CHD) family have two chromodomain

motifs. In contrast to HP1 and polycomb, CHD1 shows high affinity for methylated K4 on active genes. Moreover, the way in which the CHD1 chromodomains bind to methyl-lysine is different from HP1 and polycomb. For HP1 and polycomb, there is a three-residue aromatic cage surrounding the methyl-lysine, whereas CHD1 recognition involves two aromatic residues. Thus, subtle differences in key residues within otherwise conserved protein folds coupled with the immediate sequence environment of the methylated lysine appear to determine site specificity for the chromodomain. However, the chromodomains appear unable to distinguish between the degree of methylation at their target lysine. Given that mono, di, and trimethylation states of K4 are found in different regions of chromatin (which implies that the different states of methylation are functionally important) other strategies or protein folds for discriminating different methylation states must exist. Recent work shows an extensive network of hydrogen bonds and complementary surface interactions are responsible for the unique recognition of ARTK(me3)QT in the histone peptide by the PHD finger. Although a K4me2 histone peptide has lower affinity for the PHD domain than a similar peptide containing K4me3, this alone does not explain how specificity for K4me3 is achieved in vivo, as is observed in inhibitor of growth 2 (ING2) and bromodomain-proximal PHD finger (BPTF). The PHD finger in BPTF (like many PHD fingers) is found in close proximity to a bromodomain. Intriguingly, the histone code hypothesis predicts the existence of code-reader proteins with double recognition domains such as this PHD-bromodomain module with the potential to recognize combinatorial marks such as trimethylation and acetylation on one or multiple histone tails. As it may be too difficult to discriminate between me2 and me3 using a single protein fold, a

simpler solution might be to discriminate using a **combinatorial code** (in this case the recognition of K4me3 and acetylated lysine) and two different domains (the PHD and bromodomains) on the code reader. In this model, the NURF (nucleosome remodeling factor) complex that contains BPTF might be targeted to the beginning of active genes by the binding of the BPTF bromodomain to acetylated lysines. In this way, the BPTF bromodomain could influence the specificity of the interaction of the PHD finger with K4me3 because K4me3, like acetylated lysine, is concentrated at the beginning of active genes. The helical linker that separates the two domains could act as a molecular ruler, linking a particular combination of me3/acetyl marks to chromatin remodeling by NURF. Whether other multidomain proteins, with helical linkers of different lengths, recognize other combinations of methyl/acetyl marks remains to be determined, but it is a very attractive model for how different states of methylation are discriminated by the code readers. Also, it is clear that the biological function is determined not by the K4me3 mark per se, but by the nature of the code readers that recognize the modification (Shi *et al.,* 2006 and Wysocka *et al.,* 2006). In fact, interaction between the PHD finger and K4me3 may also lead to the repression of active genes. This is the case of ING2, which in response to DNA damage, via K4me3, stabilizes the binding of an mSin3-HDAC1 histone deacetylase complex at the promoters of genes that stimulate proliferation, such as cyclin D1, resulting in histone deacetylation, repression of the active gene and, likely, tumour suppression (reviewed in Mellor, 2006 and in Peterson & Laniel, 2004).

These and other observations, however, have led to the idea that the term “code” may be a misnomer, as it implies that a particular combination of histone marks will always dictate the same biological

function. By analogy, the genetic code is always the same no matter which gene is analyzed, in any cell type or tissue: TAG always means STOP. In the case of histone modifications, however, there are clear exceptions – a particular mark or set of marks can have different or even opposite biological consequences. Adding to the ING2 example, the generally inhibitory H3 K9 methylation can in some cases be associated with actively transcribed genes, and histone acetylation can be inhibitory rather than stimulatory for transcription. Thus, rather than a histone code there are instead clear patterns of histone marks that can be differentially interpreted by cellular factors, depending on the gene being studied and the cellular context (reviewed in Mellor, 2006 and in Peterson & Laniel, 2004).

In conclusion, histone variants, distinct patterns of posttranslational modifications of histones, and histone tail binding proteins all contribute to establishment of various 'open' or 'closed' chromatin domains that have specialized folding properties and biological functions. Some of these domains can be propagated through DNA replication and mitosis, guaranteeing the inheritance of chromatin states to progeny. Furthermore, several nonhistone proteins, such as HP1 or the PRC1 polycomb complex, not only bind to methylated histone lysines, but also recruit the methylase itself, thus providing a means for templating new histone methylation events – for example, following replication fork passage – or for spreading the domain to adjacent nucleosomes. How 'open' states are propagated through cell divisions is not clear, especially as histone lysine acetylation or serine phosphorylation can be rapidly reversed by HDACs or histone phosphatases. Future studies will no doubt continue to identify the functional and biochemical properties of

new chromatin domains as well as to elucidate the principles that govern their maintenance and propagation (reviewed in Mellor, 2006 and in Peterson & Laniel, 2004).

2. AIM OF THE WORK – SUMMARY

Epigenetic mechanisms leading modulation of gene expression are highly dynamic processes. At current stage, several data show the specific and numerous steps of such dynamics during the early stages of external stimulus-dependent gene activation. Nuclear receptors represent good candidates for dynamic analysis of epigenetic processes. However, Retinoic Acid Receptor (RAR)-dependent epigenetic dynamics are not well elucidated.

The present work wants to improve the knowledge of the dynamic epigenetic processes depending on *all-trans* retinoic acid stimulation (ATRA) on ret proto-oncogene locus during the early stages of transcriptional activation in a retinoid-sensitive neuroblastoma cell line system. We demonstrated that RARα is specifically bound to two regulatory regions of RET gene, the promoter and an upstream enhancer, in absence of its ligand. We also show that activation of RAR by addition of ATRA rapidly induces several covalent modifications to the histone tails of nucleosomes encompassing RET locus. Moreover, we provide evidence that such covalent modifications are specific for RET enhancer and promoter regulatory regions. Most of the chromatin modifications driving RET gene activation occur onto a highly methylated DNA enhancer region. We observed that such enhancer is methylated in tissues expressing and not-expression RET mRNA. Here, we show that RET enhancer DNA methylation in SK-N-BE cells does not inhibit activation of transcription per se but serves, likely, as a docking site for MeCP2/HDAC1/mSin3A repressive complex. Such repressive complex is recruited on the methylated DNA enhancer region and keeps RET gene silenced in absence of RAR ligand. ATRA treatment does not modify RARα loading on RET enhancer but induces the

displacement of MeCP2/HDAC1/mSin3A repressive complex from the enhancer region. Also, ATRA induces a specific CpG site demethylation which, together with the previous mechanisms, eventually determine activation of RET mRNA transcription.

3. RESULTS

In order to perform dynamic analysis of epigenetic processes occurring on RET locus we needed ATRA-sensitive cell line systems. Many evidences show that ATRA represents a differentiating-inducing agent in neuroblastoma SK–N–BE cell line, determining up-regulation of several genes as matrix metalloproteinase 9 (MMP-9) and p55 TNF receptor (Chambaut-Guerin AM *et al.,* 1995-2000). Also, it has been shown that SK–N–BE cell line over-expresses RET mRNA upon ATRA stimulation (Tahira *et al.,* 1991; Bunone *et al.,* 1995; Patrone *et al.,* 1997). Thus, we chose SK–N–BE cell line as dynamic system for further analysis.

3.1 All-*trans* retinoic acid induces RET mRNA transcription in SK-N-BE neuroblastoma cell line.

To validate our system, we treated SK-N-BE cells with 1 □M ATRA at different times (3, 6, 12 and 24 hours) and measured RET mRNA expression by quantitative real time PCR (Q-PCR). As expected, ATRA functions as a strong positive modulator of RET expression (Fig. 3). Maximum RET mRNA accumulation is observed after twelve hours of ATRA being about forty five-fold the untreated sample. However, mRNA accumulation is already clearly detectable in SK-N-BE after three hours of ATRA treatment. Also, Bunone and colleagues showed that induction of RET expression by retinoic acid occurred in the absence of de novo protein synthesis in SK-N-BE cell line (Bunone *et al.,* 1995). Taken together, these findings suggest that RET mRNA expression could be directly driven by a retinoic acid receptor-dependent activating complex.

3.2 RARα binds RET promoter and enhancer *in vivo*.

Since ATRA specifically binds and activates Retinoic Acid Receptors (RARs), we asked whether ATRA treatment could determine a direct recruitment of RARα receptor on DNA regulatory regions involved in activation processes of RET transcription. We analyzed the upstream flanking region on RET locus (Ensemble ID: ENST00000355710) including the proximal promoter and a known enhancer region (Lang *et al.,* 2000; Puppo *et al.,* 2002) which is located about 3.5 Kbp upstream the transcription start site (TSS). We identified at least four putative hemi-sites for RARα binding. Specifically, one hemi-site is clearly enclosed in the enhancer region. Therefore, we checked the presence of RARα on both promoter and enhancer in SK-N-BE cells incubated or not with 1 □M ATRA by chromatin immunoprecipitation (ChIP). We observed that RARα is bound to both RET promoter and enhancer even in absence of ATRA stimulation. This is in accord with other reported observations according to which RAR receptors may be resident on regulatory regions of several genes in absence of ligand (Hao *et al.,* 2003). Receptor loading on RET enhancer is significantly higher than that observed on RET promoter (Fig. 4, lanes 1 and 5). Moreover, ATRA treatment does not modify RARα loading on both the regulatory regions (Fig. 4, lanes 2 and 6). To check whether the loading of the receptor was specific for both promoter and enhancer we analyzed an intervening DNA region localized between promoter and enhancer. As shown in figure 4, only a weak signal was detected for RARα binding onto such intervening region in absence of ATRA stimulation (lane 3). Rather, a slight reduction is observed when ATRA is added (lane 4). This data indicates RARα is found to be specifically bound to RET promoter and enhancer in absence of the

external stimulus and ATRA treatment does not modify its loading on promoter and enhancer regions.

3.3 Effects of epigenetic drugs on RET mRNA transcription in SK-N-BE neuroblastoma cell line.

We asked whether chromatin remodelling enzymatic activities could be involved in the process leading to activation of RET mRNA transcription. Therefore, we treated SK-N-BE cells with epigenetic drugs in order to abrogate distinct histone modifying and DNA methylating enzyme activities. Specifically, SK-N-BE cells were pre-incubated with decitabine (or deoxyazacytidine), a DNMTs inhibitor, and pargyline, a specific mono-amino-oxidase inhibitor (MAOi), prior to 1 □M ATRA stimulation. As shown in figure 5a, both decitabine and pargyline up-regulate RET mRNA. Specifically, decitabine determines a two- to three-fold induction of RET mRNA. We have also tested decitabine in another neuroblastoma cell line, SH – SY5Y, and we observed a more potent RET up-regulation (data not shown). Interestingly, pargyline shows a remarkable effect determining a six- to eight-fold induction of RET mRNA. We also inhibited HDACs activity by trichostatine A (TSA), an unselective HDAC inhibitor (HDACi), but we did not obtain clear results (data not shown) since TSA exerts a strong pro-apoptotic effect in neuroblastoma cells (Subramanian *et al.,* 2005). However, other HDACi, as sodium butyrate, may induce RET up-regulation in cell lines displaying low levels of its mRNA (Puppo *et al.,* 2002).

