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## The Roles of Retinoic Acid and Retinoic Acid Receptors in Inducing Epigenetic Changes

**Alison Urvalek\***,

Complete Address, Weill Cornell Medical College, Department of Pharmacology, 1300 York Avenue, New York, NY 10065 USA, Phone: 212-746-6262, Fax: 212-746-8835

**Kristian Bruun Laursen\***, and

Complete Address, Weill Cornell Medical College, Department of Pharmacology, 1300 York Avenue, New York, NY 10065 USA, Phone:212-746-6262, Fax: 212-746-8835

**Lorraine J. Gudas**

Complete Address, Weill Cornell Medical College, Department of Pharmacology, 1300 York Avenue, New York, NY 10065 USA, Phone: 212-746-6250, Fax: 212-746-8858

Alison Urvalek: alu2004@med.cornell.edu; Kristian Bruun Laursen: krl2004@med.cornell.edu; Lorraine J. Gudas: ljgudas@med.cornell.edu

### 7.1 Introduction

“What determines whether a given piece of DNA along the chromosome is functioning, since it's covered with the histones? You can inherit something beyond the DNA sequence. That's where the real excitement of genetics is now.”

-James Watson, 2003

The term “epigenetics” was coined by Conrad Waddington in 1942 in a discussion of the mechanisms of cell differentiation. Waddington defined epigenetics as “the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being” (Waddington, 1968). The specific epigenetic mechanisms that regulate genetic programming were not discovered until decades after Waddington first coined the term (Griffith and Mahler, 1969; Riggs, 1975). These mechanisms are now known to include histone tail post-translational modifications, DNA methylation, ATP-dependent chromatin remodeling, and non-coding RNA pathways (Tollervey and Lunyak, 2012). With these discoveries, Waddington's original definition of “epigenetics” has changed and evolved to the currently accepted view that “epigenetics (epi- being a Greek

\*These authors contributed equally to this work

**Standardized Gene Names/Nomenclature:** KDM1: LSD1/2

KDM4A: JMJD2A

KDM5A: Jarid1A/B/C/D

KDM6: JMJD3/UTX/(UTY)

KAT3A/B: CBP/p300

KAT6A: MOZ

KAT6B: MORF

MT2A: MLL1

KMT2B/C: MLL2/3

prefix for “on top of”) refers to “the study of heritable changes in genes that are not the result of changes in the DNA sequence” (Riggs et al., 1996).

Dr. James Watson won the Nobel Prize for his seminal role in discovering the structure of the DNA double helix structure in 1953, but fifty years later he acknowledged that DNA is not the sole regulator of gene inheritance and expression. Instead, epigenetic changes that occur “above” the DNA may be just as or more important than genetics in terms of their effects on development and disease state. Retinoic acid (RA), a vitamin A derivative that functions as the active metabolite in cellular signaling, induces cell differentiation in stem cells and some cancer cells. Along with the more well known effects of RA signaling on cell lineage specification through transcriptional activation of retinoic acid receptor (RAR)-regulated genes, recent studies are demonstrating that RA also mediates cell differentiation via rapid, profound effects on the epigenome. This observation is opening up a new area of fundamental research into transcriptional regulation as well as pointing the way to new clinical applications of RA. The use of RA in combination with drugs that modify the epigenome is showing promise in the treatment and/or prevention of several types of cancer. This type of combination therapy is increasingly relevant, as many types of cancer exhibit aberrant levels of or mutations in epigenetic regulatory proteins.

## 7.2 History: Epigenetic regulation is achieved by a number of different mechanisms

Waddington nicely illustrated the idea of genotype-to-phenotype changes along cell development pathways by his drawing of an “epigenetic landscape” (Waddington, 1957) (Fig. 7.1, adapted by Hochedlinger and Plath). A more recent adaptation of this drawing updates the model to include a summary of recent epigenetic research. In this model specific epigenetic modifications are acquired as progenitor cells, depicted as marbles (Fig. 7.1), differentiate and commit to a specific cell fate, conceptualized in the figure as marbles rolling down into one of several valleys. This idea has been substantiated by experimental findings where it has been demonstrated that commitment of cells into specific differentiation pathways is associated with progressive epigenetic modifications (Hochedlinger and Plath, 2009).