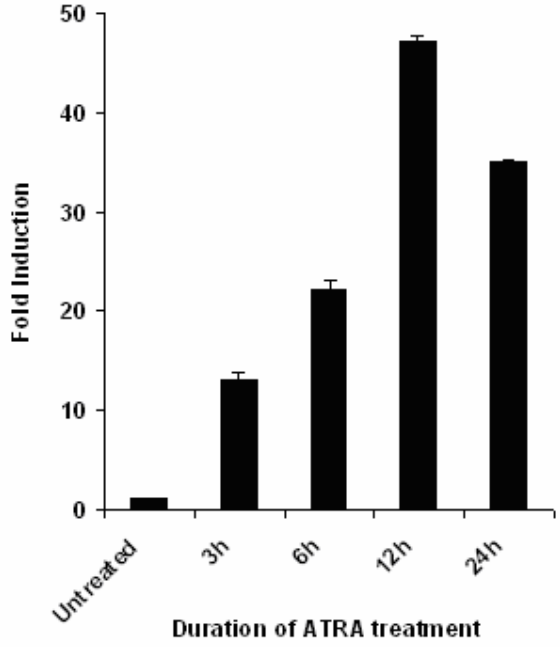

Fig. 3 Activation of transcription of RET mRNA upon ATRA treatment. SK-N-BE cells were incubated with 1 μM ATRA for indicated time periods before harvesting for total RNA collection and retro-transcription. 20 ng of cDNA was used as template for each reaction. The experiment was repeated three times and quantitative PCR analysis were performed in triplicates.

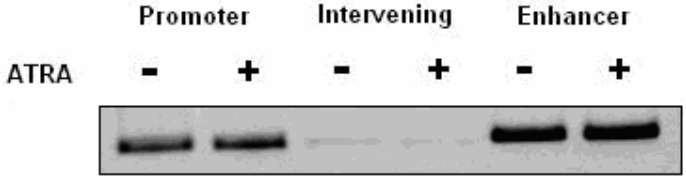

Fig. 4 Occupancy of RARα onto the RET locus. SK-N-BE cells were incubated with 1 μM ATRA for 45 minutes before harvesting for ChIP assays. Chromatin samples were immunoprecipitated with anti-RARα antibody and amplification was performed with enhancer- (-4002 to -3820), intervening region- (-1860 to -1692) and promoter-specific (-513 to -373) primers. The experiment was performed keeping dNTPs in excess.

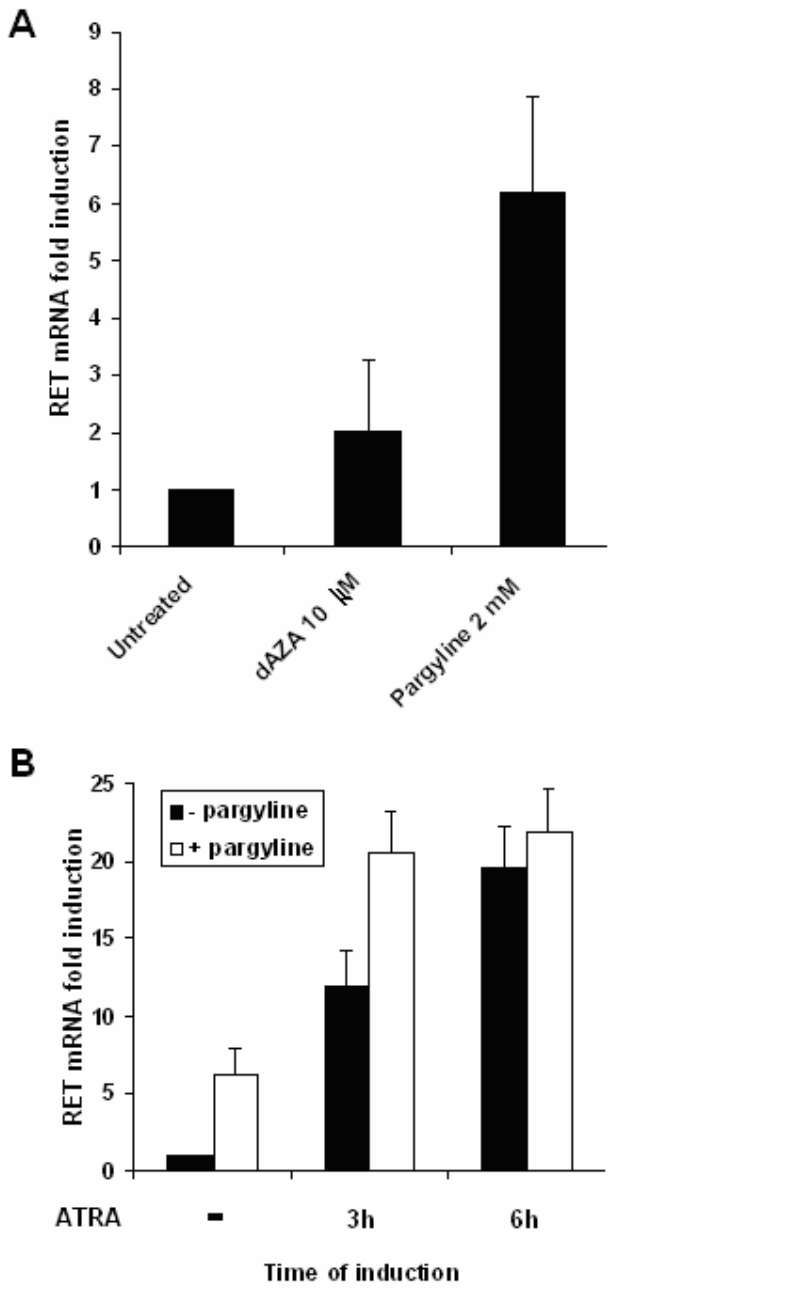

Fig. 5 Effects of epigenetic drugs on RET mRNA levels. (A) Decitabine and pargyline up-regulate RET mRNA. SK-N-BE cells were incubated with 10 µM decitabine for 48h and 2 mM pargyline for 3h. Optimal drug concentration was obtained by performing curves of concentration (data not shown). (B) Pre-incubation with pargyline enhances RET mRNA transcription induced by ATRA treatment. SK-N-BE cells were pre-incubated (3h) with 2 mM pargyline before the incubation with 1 µM ATRA for indicated time periods. After the treatment, cells were harvested for total RNA collection and retro-transcription. 20 ng of cDNA was used as template for each reaction. The experiment was repeated three times and quantitative PCR analysis were performed in triplicates.

Moreover, while decitabine seems to have no relevant effect on ATRA treatment (data not shown), pargyline enhances the effect of ATRA (Fig. 5b) in terms of RET up-regulation. This latter observation, together with the basal RET mRNA induction due to pargyline, cannot be explained since we have no evidence of any molecular mechanism involved in the process. However, these observations remain much interesting since pargyline, in ATRA-stimulated neuroblastoma cells, acts contrary to what has been largely observed for androgen and estrogen receptors. Also, the observed different responsiveness to decitabine among the two cell lines suggests the importance of factors, which may be missing in either cell line, regulating the mechanism of transcription control (see discussion).

3.4 *all-trans* retinoic acid induces multiple epigenetic modifications restricted on RET promoter and enhancer.

Since ATRA induces RET mRNA transcription soon after the treatment and epigenetic drug treatment modifies RET mRNA levels, it is likely that activation of RET transcription goes with epigenetic modifications. We though that such modification might occur upon nucleosomes encompassing RET locus. Therefore, we treated SK-N-BE cells with ATRA at different times (0,5 – 1 – 3 – 6 hours) and prepared chromatin. Further, we quantitatively determined the major histone modifications of the histone tails of nucleosomes encompassing RET promoter and enhancer by coupling ChIP assays and Q-PCR.

H3 Lysine 4 Methylation. We analyzed the three methylated form of lysine 4 on histone H3 (H3K4me, H3K4me2, H3K4me3) by immunoprecipitating chromatin with antibodies directed against the three specific modifications. We measured H3K4me2 levels on both

RET promoter and enhancer observing a strong decrease of the specific signal. Specifically, after thirty minutes of ATRA stimulation H3K4me2 levels are about 14% and 26% of the relative untreated sample chosen as reference for promoter and enhancer, respectively (Fig. 6a). After the sharp reduction, H3K4me2 signal slightly increases until the end of ATRA stimulation for both promoter and enhancer (Fig. 6a). We demonstrated the specificity of the observed process by analyzing an intervening DNA region located at half distance between promoter and enhancer. Figure 6a clearly shows the H3K4me2 signal for the intervening region was much lower than that observed for promoter and enhancer. Since H3K4me3 modification is associated with the 5' region of activated genes (reviewed in Mellor, 2006 and in Peterson & Laniel, 2004) we asked weather H3K4me2 could be methylated and transformed in H3K4me3 or alternatively demethylated, giving H3K4me. We measured, then, H3K4me3 levels onto both promoter and enhancer. As expected, H3K4me3 signal levels on RET promoter increase soon after ATRA stimulation. Specifically, H3K4me3 levels increase after thirty minutes of ATRA treatment and maximum levels, which are three-fold the untreated sample, are observed at 30 and 180 minutes (Fig. 6b). Conversely, H3K4me3 signal on RET enhancer only slightly increases in respect to the untreated sample after thirty minutes of ATRA treatment, remaining unchanged until the end of the treatment. Quantitatively, H3K4me3 signal on the promoter is two-fold the H3K4me3 signal on the enhancer (Fig. 6b). Figure 6b clearly shows the H3K4me3 signal for the intervening region was much lower than that observed for promoter and enhancer (1 – 10 % input DNA values), indicating the process is specific for both the regulatory regions. H3K4me signal was detected predominantly on the enhancer

at the steady state (Fig. 6c). However, ATRA stimulation induces a sharp increase in H3K4me levels on both promoter and enhancer after 1 – 3 hours followed by a decrease at 6 hours (Fig. 6d). The process is not specific for the regulatory regions as we found that after 1 hour of ATRA treatment (but not after 30 minutes) a strong H3K4me signal was detectable by Q-PCR for the intervening region (Fig. 6d).

H3 Lysine 27 Methylation. Methylation of histone H3 lysine 27 (H3K27) is highly correlated with genomic silencing (Margueron *et al.,* 2005; Schubert *et al.,* 2006) and, recently, with retinoic acid signaling (Lee MG *et al.,* 2007; Villa *et al.,* 2007). Since RARα is loaded on both RET promoter and enhancer during the activation of RET mRNA transcription, we asked whether H3K27me3 was present on the histone tails of nucleosomes encompassing RET locus and whether this modification decreased after ATRA stimulation. We detected H3K27me3 signal on both RET enhancer and promoter in absence of stimulation. However, H3K27me3 signal detected on the enhancer was much higher than that detected on the promoter (Fig. 7a). After ATRA treatment, H3K27me3 levels decreased during the activation process both on enhancer and promoter (Fig. 7a-b). However, the observed reduction occurring on the enhancer is much evident than that observed on the promoter. The process is much more specific for the enhancer region than the promoter region, as the signal for the intervening sequence is similar to that detected for the promoter (Fig. 7a-b).