### Histone protein tail modifications and transcriptional regulation

Cellular chromatin is composed of DNA-wrapped nucleosomes packed into regions of either compacted or loose nucleosomal structure, referred to as hetero- and eu- chromatin, respectively. In general, genes residing in heterochromatic regions are silenced, whereas genes located in euchromatic regions are actively transcribed (Fig. 7.2).

The nucleosome is a histone octamer composed of two of each of the core histones H2A, H2B, H3, and H4, and one auxiliary H1 linker histone (Dawson et al., 2012). The histone proteins are each composed of a globular domain with an extended, positively charged N-terminal tail that interacts with the phosphodiester backbone of the DNA. Histone proteins were discovered in 1884, but it was not until 1963 that histone tails were shown to be post-transcriptionally modified (Phillips, 1963). Subsequently, the effects of these histone

modifications on gene regulation began to be elucidated (Allfrey et al., 1964). Importantly, the histones, in particular the lysine/arginine rich tails, were shown to be targets for extensive post-transcriptional modifications (Fig. 7.2). Recently, Yuan et al (Yuan et al., 2012) have shown that RA-mediated transcriptional activation of the Cytochrome P450 26a1 gene is associated with a loosening of the chromatin structure, which is required for transcriptional activation.

Histone protein tail regulation is highly complex, and numerous post-translational modifications can regulate different aspects of gene transcription; these include phosphorylation, sumoylation, ubiquitination, ribosylation, neddylation, ADP-ribosylation, citrullination, and others (Tan et al., 2011; Yang, 2005). The enzymes that regulate these modifications can be divided into three groups of epigenetic regulators: “writers”, “readers”, and “erasers”.

Histone modifications are generated by “writer” enzymes, which include families of lysine/arginine methyltransferases (KMTs/PRMTs), histone lysine acetyltransferases (KATs), and serine/threonine kinases. Methylation of histone tails, including trimethylation of histone 3 lysine 9 (H3K9me3) and 27 (H3K27me3), is generally associated with gene repression, whereas the acetylation of the same residues (H3K9 and H3K27) correlates strongly with gene activation (Guillemette et al., 2011; Verdone et al., 2006). There are also instances in which methylation of certain residues is associated with gene activation, such as methylation of H3K4 and H3K36 (Schneider et al., 2004; Voigt et al., 2012). Acetylation by KAT proteins, such as KAT3A(p300), KAT3B (CBP), PCAF, or KAT13B (pCIP), promotes the activation of gene expression by neutralizing the positive charges of histone tails (Verdone et al., 2006), leading to loosening of the negatively charged chromatin and subsequent binding of DNA binding factors that promote gene transcription.

The actions of “writer” enzymes are countered by a group of enzymes known as “erasers”. “Erasers” are responsible for the removal of specific histone modifications. This group includes lysine/arginine demethylases (KDMs/PRDMs), histone deacetylases (HDACs), and serine/threonine phosphatases (summarized in Table 7.1). Typically, these “erasers” counteract the actions of “writer” enzymes, having direct effects on gene transcription. For example, “writer” enzymes of the KAT family mediate the deposition of acetylation marks onto lysine residues of histone tails, thereby neutralizing the attraction of positively charged histones with negatively charged DNA. This allows for the unraveling of DNA, thereby allowing general transcriptional machinery and other proteins to bind that mediate gene activation. Conversely, “eraser” proteins of the HDAC family remove the acetylation mark. This allows the DNA to again wrap around histones, preventing the bindings of general transcription machinery, leading to gene repression.

“Reader” proteins specifically bind to post-translationally modified chromatin, and recognize these specific histone modifications to alter chromatin structure and dynamics. Often, “reader” proteins are part of larger protein complexes that contain “reader” and/or “eraser” proteins. Without “reader” proteins, posttranslational modifications would not be recognized, and the protein complex or specific “writers” or “erasers” would not be recruited. Alternatively, “writer” or “eraser” proteins themselves can also serve as “readers”

proteins. For example, KAT proteins possess a bromodomain that recognizes and binds acetylated lysine residues on histone tails. This allows for KAT proteins to further mediate acetylation at these specific DNA regions. Because of this, these “reader” proteins also mediate changes in transcription or DNA replication (Cohen et al., 2011).

The distinction between “writers”, “readers”, and “erasers” is complicated by the fact that protein complexes that add marks (“writer” complexes) are frequently composed of several subunits with different enzymatic properties. For example, the polycomb repressive complex 2 (PRC2) is comprised of at least four subunits which include the Suz12 (zinc finger), Eed, Ezh2 (SET domain with histone methyltransferase activity) and RbAp48 (histone binding domain) proteins. Importantly, the Ezh2 protein has enzymatic activity and can add methyl groups specifically to the H3K27 resulting in trimethylation of this histone residue. This posttranslational modification is deposited onto histone tails at lysine 27 by the PRC2 complex – a “writer”, but is recognized by the polycomb repressive complex 1 (PRC1) – a “reader” (Min et al., 2003). However, PRC1 mediates the deposition of ubiquitin, another histone modification, onto histone 2A lysine 119. PRC1 can in other words read the H3K27me3 and write the H2A.K119Ub, and can thus be considered both a “reader” and a “writer” enzyme (Stock et al., 2007). Furthermore, additional modifications of the histone tail (e.g. H3S28ph) proximal to the site of the initial modification (H3K27me3) add another layer complexity. In this example the recognition of H3K27me3 by the INHAT “reader” protein is prevented by phosphorylation of Serine 28 (H3S28ph) (Kim et al., 2012). This illustrates how modification of nearby residues can interfere with the recognition of specific histone marks by “reader” proteins. The effect of combinatorial histone modifications is commonly referred to as the histone code, a term coined by Charles D. Allis in 2001 (Jenuwein and Allis, 2001). As exemplified above by the context dependent recognition of H3K27me3, the emerging view is that the recognition by “reader” proteins is not dictated only by specific histone modifications, but rather by an interplay between different histone modifications

### DNA methylation and gene silencing

In contrast to histone modifications, which are relatively transient in nature, DNA methylation provides a more persistent, long-term gene silencing. DNA methylation occurs when a methyl group is deposited on the cytosine of a phosphodiester-bonded cytosine-guanine dinucleotide (CpG) sequence. DNA methylation, e.g. the formation of 5-methylcytosine (5mC), was first proposed as a mechanism for changing gene expression in 1975 (Holliday and Pugh, 1975; Riggs, 1975). CpG sequences are typically concentrated in large clusters called CpG islands, predominantly located at or near gene promoters, but CpG islands are also found in intergenic regions. A family of DNA methyltransferase (DNMT) enzymes transfer methyl groups to DNA and this engenders stable, long term gene silencing (Coskun et al., 2012). DNA methylation is introduced by the recruitment of DNMT3a and 3b by sequence specific repressors that silence gene transcription (Fuks et al., 2001). Newly replicated DNA is transiently hemi-methylated until DNMT1 uses the methylated parent strand to direct deposition of corresponding methylation on the daughter strand, thus maintaining the overall pattern of DNA methylation (Kulis and Esteller, 2010). In the context of DNA methylation, the DNMTs function as “writers”, whereas methyl-CpG-

binding domain proteins (MECP), which recognize methylated CpGs, function as “readers” (Sasai and Defossez, 2009).

Recently, researchers have determined that DNA methylation is reversible (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009), which suggests that DNA methylation is a dynamic process rather than a one-way mechanism of gene silencing, as was previously thought to be the case.

Selected groups of epigenetic regulators are listed in Table 7.1, where families of “writers”, “erasers”, and “readers” are listed for each type of epigenetic modification (individual rows). In Table 7.2 are listed a number of commonly investigated epigenetic modifications (individual rows), and their effects on transcription.

### Other epigenetic regulators of gene expression

ATP-dependent remodeling of chromatin structure and long intergenic non-coding RNAs (lincs) are other major epigenetic regulators of gene expression, but to date, little is known about their roles, if any, in RA regulated gene transcription. Here, we will focus on what is known about RA involvement in histone modifications and DNA methylation.