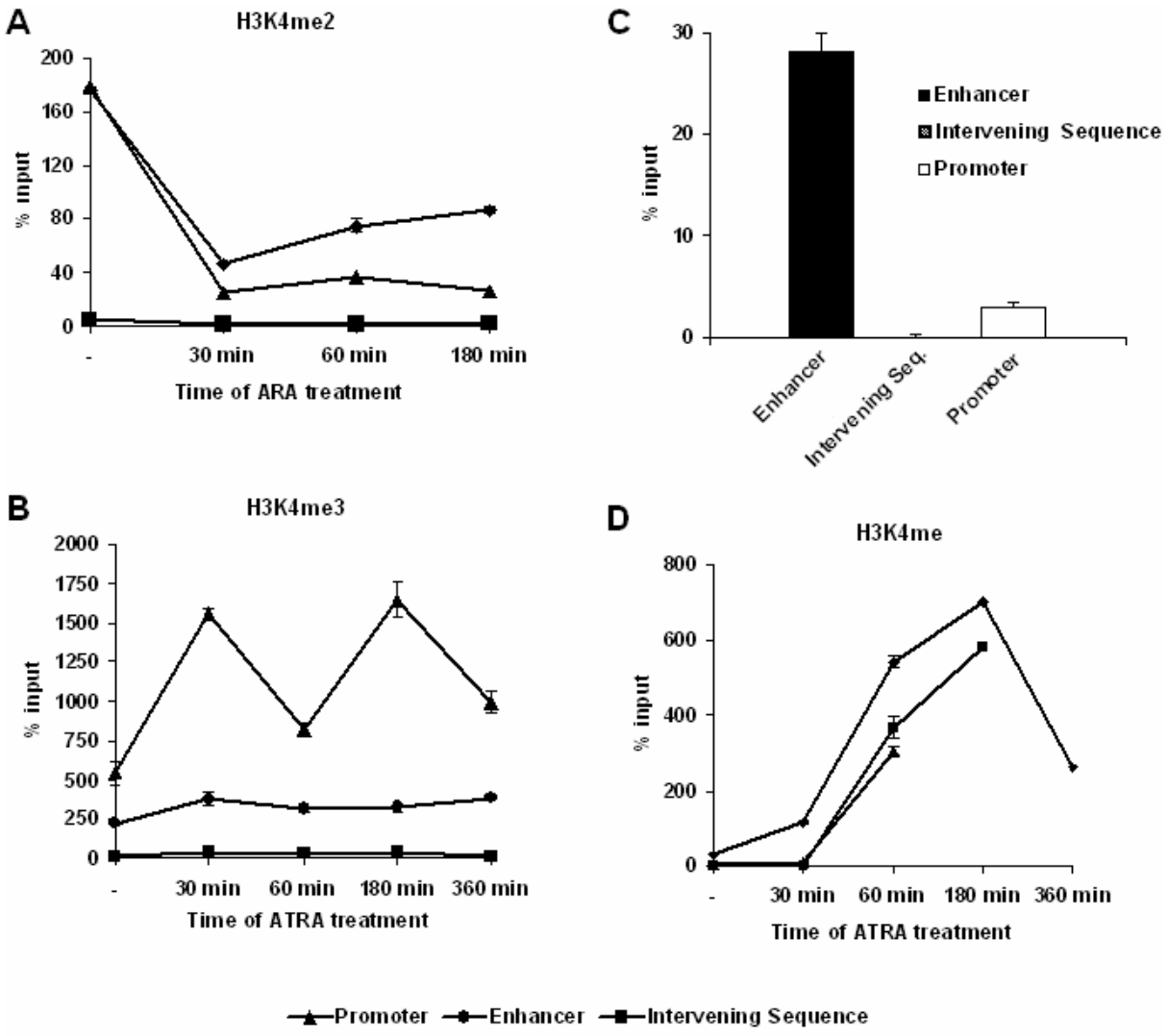

Fig. 6 H3K4 methylation on RET locus. SK-N-BE cells were incubated with 1 μM ATRA for indicated time periods before harvesting for ChIP assays. 80 μg of chromatin were immunoprecipitated with anti-H3K4me2 (**A**), anti-H3K27me3 (**B**), anti-H3K4me (**C** and **D**) antibodies for each analyzed time. Amplifications were performed with enhancer- (-3458 to -3398), intervening region- (-1678 to -1605) and promoter-specific (+654 to +718) primers. ATRA induces reduction of H3K4me2 signal (**A**). This is consistent with H3K4me3 signal increase (**B**). H3K4me signal is found to be predominant on RET enhancer in untreated sample (**C**). H3K4me2 and H3K4me3 signals are specific for both promoter and enhancer. Conversely, H3K4me signal is found on the intervening sequence after 60 minutes of ATRA treatment (**D**). Data are presented as percentages of input DNA before immunoprecipitation (mean ± SE).

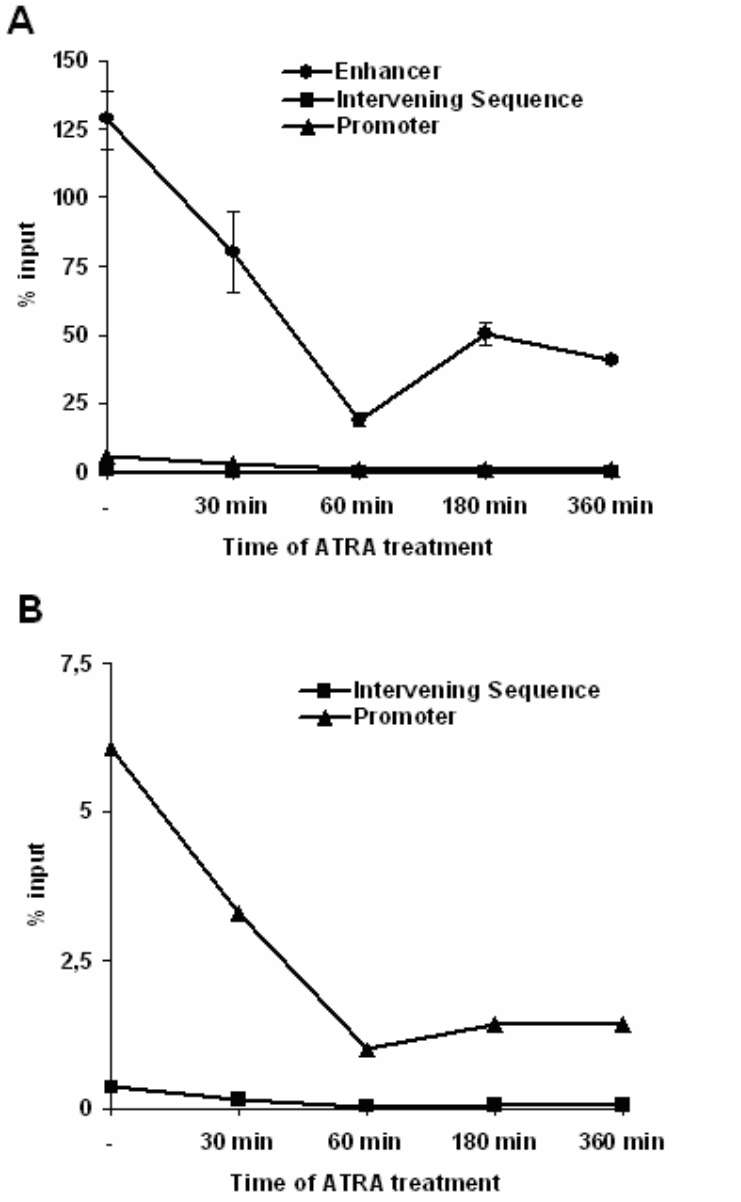

Fig. 7 H3K27 demethylation on RET locus upon ATRA treatment. SK-N-BE cells were incubated with 1 μM ATRA for indicated time periods before harvesting for ChIP assays. 80 μg of chromatin were immunoprecipitated with anti-H3K27me3 antibody for each analyzed time. Amplifications were performed with enhancer-, intervening sequence- and promoter-specific primers (see Fig. 6 and Materials and Methods). ATRA treatment induces demethylation of lysine 27 on histone H3 upon RET enhancer (**A**) and promoter (**B**). However, the process is much more specific for the enhancer region than the promoter region, as the signal for the intervening sequence is similar to that detected for the promoter (**B**). Data are presented as percentages of input DNA before immunoprecipitation (mean ± SE).

H3 Lysine 9 Methylation. H3K9 methylation is usually associated with gene silencing (reviewed in Mellor, 2006 and in Peterson & Laniel, 2004). However, we found that nucleosomes encompassing the enhancer and the promoter regions were di-methylated in lysine 9 in untreated samples. Quantitatively, we found that H3K9me2 signal on RET enhancer is two-fold higher than that observed on RET promoter, in absence of ATRA stimulation. Also, ATRA treatment induces a general increase of H3K9me2 signal, though the process mainly involves the enhancer region. Specifically, after thirty minutes of treatment, H3K9me2 signal is two-fold and four-fold the related untreated sample for RET promoter and enhancer, respectively (Fig. 8a). A subsequent reduction of the signal is also observed (1h and 3h). While H3K9me2 signal on RET promoter continues decreasing until the end of the treatment, a sharp increase is observed on RET enhancer after six hours of ATRA stimulation (Fig. 8a). The observed trend for H3K4 methylation could be explained by a process of demethylation starting from H3K9me3 that leads to an enrichment of H3K9me2, followed by a further demethylation that leads to H3K9me and a concomitant reduction of H3K9me2 signal (see discussion).

H3 Acetylation. Histone acetylation is highly associated with transcriptionally active chromatin (reviewed in Mellor, 2006 and in Peterson & Laniel, 2004). Therefore, we checked whether histone H3 could be acetylated during the process of activation of transcription triggered by ATRA. We found global acetylation on both promoter and enhancer prior to ATRA stimulation (Fig 9). The levels of acetylated H3 on both regions increased rapidly in SK-N-BE cells after inclusion of ATRA in culture medium (Fig. 9). However, there were clear differences in the dynamics of H3 acetylation between

promoter and enhancer. In general, these modifications peaked on both enhancer and promoter after one hour of ATRA treatment. However, the enhancer signal increases more gradually than that observed on promoter, which, in turn, rapidly increases from 30 to 60 minutes. This is also true for the reduction of the signal: while H3Ac signal decreases gradually on the enhancer, a rapid decrease is observed on the promoter after three hours of ATRA treatment. However, at six hours a peak is observed on promoter but not on the enhancer region (Fig. 9).

Figure 10 shows the overall distribution of histone modifications occurring on RET locus during ATRA treatment. Every observed modification clearly shows specificity for distinct DNA regions which are involved in modulation of gene expression. Specifically, enhancer region is predominantly involved in demethylation of K27, while promoter region is predominantly involved in trimethylation of K4. Additionally, acetylation involves both promoter and enhancer but shows different kinetics depending on which regulatory region is observed. Moreover, all the modifications analyzed do not involve the intervening region (except for H3K4me), underscoring the specificity of the observed phenomenon and the importance of the two regulatory regions.

Taken together, increasing in H3K4 tri-methylation and H3 global acetylation levels concomitant to H3K27 demethylation and RET mRNA accumulation upon ATRA treatment indicate that *all-trans* retinoic acid, likely via RARα-dependent complexes, induces activating epigenetic modifications on nucleosomes encompassing RET locus activating RET mRNA transcription in SK-N-BE cells.

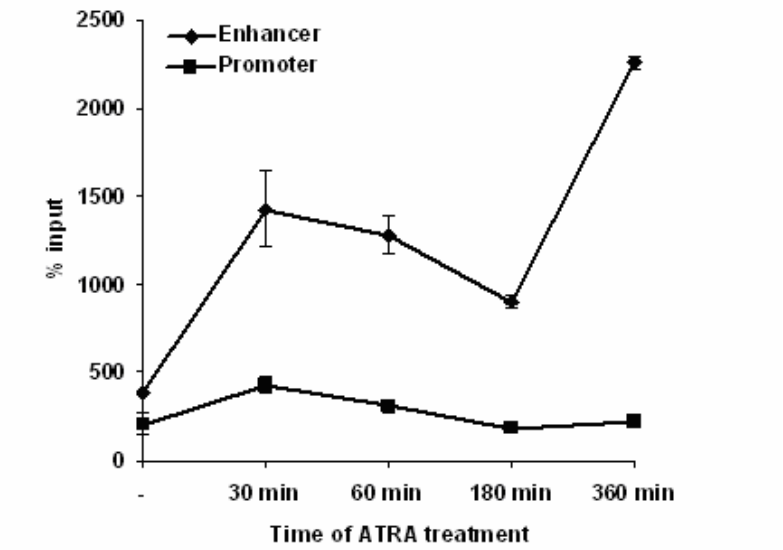

Fig. 8 H3K9 methylation levels. SK-N-BE cells were incubated with 1 μM ATRA for indicated time periods before harvesting for ChIP assays. 80 μg of chromatin were immunoprecipitated with anti-H3K9me2 antibody for each analyzed time. Amplifications were performed with enhancer-, intervening sequence- and promoter-specific primers (see Fig. 6 and Materials and Methods). ATRA treatment induces increase followed by decrease of H3K9me2 signal. This could be explained by a process of demethylation starting from H3K9me3 (see text and discussion). Data are presented as percentages of input DNA before immunoprecipitation (mean ± SE).

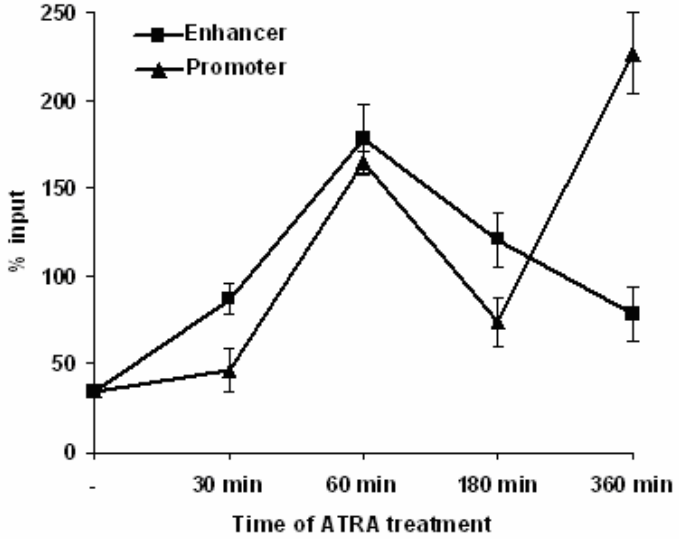

Fig. 9 H3 acetylation levels. SK-N-BE cells were incubated with 1 μM ATRA for indicated time periods before harvesting for ChIP assays. 80 μg of chromatin were immunoprecipitated with anti-H3Ac antibody for each analyzed time. Amplifications were performed with enhancer-, intervening sequence- and promoter- specific primers (see Fig. 6 and Materials and Methods). ATRA treatment clearly induces H3 acetylation. Maximum effect is observed after one hour of treatment. Data are presented as percentages of input DNA before immunoprecipitation (mean ± SE).

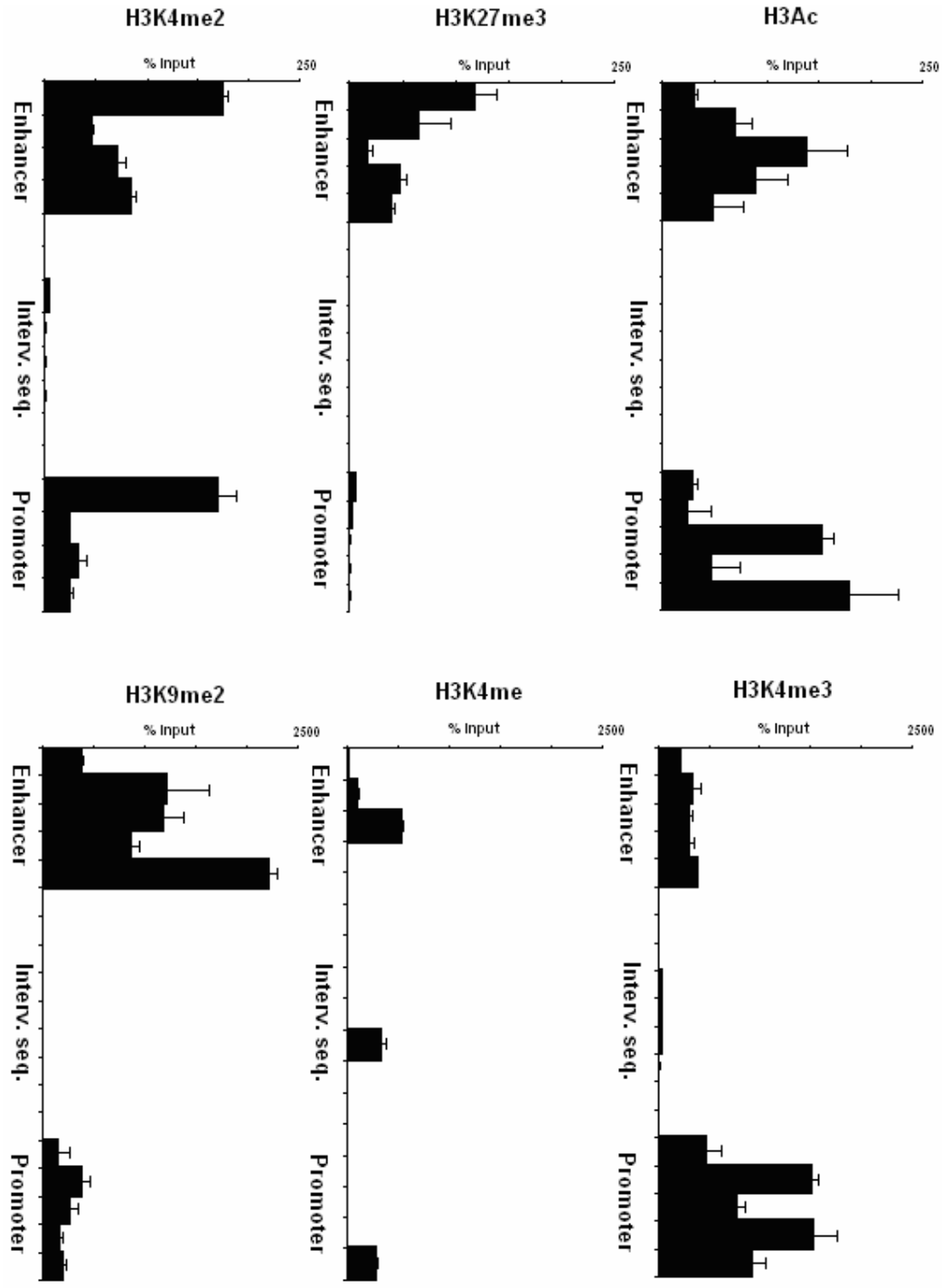

Fig. 10 Overall scheme of histone modifications occurring on nucleosomes encompassing RET locus.

3.5 DNA methylation pattern is slightly but rapidly modified by *all-trans* retinoic acid treatment.

Since RET locus undergoes remarkable epigenetic modifications on histones following ATRA treatment, we asked whether also DNA methylation could be involved somehow in the modulation of the epigenetic processes leading the activation of RET mRNA transcription. Therefore, we analyzed the DNA methylation profiles of the promoter region and the enhancer region. We collected genomic DNA from SK-N-BE cells and analyzed DNA methylation profiles by bisulfite treatment and sequencing. First, we looked at promoter methylation. RET promoter is embedded in a CpG island that was shown to be unmethylated in peripheral white blood cells (Munnes *et al.,* 1998). We analyzed a region spanning from -739 to -358 in respect to Transcription Start Site (TSS) which likely represent the CpG island edge and contains 23 CpG sites. Only the more distal three sites were partially methylated, while the remaining sites were almost completely unmethylated without ATRA stimulation: observed promoter methylation index (MI) was 3.62% (Fig. 11). The same analysis was performed on the enhancer region. RET enhancer is localized about 3.5Kb upstream TSS. Specifically, we analyzed a region spanning from -3459 to -3038 which includes 10 CpG sites and two previously described binding sites for Pax3 and Sox10 factors (Lang *et al.,* 2000, 2003; Puppo *et al.,* 2002). Figure 11 shows that unstimulated cells have a heavily methylated RET enhancer whose MI is 98,3%. These data are supported by the observation that the enhancer region which was analyzed in tissues expressing or not-expressing RET, showed a lower but comparable MI of about 75% (data not shown). Since ATRA induces RET mRNA transcription in SK-N-BE cells modifying the chromatin configuration of RET locus,

we asked whether DNA methylation might be modified by the treatment as well. We stimulated SK-N-BE cells with ATRA at different times (6, 12, 24 hours) and analyzed promoter and enhancer DNA methylation profiles by bisulfite treatment and sequencing. We found that promoter DNA methylation is only poorly affected by ATRA treatment. The highest variation of promoter MI is observed after six hour of ATRA treatment and consists of a 1.2% reduction in respect of the untreated sample (Fig 11). Conversely, a more evident variation of MI is observed on the enhancer region after ATRA treatment. Specifically, the enhancer MI is reduced by 14% after twelve hours of treatment. However, the starting MI of the enhancer region is restored after twenty four hours of ATRA treatment (Fig. 11). We considered the observed reduction of enhancer MI a remarkable phenomenon to be further analyzed. However, bisulfite treatment and sequencing is not quantitative and may be biased by some intrinsic features of the technique itself. Thus, in order to quantitatively evaluate the MI reduction observed in SK-N-BE cells upon ATRA treatment we used Pyrosequencing™ technology (reviewed in Ronaghi, 2001 and Fakhrai-Rad, Pourmand and Ronaghi, 2002). Specifically, we analyzed three CpG sites in the enhancer region, which correspond to CpG 1 (-3455-4), 2 (-3358-7) and 3 (-3339-8) analyzed by cloning. We found that a specific CpG site, which is 100% methylated in untreated samples, undergoes a 16% loss of methylation after six hours of ATRA treatment (MI: 84%) (Fig. 12a). Such MI index reduction is observed until the end of treatment (Fig. 12a). Concomitantly, we analyzed four CpG sites on the promoter region, which correspond to CpG 4 (-567), 5 (-555), 6 (-539), 7 (-537) analyzed by cloning. As expected, DNA methylation is very low and is consistent with the CpG island spanning the promoter region (Fig.

12b). Higher MI, which is 12.6%, is observed in the first CpG site analyzed, in absence of ATRA. This site undergoes a 2% demethylation upon ATRA treatment. Conversely, sites 3 and 4, which are unmethylated in absence of ATRA, get methylated (4% and 1.4%, respectively) after six hours of ATRA treatment (Fig. 12b). However, such differences are similar to that observed for the assay control site, suggesting they may be background signals.

Taken together, these findings indicate that ATRA poorly affect promoter DNA methylation but induces a moderate demethylation of a specific CpG site on RET enhancer though the overall enhancer DNA methylation remains remarkable. Because we cannot say this CpG demethylation is sufficient to contribute to activation of RET mRNA transcription upon ATRA stimulation, further analysis addressing the behaviour of transcriptional mediators depending on DNA methylation are needed.