## 7.3 Development of the Field: Retinoids and Rars mediate histone modifications

RA functions as the ligand for retinoic acid receptors (RARs), and can regulate several developmentally important genes, including the Hox (homeobox) gene clusters (Kashyap et al., 2011; Kashyap et al., 2013; Langston et al., 1997). At these gene clusters as well as at other RA regulated genes, heterodimers of RAR $\gamma$  and retinoid X receptor  $\alpha$  (RXR $\alpha$ ) recognize and bind to RA responsive DNA elements (RAREs), then inducing epigenetic changes and transcriptional induction in response to RA (Fig. 7.3) (Gillespie and Gudas, 2007a, b). The transcriptional induction by RA is associated with increased levels of the co-activator proteins KAT3A (p300), KAT13B (pCIP), and of RNA polymerase II at target RAREs. Conversely, co-repressor proteins such as SUZ12, a key protein component of PRC2, are associated with specific RAREs, but dissociate in response to RA (Figs. 7.3 and 7.4) (Kashyap et al., 2011; Laursen et al., 2013). Furthermore, the re-association of SUZ12 with RAREs upon RA removal (Gillespie and Gudas, 2007a, b) exemplifies the highly reversible nature of cofactor association. Extensive changes in histone marks can be observed in response to RA, as illustrated by a heat-map showing RA-associated changes in H3K27me3, H3K4me3, and H3ac levels at the Hoxa cluster (Fig. 7.3). Importantly, for the Hoxa cluster the levels of activating marks (H3K4me3 and H3ac) increase, whereas the levels of repressive marks (H3K27me3) decrease in response to RA (Fig. 7.3) (Kashyap et al., 2011). This is not the case for all RA inducible genes; for Nr2F1 the levels of both activating (H3K4me3) and repressive (H3K27me3) marks show an initial increase in response to RA (Fig. 7.4). However, the H3K27me3 levels then start to decline, thereby increasing the extent of the induction (Laursen et al., 2013). The simultaneous presence of active H3K4me3 marks and repressive H3K27me3 marks is referred to as a bivalent domain, and this chromatin structure can often be found at promoters of RA inducible genes

(Kashyap et al., 2011; Laursen et al., 2013; Mark et al., 2006; Pan et al., 2007). The bivalent chromatin structure signifies that these genes are in a poised state in which changes in the H3K4me3/H3K27me3 ratio are associated with transcriptional induction (presence of RA; H3K4me3 ↑, H3K27me3 ↓) or silencing (absence or removal of RA; H3K27me3 ↑, H3K4me3 ↓), as illustrated in Fig. 7.4 (Bernstein et al., 2006; Kashyap et al., 2011; Kashyap et al., 2013; Langston et al., 1997; Laursen et al., 2013).

Now the question is; what regulates the levels of histone marks? Since histone marks are actually covalent modifications of histones, the levels need to be regulated by enzymes located in proximity to the histone. This brings us back to the “writers”, “readers”, and “erasers” mentioned in the beginning of the chapter. The levels of the active H3K4me3 mark are regulated by lysine methyl transferases (KMT) “writers” and lysine demethylases (KDM) “erasers”. Specifically, MLL proteins, which are KMT “writers” of the trithorax family, trimethylate H3K4 (Pasini et al., 2008), and KDM5 proteins, which are H3K4me3-specific “erasers,” can subsequently convert the H3K4me3 mark into H3K4me2 (Iwase et al., 2007; Kouzarides, 2007; Pasini et al., 2008). Once H3K4me3 has been converted to H3K4me2, the KDM1A/B “eraser” proteins can remove the remaining methyl marks, thus returning H3K4 to the unmethylated state (Ciccone et al., 2009). Pharmacological inhibition of KDM1A (LSD1) reactivates the RA differentiation pathway in leukemia cells (Schenk et al., 2012), indicating that enzymatic conversion of H3K4 to the unmethylated state by KDM1A plays a key role in antagonizing RA signaling. Curiously, H3K4me3 and H3K27me3 can be found on the same nucleosome, composed of eight histones, but not on the same histone tail (Voigt et al., 2012). Thus, each of the two histone 3 components of the nucleosome can be differentially modified. This specification of a histone as simultaneously activating and repressive is the core of the bivalent domains.