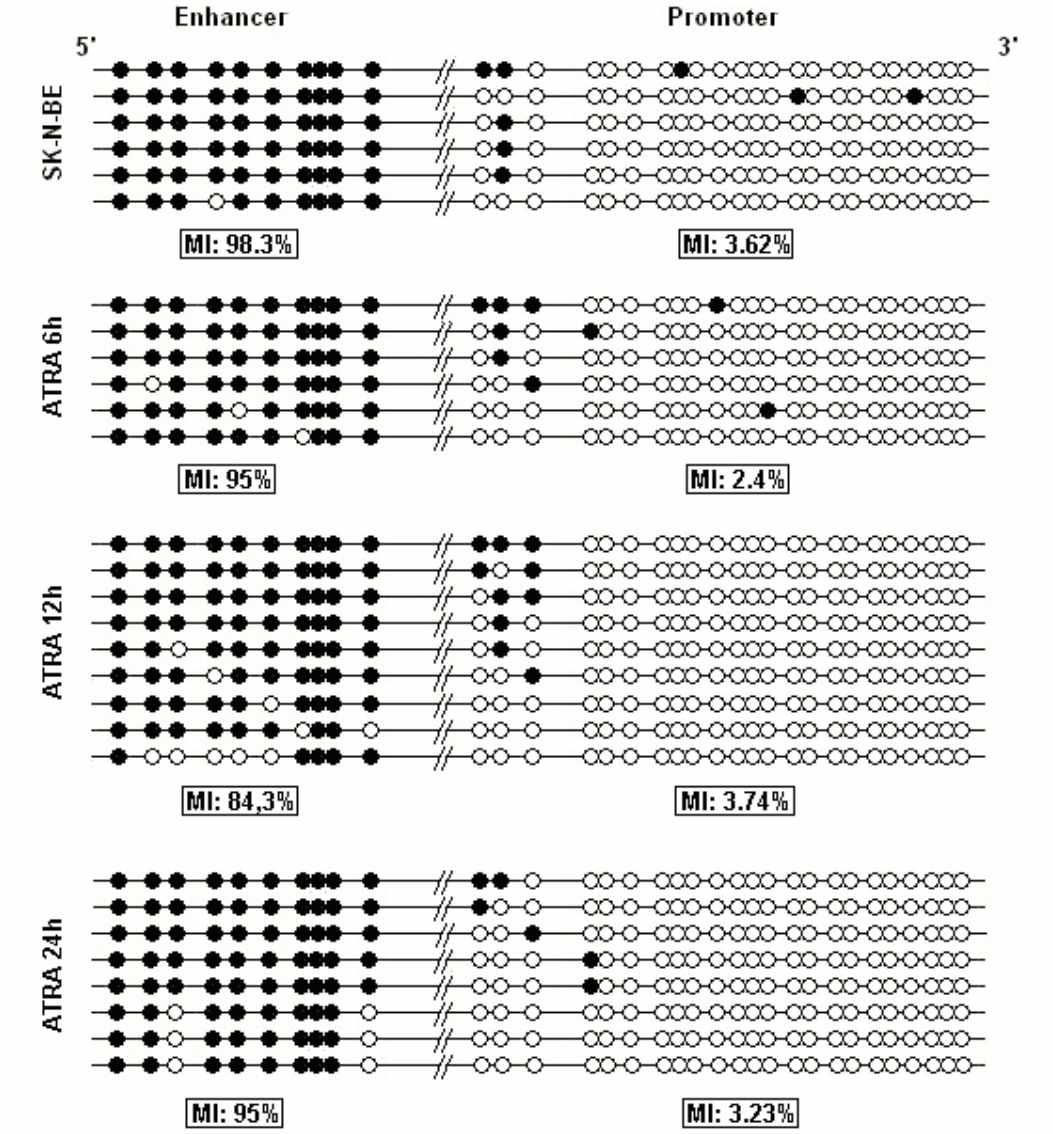

Fig. 11 Promoter and Enhancer DNA methylation profiles upon RET. Full circles represent methylated CpGs, empty circles represent unmethylated CpGs. Methylation Index (MI) for both promoter and enhancer is also shown. SK-N-BE cells were incubated with 1 μM ATRA for indicated time periods before harvesting for genomic DNA collection. Samples were treated with sodium bisulfite and amplified by PCR with enhancer- (-3459 to -3038) and promoter-specific (-739 to -358) primers. 40 ng of bisulfite converted DNA were used for each amplification. Samples were cloned and each clone was subsequently sequenced. At least 10 molecules were analyzed for each sample.

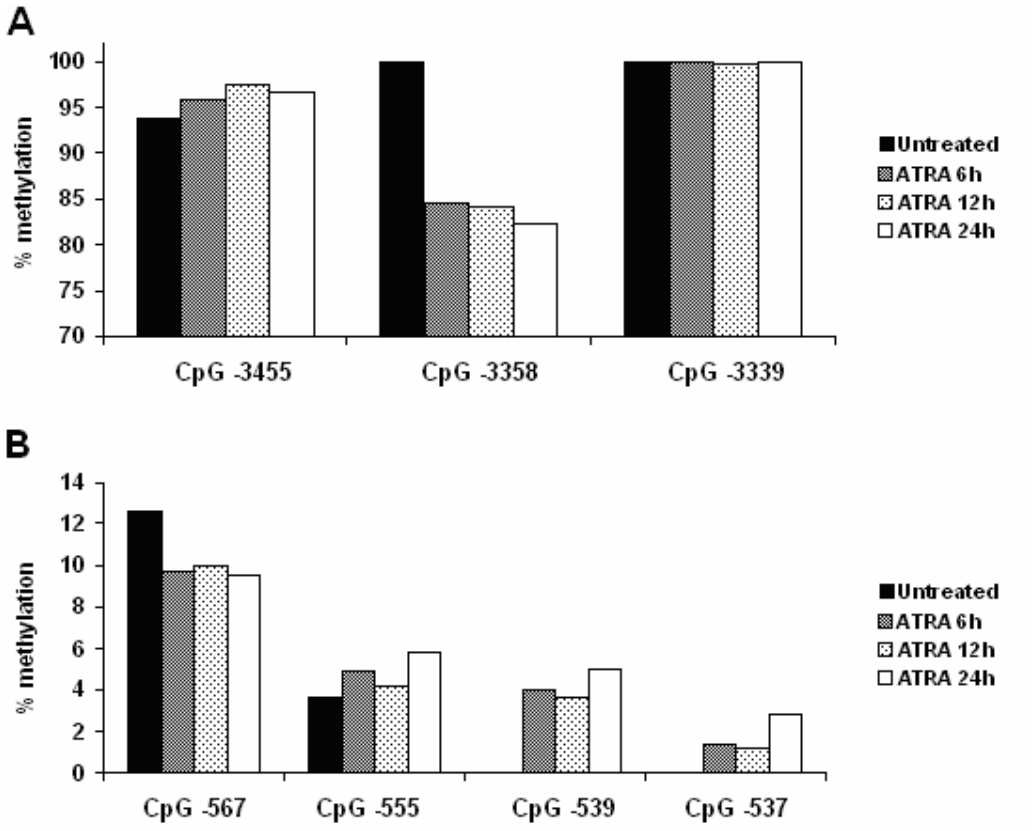

Fig. 12 Pyrosequencing™ analysis of RET DNA enhancer and promoter methylation. SK-N-BE cells were incubated with 1 μM ATRA for indicated time periods before harvesting for genomic DNA collection. Samples were treated with sodium bisulfite and enhancer (-3459 to -3038) and promoter (-739 to -358) regions were amplified by PCR with pyrosequencing-specific primers. Samples were subsequently sequenced with enhancer- and promoter-specific sequencing primer. ATRA treatment induces a 16% reduction of methylation of CpG -3358 on RET enhancer (**A**) but only poorly affects DNA methylation on RET promoter (**B**). Data are presented as percentages of DNA methylation per CpG site.

3.6 All-*trans* retinoic acid induces MeCP2/HDAC1/mSin3A complex displacement from RET enhancer facilitating transcription.

We asked whether Methylated DNA Binding Proteins (MBPs) might be involved somehow in the process. MBPs are generally associated with gene silencing, so we postulated that some MBPs could be involved in keeping RET gene silenced. Therefore, we collected chromatin from SK-N-BE cells and checked the presence of MBD2 and MeCP2 on both RET enhancer and promoter by quantitative ChIP assays. We already knew that RET promoter was unmethylated and we would have not expected to find MBPs bound to it. In fact, we could not detect any MBD2 signal on promoter. However, MBD2 signal was undetectable also on the enhancer region (data not shown). Conversely, while we could detect only a very low MeCP2 signal on RET promoter, we found a remarkable MeCP2 signal on the enhancer region (Fig. 13a). No signal was detected on the intervening region (Fig 13a). These data indicates that MeCP2, but not MBD2, strictly binds RET enhancer but not the promoter region, which is embedded into an unmethylated CpG island. Further, we asked whether ATRA, which induces a CpG specific-demethylation, could determine any alteration of MeCP2 binding on RET enhancer during the process leading RET mRNA transcription. We stimulated SK-N-BE cells with ATRA for sixty minutes and then we removed ATRA from cell culture medium, keeping cells in culture for additional four hours (treatment lasted totally 5 hours). Interestingly, MeCP2 signal decreases after five hours of incubation. Quantitatively, MeCP2 signal in untreated cells was at least five-fold the signal detected in SK-N-BE cells after five hours of ATRA treatment (Fig. 13b). No MeCP2 signal is detected on

the intervening sequence (Fig. 13b) indicating that the process is specific for RET enhancer. Also, since no MBPs signal has been detected on the intervening sequence, we think it is *bona fide* unmethylated. This data indicates that ATRA induces MeCP2 displacement from RET enhancer concomitantly to activation of RET mRNA transcription.

MeCP2 is a component of a repression complex which is often constituted by HDAC1 and mSin3A. First, we asked whether HDAC1 and mSin3A were part of the complex including MeCP2. Thus, we checked HDAC1 and mSin3A presence on RET promoter and enhancer by quantitative ChIP assays. Both HDAC1 and mSin3A signals were found on RET enhancer (Fig. 14a-b, untreated). Quantitatively, HDAC1 and mSin3A signals on RET enhancer were comparable to that observed for MeCP2. A four-fold lower signal was detected for both HDAC1 and mSin3A on the promoter region (Fig. 14a-b, untreated). These data indicate that the two repressor molecules are present on RET enhancer and, partially, on RET promoter. Also, their levels on RET enhancer are quantitatively comparable and this suggests the possibility they form a complex. Further, since ATRA treatment induces MeCP2 displacement from RET enhancer, we asked whether it could also modify the levels of HDAC1/mSin3A, thus contributing to activation of gene transcription. We stimulated SK-N-BE cells with ATRA as described for MeCP2 experiments and quantitatively measured HDAC1 and mSin3A levels on RET enhancer. We found that both HDAC1 and mSin3A levels decrease on RET enhancer upon ATRA treatment (Fig. 14a-b).

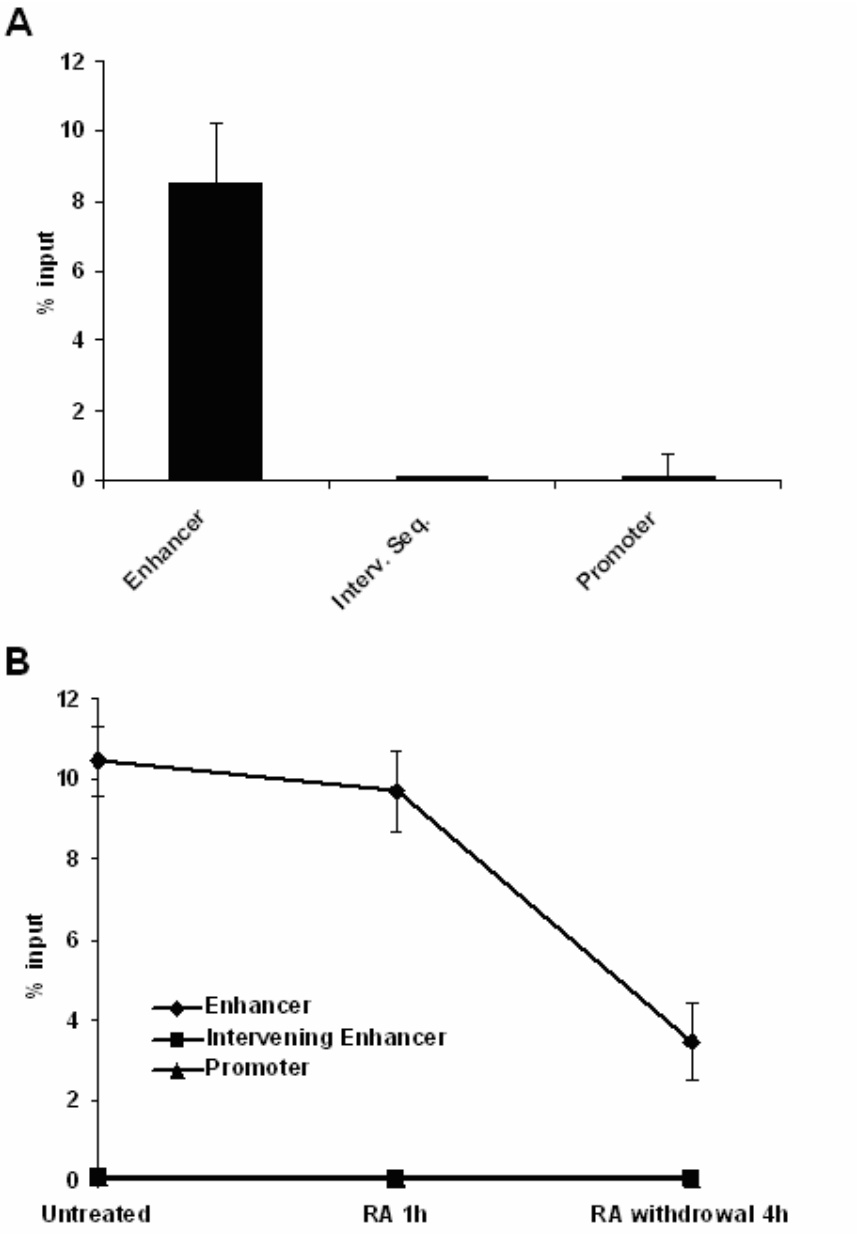

Fig 13 Occupancy of MeCP2 onto the RET locus. (**A**) MeCP2 is strictly bound to RET enhancer. 80 μg of chromatin, collected from SK-N-BE cells, were immunoprecipitated with anti-MeCP2 antibody, and bound DNA was quantified as described in Fig. 6. Values, expressed as percentages of input DNA, are the mean – SE. Error bars are 2*SE. (**B**) ATRA treatment induces a displacement of MeCP2 from RET enhancer. SK-N-BE cells were incubated with 1 μM ATRA for 60 minutes. Then, ATRA was washed away with PBS and cells were subsequently cultured for additional 4 hours. Chromatin samples were immunoprecipitated with anti-MecP2 antibody and bound DNA was quantified as described in Fig. 6. Values, expressed as percentages of input DNA, are the mean ± SE.

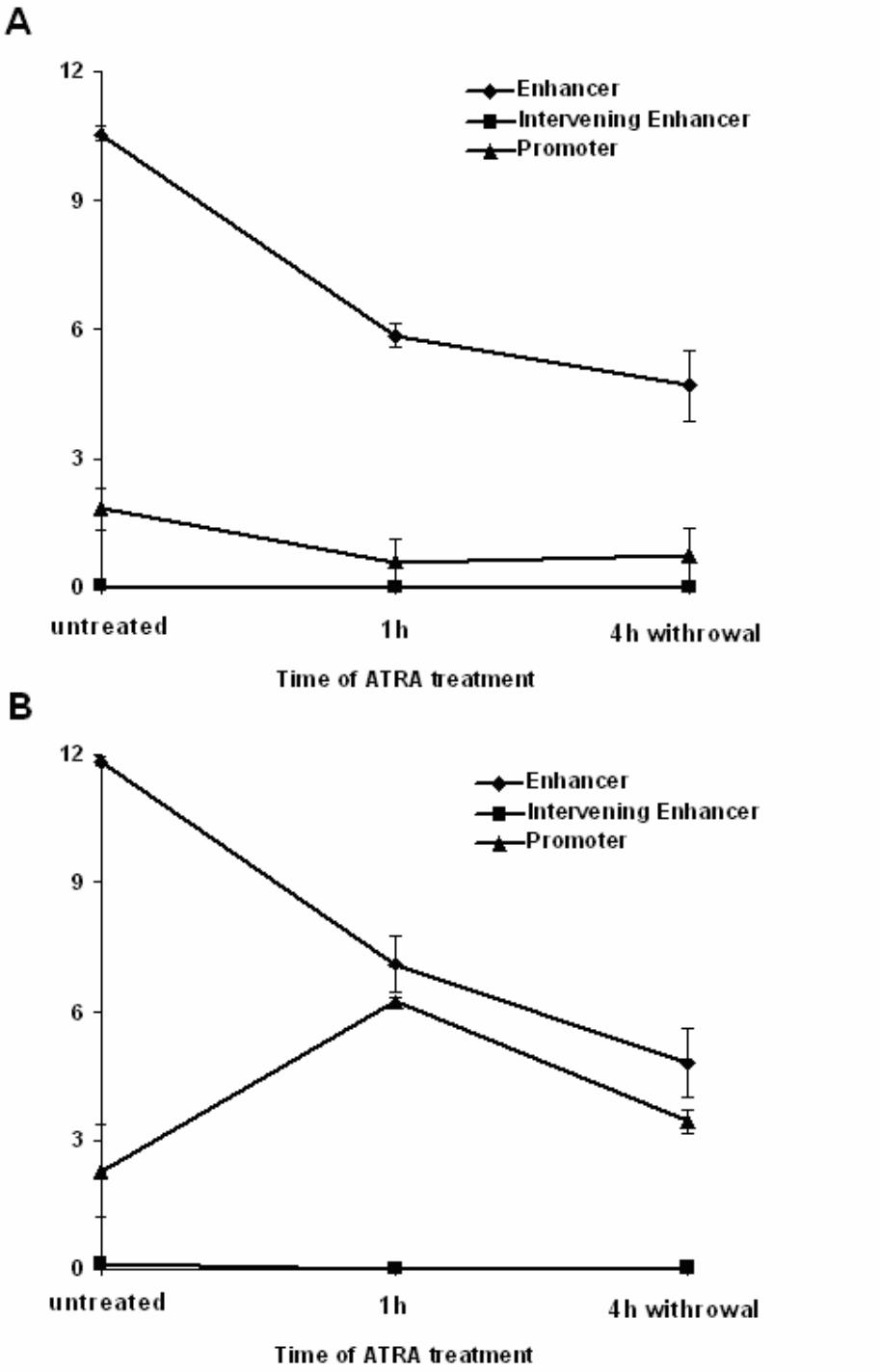

Fig. 14 Occupancy of HDAC1 and mSin3A onto the RET locus after ATRA treatment. HDAC1 (**A**, untreated) and mSin3A (**B**, untreated) are recruited on RET enhancer and, partially, on RET promoter in absence of ATRA. ATRA induces a two-fold reduction of HDAC1 (**A**) and mSin3a (**B**) levels on RET enhancer. SK-N-BE cells were incubated with 1 μM ATRA for 60 minutes. Then, ATRA was washed away with PBS and cells were subsequently cultured for additional 4 hours. Chromatin samples were immunoprecipitated with anti-HDAC1 (**A**) and anti-mSin3A (**B**) antibody and bound DNA was quantified as described in Fig. 6. Values, expressed as percentages of input DNA, are the mean ± SE.

Quantitatively, a two-fold reduction is observed for both HDAC1 and mSin3A on RET enhancer. HDAC1 and mSin3A levels on RET promoter behave differently: HDAC1 signal is poorly reduced but, interestingly, mSin3A signal rises after one hour and then decreases at the end of the treatment. Quantitatively, mSin3A levels are three-fold and two-fold the levels observed in the untreated sample after one hour and five hours of ATRA treatment, respectively.

Taken together, these data indicate that ATRA induces the displacement of MeCP2/HDAC1/mSin3A repressive complex from RET enhancer facilitating the concomitant activity of positive chromatin modifying enzymes which, in turn, leads to activation of RET gene transcription.

4. DISCUSSION

Nuclear receptors represent good candidates for dynamic analysis of epigenetic processes. Specifically, RAR dynamics are not well understood and only recent data have elucidated some epigenetic mechanisms involving such receptors. The recent findings that unliganded RAR receptors are bound to corepressor/histone deacetylase complexes and that, upon ligand addition, several histone acetylases are recruited to the receptor support the idea that transcriptional activation by RAR/RXR heterodimers coincides with an alteration of chromatin structure (Chen *et al.,* 1997; Nagy *et al.,* 1997). In addition, acetylation or removal of histone tails increases RAR/RXR affinity for a RARE assembled into a nucleosome in vitro (Lefebvre *et al.,* 1998). However, besides RA-dependent promoters, acetylation of local histones has been reported to be concomitant to increased transcription in other hormone-sensitive promoters (Chen *et al.,* 1999; McKenna *et al.,* 2002).

In the present study, we show how *all-trans* retinoic acid induces rapid and dynamic epigenetic changes on RET gene locus, leading to activation of transcription of RET gene.

We demonstrated that RARα is loaded on both RET promoter and enhancer even in absence of its ligand (Fig. 4). This is in accord with reported observations according to which RAR receptors may be resident on regulatory regions of several genes in absence of their agonist (Hao *et al.,* 2003). Also, we demonstrated that ATRA does not increase the loading of the receptor on RET promoter and enhancer. However, even though the fraction of receptor bound to the regulatory regions remains unmodified, ligand binding leads to the release of nuclear receptor

corepressors SMRT/TRAC and NCoR/RIP13 (Horn *et al.,* 1996; Nagy *et al.,* 1999; Perissi *et al.,* 1999), remarking the activating effect on transcription the retinoid may exerts by switching the interactors of the receptor itself.

Abrogation of chromatin remodelling enzymatic activities by epigenetic drugs treatment clearly affects RET mRNA transcription in SK-N-BE neuroblastoma cell system (Fig. 5). Puppo and colleagues demonstrated that sodium butyrate, which is a non-specific HDACi, induces RET up-regulation in cell lines displaying low levels of its mRNA (Puppo *et al.,* 2002), indicating that histone acetylation is mainly involved in activation of RET gene.