Conversely, the levels of the repressive H3K27me3 mark are regulated by Polycomb group proteins, which are H3K27me1/2 specific KMT “writers” (Cao et al., 2002; Kuzmichev et al., 2002), and the H3K27me2/3-specific KDM “erasers” KDM6A/B (Agger et al., 2007; Lee et al., 2007). The antagonistic effects of H3K4me3 and H3K27me3 are supported by the observation that RA-induced transcription leads to a concomitant decrease in H3K27me3 levels as well as an increase in H3K4me3 levels along the *Hoxa* cluster (Fig. 7.4). In this respect, it is interesting that the MLL2 complex contains KDM6A, an H3K27 demethylase (Agger et al., 2007). Consequently, the MLL complex combines methyltransferase activity targeting H3K4 with demethylase activity targeting the opposing H3K27me3 mark. A similar “push-pull” effect is observed with the PRC2 complex which contains EZH2, an H3K27 methyltransferase, and KDM5B, an H3K4 demethylase (Zhou et al., 2009). The depletion of H3K27me3 through knockdown of the EZH2 methyltransferase failed to induce *Hoxa1* expression (Lee et al., 2007). This suggests that without increased H3K4 methylation the loss of H3K27 methylation is insufficient to induce transcription of *Hoxa1*. Consequently, the combined actions of H3K4 methyltransferases and H3K27 demethylases may be required for gene transcriptional activation of at least some genes. Also, the transcriptional activation of *Hoxa1* precedes the removal of the H3K27me3 mark by many hours, showing that the removal of the H3K27me3 mark is not required for *Hoxa1* transcriptional activation by RA (Kashyap et al., 2011).

A different scenario of the “push-pull” effect is observed when RA induces the CoupTF1 (Nr2F1) gene. In this case, activating H3K4me3 and repressive H3K27me3 marks are simultaneously recruited to the CoupTF1 promoter (Fig. 7.4), initiating a repressed or dampened induction characteristic of several late RA target genes (Laursen et al., 2013). While the functional depletion of PRC2 did not enhance RA induction of *Hoxa5* and *Hoxa1* (early genes), the depletion potently enhanced RA mediated induction of CoupTF1 and CoupTF2 (late genes) (Laursen et al., 2013). This finding is important since it provides a mechanistic rationale for distinguishing between early and late targets of RA induction. It has been shown that PRC2 can sense chromatin density, and thereby distinguish active chromatin (marked by H3K4me3 and H3K36me2/3) from inactive chromatin, on which PRC2 will target H3K27 for methylation. This helps to explain how PRC2 maintains target genes in an inactive, compacted chromatin state for long periods (Yuan et al., 2012). Taken together, these data further point to the presence of a combined “push-pull” effect, wherein the effects of specific KMTs are supported by the effects of specific KDMs, which together place and remove specific lysine methylation marks in a coordinated manner.

RA induced transcription of the Hox genes increases not only histone H3K4 methylation, but also histone H3K9 and H3K14 acetylation (Kashyap et al., 2011) (Fig. 7.3 and 7.4). In addition, H3K27 can be modified by either acetylation or methylation, with opposite effects on the chromatin environment, and thus on the transcriptional activity. Acetylation (Creyghton et al., 2010; Rada-Iglesias et al., 2011) and methylation of H3K27 are mutually exclusive marks positioned by KAT3A/B (CBP/p300) and PRC2 (EZH2), respectively (Pasini et al., 2010; Tie et al., 2012). H3K27 thus provides an example in which the enzymatic activities of KATs/KMTs and HDACs/KDMs converge in regulating gene activity. However, H3K27 is not the only target of acetylation; H3K9 and H3K14 are acetylated concurrently with RA induced transcriptional activation (Figs. 7.3 and 7.4) (Kashyap et al., 2011; Kashyap et al., 2013). The RA-dependent recruitment of the acetyltransferases KAT3B (p300) and KAT13B (NCoA3, Actr, pCIP, Src3) to the RAREs of *Hoxa1* (Fig. 7.3) and *Cyp26a1* in F9 teratocarcinoma stem cells and ES cells suggests that these KATs also play key roles in RA-induced transcription (Gillespie and Gudas, 2007a, b; Kashyap and Gudas, 2010). Finally, KAT6A (Moz) is involved in H3K9 acetylation of the Hox gene loci, yet RA can activate the Hox loci independently of KAT6A (Voss et al., 2009). The plethora of coregulators involved in RA induced transcription allows for fine-tuning of a highly gene specific response (Fig. 7.3).