Also, we showed that decitabine, despite its unspecific effects, slightly increases RET mRNA levels indicating that DNA methylation may play a role in modulation of RET gene expression. This is supported by the different responsiveness to decitabine we observed among SK-N-BE and another neuroblastoma cell line, SH – SY5Y (data not shown). In fact, decitabine functions as an activator of RET transcription in both cell lines but SH – SY5Y cells are much more sensitive than SK-N-BE cells, being the effect of decitabine in SH – SY5Y comparable to that exerted by ATRA (data not shown). These observations suggest that this system presents a multiple level-regulation and the two cell lines are clearly set to different levels of regulation. In general, SK-N-BE cells would be subjected to a stricter control of RET transcription, being DNA demethylation sufficient for SH – SY5Y, but not for SK-N-BE cells, to trigger a remarkable RET gene activation. We postulate such levels of regulation may be represented by factors, which, possibly, are

missing in either cell line, determining the different responsiveness to the demethylating agent.

Pargyline exerts a more interesting effect determining a remarkable activation of RET mRNA transcription. Pargyline is a selective inhibitor of mono-amino-oxidases (MAO). Such enzymatic activities are present in many chromatin remodelling factors which are committed in demethylating histones. Specifically, the histone demethylase LSD1, which catalyzes demethylation of lysine 4 and 9 on histone H3, is mainly involved in activation of gene transcription and is inhibited by pargyline. If LSD1 played a key role in RET gene activation, we would have expected that its inhibition leads to absence of RET activation, in presence of ATRA. Conversely, we demonstrated that pargyline induces activation of RET mRNA transcription. We wonder how that molecule could do that and we speculated a possible mechanism (see below, model). As extensively stated before, epigenetic dynamics are highly complex and involve hundreds of factors. Among them, histone demethylases which have a non-MAO demethylating activity and are not inhibited by pargyline may be taken into account. Specifically, histone demethylases bearing jmjC domain are flavo-oxidases that remove methyl groups from lysine 27 of histone H3 triggering activation of transcription (see below, model).

Our data, together with previous observations, indicate that a crosstalk between activated RARα and chromatin remodelling factors of RET locus must exist. In fact, we demonstrated that the activation of RARα by ATRA treatment induces several rapid histone modifications (Fig. 10).

Histones H3 underwent a remarkable acetylation both on promoter and enhancer upon ATRA stimulation, indicating that a HAT activity is recruited. Acetylation levels are comparable for both the analyzed regions. However, there were clear differences in the dynamics of H3 acetylation between promoter and enhancer. In general, enhancer acetylation gradually raises and decreases, while promoter acetylation peaks rapidly after one hour and six hours of ATRA treatment.

Histone core tails are also subject to methylation. Lysine methylation of H3 is generally associated with transcriptionally active genes (Santos-Rosa *et al.,* 2002). Also, yeast studies have indicated that tri-methylated lysine of H3 is present solely on active genes and that the occurrence of di-methylated K4 correlates with a poised state of chromatin, in which genes are either active or potentially active (Santos-Rosa *et al.,* 2002). Our data are consistent with previous observations since we demonstrate that di-methyl-lysine 4 is present at comparable levels on both RET promoter and enhancer in absence of stimulus. ATRA induces a potent reduction of di-methyl-lysine 4 signal on both regions and a concomitant increase of tri-methyl-lysine 4 which mainly involve the promoter region. Taken together, these observations indicate that an active lysine tri-methylation is occurring on K4 upon ATRA stimulation and the major effect of this process takes place on the promoter concomitantly to RET gene activation.

Conversely, lysine 9 methylation is linked to gene silencing and generation of chromodomain-binding sites for heterochromatin protein 1 (Bannister *et al.,* 2001; Santos-Rosa *et al.,* 2002). Our data indicate that di-methyl-lysine 9 is present on

both promoter and enhancer at comparable levels. ATRA treatment induces specifically on RET enhancer a rapid and potent increase of this modification signal which gradually decreases and, finally, peaks again after six hours. Our findings are in contrast with previous observations and we cannot explain the phenomenon. However, we speculate that increase of di-methyl-lysine 9 signal could depend on a demethylation from a tri-methylated form of lysine 9 which determines a transient accumulation of H3K9me2. Further demethylation would explain the subsequent decrease of H3K9me2 signals. Performing the analysis with an antibody recognizing H3K9me3 may elucidate the process. However, as previously stated, the phenomenon is specific for the enhancer region, being H3K9me2 levels on RET promoter almost unaffected by ATRA treatment.

Methylation of lysine 27 is also associated with gene silencing (*Cao et al.,* 2002; reviewed in Swigut and Wysocka, 2007). Specifically, E(Z)/ESC complex, also known as Plycomb repressive complex 2 (PRC2), methylates lysine 27 of H3 through the intrinsic HMTase activity of the E(Z) SET domain (Tie *et al.,* 2007), leading to gene silencing. On the other hand, Agger and colleagues identified UTX and JMJD3 as histone H3K27 demethylases involved in activation of gene transcription (Agger *et al.,* 2007). Our data are consistent with previous observations since we demonstrated that lysine 27 is tri-methylated upon RET enhancer, but not RET promoter, in absence of ATRA. Ligand addition induces a rapid demethylation followed by a gradual increase of H3K27me3 signal, which is specific for enhancer region and concomitant with RET gene activation.

Our findings also indicate that the observed processes are very specific for both the regulatory regions. In fact, we demonstrated that no signal, or at least a much lower signal, for any analyzed histone modification was detected in the intervening region, which is about 1.7Kb distant from both promoter and enhancer.

Taken together, all the described histone modifications indicate that ATRA, likely via RAR, induces the recruitment of specific chromatin modifying enzymes determining H3 general acetylation, tri-methylation of lysine 4, tri-methyl-lysine 27 demethylation and, *bona fide*, tri-methyl-lysine 9 demethylation, which eventually lead to gene activation.

DNA methylation has been widely associated with gene silencing and one example is CpG island hypermethylation in cancer. CpG islands are CG reach DNA regions which are almost completely overlapped to gene promoters and do not present methylation in physiologic conditions (Bird, 2002). This is the case of RET gene. In fact, we showed that its promoter is completely unmethylated in SK–N–BE cells prior and after ATRA treatment (Fig. 11). This data are supported by the observations that RET promoter has been shown to be unmethylated in peripheral white blood cells (Munnes *et al.,* 1998). Conversely, we showed that RET enhancer is heavily methylated in SK–N–BE cells. Such observations are supported by the analysis we performed in tissues expressing or not-expressing RET that showed a lower but comparable DNA methylation (data not shown). Besides, we demonstrated that ATRA treatment induce a rapid and quantitatively significant demethylation at CpG -3358 (Fig. 12) which is concomitant to RET gene activation. However, we

observed that CpG -3358 demethylation is specific for SK–N–BE cells since we found it did not occur in SH – SY5Y cells incubated with ATRA (data not shown). Also, SH – SY5Y cells showed an unmethylated promoter and a methylated enhancer which is, however, less methylated than RET enhancer in SK–N–BE cells (data not shown).

Thus, we speculated that DNA methylation could be somehow involved in RET gene regulation and the enhancer could be the regulatory region exerting such control which is modulated by ATRA. This hypothesis was supported by some early observations: (i) RET promoter is unmethylated and the retinoid does not affect its methylation status (in both analyzed cell lines) but induces a specific CpG site demethylation on RET enhancer concomitantly with gene activation. (ii) ATRA induces RET mRNA transcription in SH – SY5Y cells. (iii) SH – SY5Y cells, which have a less-methylated RET enhancer, are more sensitive than SK–N–BE cells to demethylating agents as decitabine.

Then, we theorized that RET regulation via histone modifications might be linked to DNA methylation.

Methylated DNA Binding Proteins (MBPs) have been described as mediator of gene silencing via the recruitment of repressor complexes, once they bind methylated DNA. We demonstrated that MeCP2 specifically binds RET enhancer, but not RET promoter, in untreated SK–N–BE cells (Fig. 13a). In addition, we demonstrated that MeCP2 recruits the repressor molecules HDAC1 and mSin3A at RET enhancer (Fig.14) which likely exert their repressor activities, keeping RET gene silenced. These observations support our previous hypothesis according to

which RET enhancer exerts a key role in RET gene regulation being the platform that links DNA methylation to histone modifications. In fact, MeCP2, which binds the methylated enhancer region and recruits HDAC1/mSin3A, likely represents the link between DNA methylation and histone modifications, thus playing a central role in RET gene regulation in untreated SK–N–BE cells. To strengthen our hypothesis, we demonstrated that ATRA induces a remarkable displacement of the repressor complex MeCP2/HDAC1/mSin3A from RET enhancer (Fig. 14), concomitantly with a specific CpG site demethylation and RET up-regulation. Our data are consistent with the observations of Martinowich and colleagues (Martinowich *et al.,* 2003) which report that increased synthesis of brain-derived neurotrophic factor (BDNF) in neurons after depolarization correlates with a decrease in CpG methylation within the regulatory region of the BDNF gene. Moreover, increased BDNF transcription involves dissociation of the MeCP2-histone deacetylase-mSin3A repression complex from its promoter (Martinowich *et al.,* 2003). Other remarkable recent observations also stress the importance of specific and direct CpG site demethylation: consistent with Bruniguel's observations (Bruniguel and Schwarts, 2003), Murayama and colleagues demonstrated that stimulation of human CD4+ T cells induces IL2 expression following epigenetic changes, including active demethylation of a specific CpG site, recruitment of Oct-1, and changes in histone modifications (Murayama *et al.,* 2006). However, in our study we cannot distinguish whether DNA demethylation is a prerequisite for transcription or demethylation is merely induced by transcription, since, up to now, we lack observations of DNA methylation

patterns during the early phases of RET gene activation. Moreover, we do not know if demethylation is subsequent to MeCP2 displacement or it could directly determine the repressor complex displacement. To elucidate this critical phenomenon we are going to perform further DNA methylation pattern analysis during the early phases of gene activation. Much interesting observations by Chen and colleagues (Chen *et al.,* 2003) indicate that MeCP2, which binds and silence BDNF promoter, undergoes phosphorylation upon physiological stimuli as neuron membrane depolarization concomitantly with displacement from BDNF promoter. Zhou and colleagues (Zhou *et al.,* 2006) demonstrated that CaMKII rapidly phosphorylates MeCP2 on S421 and such post-translational modification determines the displacement from BDNF promoter. This hypothesis has been taken into account in our system and ATRA induced-MeCP2 post-translational modifications such as phosphorylation and acetylation are going to be checked. Confirmation of such mechanism would validate and extend our hypothesis according to which the enhancer region, via DNA methylation/MeCP2 binding, plays a key role in ATRA-dependent RET gene regulation.

Finally, we propose an overall model which can be applied in ATRA sensitive cell systems and take into account the crosstalk that may exist between DNA methylation, histone modifications and RAR kinetics (Fig. 15). In our unstimulated system, RET gene is poised, showing di-methylation at lysine 4 on H3 histones encompassing the promoter region. However the gene is not expressed and the main regulatory region is the enhancer which binds RAR and is methylated. In absence of its ligand, RAR interacts with corepressors which may contribute to keep the gene

silenced. Enhancer DNA methylation, acting as a docking site, allows MeCP2 to bind and recruits its reppressorial apparatus (HDAC1/mSin3A). Enhancer DNA methylation is crucial to maintain the gene silenced as MeCP2 binding is essential. A key role may be played by lysine 27 on H3. In an unstimulated system, lysine 27 on H3 is tri-methylated and this is in accord with the silent status of the gene. PcG is mainly involved in H3K27 methylation and can be recruited on the enhancer. Moreover, PcG can directly interact with DNMTs and, therefore, may contribute via such recruitment, to keep the enhancer methylated which, in turn, will keep the gene silenced. When the retinoid is added, RAR undergoes a conformational change that leads to the displacement of the corepressors which were bound to it. ATRA also determines, likely via cAMP, MeCP2 phosphorylation which, in turn, leads to the displacement of MeCP2/HDAC1/mSin3A complex from the promoter. Concomitantly, a sharp reduction in methylation of lysine 27 and a methylation of di-methyl-lysine 4 happen on the enhancer and promoter, respectively, shifting the gene status from poised to active. H3K27 demethylation likely depends on jmjC domain-demethylases which are specific for K27 (UTX or JMJD3), while H3K4 methylation likely depends on SET domain-methyltransferases which are specific for K4 (SET1). ATRA also induces a specific active CpG site demethylation which can be read in this context as a possible mechanism to set a “memory” of the previous event which can facilitate further processes triggered by the specific external stimulus.

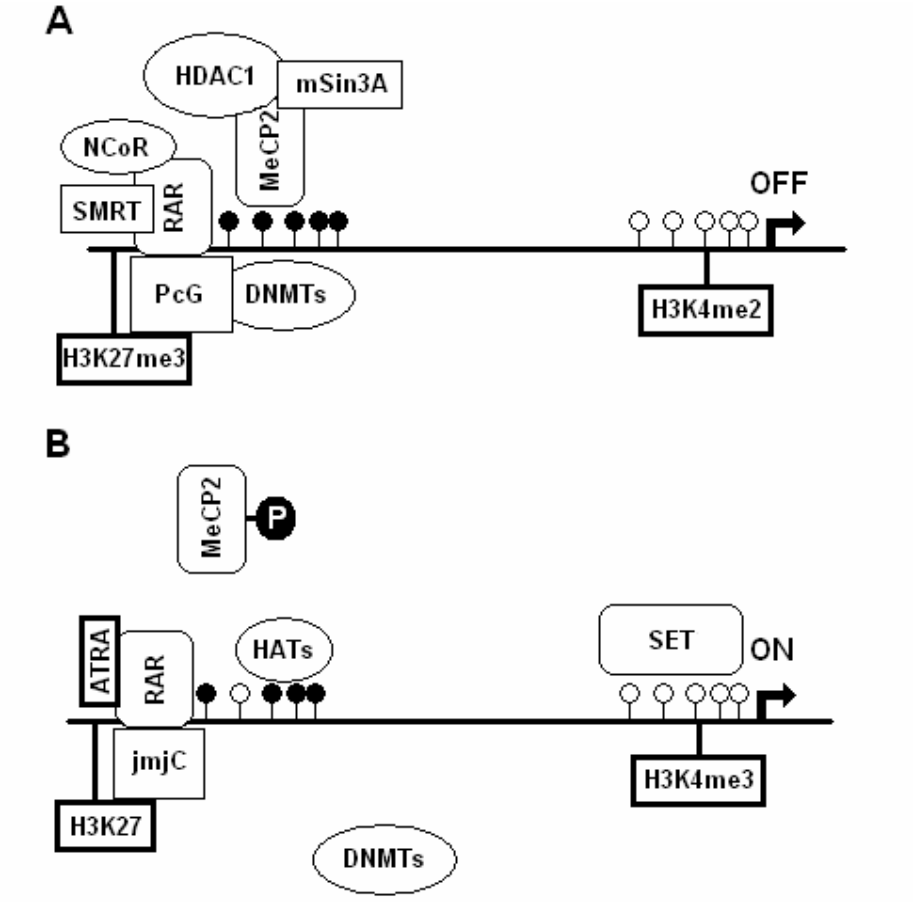

Fig. 15 General model of crosstalk between DNA methylation, histone modifications and RAR kinetics in ATRA sensitive cell systems applied to RET locus. (**A**) RET locus In absence of retinoid (see text for details). (**B**) RET locus upon retinoid addition (see text for details).

5. MATERIALS AND METHODS

5.1 Cell culture

SK-N-BE (http://www.biotech.ist.unige.it/cldb/cl4332.html) cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% foetal bovine serum (Life Technologies), 2 mM glutamine, penicillin (25 U/mL) and streptomycin (25 μg/mL) in a 5% CO_2 atmosphere at 37 °C.

5.2 Chemicals and Antibodies

All the treatments with all-trans retinoic acid (ATRA) (Sigma-Aldrich) were performed using a 1 μM final concentration. Trichostatine A (TSA, Invitrogen) pre-treatments were performed at 5 μM, 10 μM, 30 μM, 50 μM, 100 μM and 300 μM final concentration. Pargyline (Sigma) pre-treatments were performed at 2 mM final concentration. Decitabine (ICN Biomedical Inc.) pre-treatments were performed at 10 μM and 100 μM final concentration. TSA and pargyline pre-treatments were three hours long while Decitabine pre-treatments were 48 hours long. Anti H3Ac (#06-599) antibody was from Upstate as well as anti di-methyl-H3K9. Anti RARα (N-20, sc-551) antibody was from Santa Cruz, as well as anti HDAC1 (C-19, sc-6298) and anti mSin3A (AK-11, sc-767). Anti mono-methyl-H3K4 (ab-8895-100), anti di-methyl-H3K4 (ab7766-100), anti tri-methyl-H3K4 (ab8580-100) were from Abcam. Anti tri-methyl-H3K27 (AM-0174-200) was from Lake Placid Biologicals (LP BIO).

5.3 Real Time RT-PCR

Total RNA was isolated with RNeasy extraction kit (QIAGEN) according to the manufacturer instructions. QuantiTect Reverse Transcription kit (QIAGEN) was used to generate cDNA. 20 ng of cDNA for each sample was used as template for gene expression assays. Quantitative PCR amplifications were then performed using QuantiTect SYBR Green (QIAGEN) in a Chromo4 Real Time thermocycler (BIORAD). Following primers were used for RET amplifications: (forward) 5'-ACCAGGTCTCCGTGGATG-3' and (reverse) 5'-CCAAGTTCTTCCGAGGGAAT-3'. Each reaction was performed in triplicates. Calculations of relative expression levels were performed using

the $2^{-\Delta\Delta Ct}$ method (Livak *et al.,* 2001) and take into account the values of three independent experiments.

5.4 Quantitative ChIP analysis

Cells were cross-linked by adding 1% formaldehyde for 15 minutes at room temperature in shaking. Glycine was added to a final concentration of 125 mM for 5 minutes at room temperature in shaking. The cells were then rinsed twice with cold PBS supplemented with 500 μM PMSF and harvested in five pellet-volumes of Cell Lysis Buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP40)) supplemented with 1 mM PMSF and Complete™ protease inhibitors mix (Roche). Lysates were incubated for 30 minutes at 4 °C and then passed through ten dounce cycles. They were subsequently centrifuged and nuclei were collected. Nuclei were then resuspended in 250 μL Sonication Buffer (0.3% SDS, 10 mM EDTA, 50 mM Tris-HCl ph 8.0) supplemented with with 1 mM PMSF and Complete™ protease inhibitors mix (Roche) and incubated for 45 minutes at 4 °C. Chromatin was sonicated to an average DNA length of 400-800 bp using a 3 mm (small size) tip equipped Bandelin Sonoplus UW-2070 sonicator with 7 X 15 seconds cycles of pulses (specific cycle 0.3, Power 30%) alternated by 60 seconds of rest. Sonicated samples were centrifuged and the supernatant was collected. 80 μg of chromatin were diluted with Dilution Buffer (0.01% SDS, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.0), precleared by incubation with 20 μL Salmon Sperm DNA/Protein A Agarose-50% Slurry (#16-157, Upstate) and subjected to immunoprecipitation with specific antibodies with rotation over-night at 4 °C. Immunocomplexes were collected by adsorption onto 30 μL Salmon Sperm DNA/Protein A Agarose-50% Slurry (#16-157, Upstate) and the beads were washed sequentially with Low Salt Washing Buffer (0.1% SDS, 2mM EDTA, 20 mM Tris-HCl pH 8.0, 1% Triton X-100, 150 mM NaCl) (4 times), High Salt Washing Buffer ((0.1% SDS, 2mM EDTA, 20 mM Tris-HCl pH 8.0, 1% Triton X-100, 500 mM NaCl) (4 times) and LiCl Washing Buffer (#20-156, Upstate). Precipitates were washed with TE Buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA), and antibody-chromatin fragments were eluted from the beads with 1% sodium dodecyl sulphate in 0.1 M $NaCO_3$. Cross-links were reverted by adding 200 mM NaCl and heating at 65 °C overnight. 40 mg/mL RNase A

and 20 mg/mL proteinase K, 10 mM EDTA and 40 mM Tris-HCl pH 6.5 were added and samples were then incubated 2 hours at 45 ºC. Samples were then extracted in phenol-chloroform-isoamylic acid (25:24:1), ethanol-precipitated and finally centrifuged for 45 minutes at 4 ºC. Air-dry pellet was resuspended in 60 μL of H_2O and 2 μL of each sample were used as template. Input DNA was the unbound fraction of the non-immunoprecipitated sample (NoAb). Proper dilutions were set in order to get a standard curve for absolute quantification. Following primers were used for amplification: enhancer region (forward) 5'-CACCGACCACTTTGCTAACAG-3' and (reverse) 5'-GGTGGTTGGAAGCACAGACT-3'; intervening region (forward) 5'-AGGAGCACAGCCCCAGAT-3' and (reverse) 5'-GCCCTTGGCTGACATTGA-3'; promoter region (forward) 5'-TTACGTGCGGAGAGTTCTGTT-3' and (reverse) 5'-CTGAGCGGGAAAAGGAAAC-3'. Each ChIP assay was performed at least on three independent occasions.

5.5 DNA methylation analysis

Genomic DNA was isolated with DNeasy extraction kit (QIAGEN) according to the manufacturer instructions. Up to 2 μg of genomic DNA were bisulfite-treated with EZ DNA Methylation Kit (ZYMO RESEARCH) and eluted in 30 μL of H_2O. 2 μL of each sample were used as template in PCR reactions. HotStar Taq DNA Polymerase (QIAGEN) was used in the PCR reaction to avoid possible room temperature mispriming.

DNA methylation patterns: PCR products were cloned with pGEM-T-Easy Kit (Promega) according to the manufacturer instructions. Ligations were transformed in supplied competent cells. Colonies were screened by PCR colony protocol and positive clones were cultured for further plasmidic DNA collection. Plasmids were isolated with NocleoSpin Kit (Macherey-Nagel) and subsequently sequenced for analysis of DNA methylation patterns.

Pyrosequencing™ analysis: bisulfite-treated genomic DNA was amplified with the following pyrosequencing-specific primers: promoter (biotinylated-forward) 5'- GAGAGTTTTTTTGTGTAAGGGATGTAAGGG-3', (reverse) 5'- CCCACTTACAATCCCTACCTTTTACCCTTTCC-3',

(sequencing) 5'- TTTTTTTGTTTGTTTTTTT-3'; enhancer (forward) 5'-AGTGGTAGATAGATGGGAAATTGA-3', (biotinylated-reverse) 5'-ACCCAACTCCATCCTAATAATACT-3', (sequencing 1) 5'-CAAATCCCTCCCATAA-3' and (sequencing 2) 5'-AACTAACTATATACACTATT-3'.

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