## 7.4 Current State of the Field: DNA demethylation is involved in the RA transcriptional response

Passive DNA demethylation takes place when maintenance methylation is inhibited during DNA replication, while active DNA demethylation requires specific enzymes and can occur without DNA replication (Zhu, 2009). Activation induced cytidine deaminase (AICDA, AID) is an active, reprogramming DNA demethylase expressed in embryonic stem cells and other cell types (Morgan et al., 2004). A second, more recently discovered DNA demethylase family, Tet 1, 2 and 3, removes DNA methylation through oxidative demethylation, a mechanism also employed by JmjC proteins to demethylate histones (Ito et

al., 2010; Tahiliani et al., 2009; Tsukada et al., 2006). Tet1 mediated hydroxylation of 5mC to 5-hydroxymethylcytosine (5hmU) is enhanced by AICDA, which generates 5hmU as a step towards the demethylation of 5mC. This requires thymine DNA glycosylase (TDG), a base excision repair enzyme, which excises the 5hmU (Guo et al., 2011). Through the active prevention of DNA methylation, TDG maintains bivalent chromatin domains in embryonic stem cells (Cortázar et al., 2011). Considering that several RA primary target genes reside in bivalent domains, it is worth noting that Um et al. (Um et al., 1998) identified interactions between TDG and the RARs/RXRs which may link RA to active demethylation of DNA. TDG forms a complex with AICDA and GADD45a, and is required for the recruitment of the coactivator protein KAT3B (p300) to the promoters of RA-inducible genes (Cortellino et al., 2011). Thus, a loss of TDG activity could result in a decrease in RAR/RA-associated gene transcription and a resultant block in cell differentiation, which would be consistent with the observed increase in DNA methylation of the Mest promoter region in response to knockout of RAR $\alpha$  (Laursen et al., 2012). This indicates that RAR $\alpha$  (and possibly other RARs) plays a direct role in maintaining gene expression by keeping specific promoters in a hypomethylated state, and conversely, underscores the fact that reduced expression of RAR $\alpha$  can have adverse consequences, such as leukemogenesis (Glasow et al., 2008). A reduction in RAR $\alpha$  signaling also impairs the survival of tumor reactive CD8 (+) T-cells within the tumor microenvironment (Guo et al., 2012). Whether this is related to RAR $\alpha$ 's ability to control the methylation state of certain genes has not yet been elucidated.

During gametogenesis, maternal or paternal genomes can be modified so that one parental allele is expressed, whereas the other is transcriptionally silenced. This genomic imprinting typically occurs through DNA methylation of CpG islands (Prickett and Oakey, 2012). An exciting, recent, findingsuggests that RAR $\alpha$ , independently of RA, maintains the DNA methylation status of specific imprinted genes (Laursen et al., 2012). This was highlighted by the identification of several aberrantly expressed, imprinted, genes in RAR $\alpha$ <sup>-/-</sup> F9 stem cells (Laursen et al., 2012). Under normal conditions RAR $\alpha$  associates with the promoter region of the paternally expressed gene, Mest; upon RAR $\alpha$  knockout, resulting in the absence of RAR $\alpha$ , the levels of H3K9me3 and the DNA methylation of the Mest promoter region significantly increase (Laursen et al., 2012) (Fig. 7.4). Several of the changes in gene expression associated with the RAR $\alpha$  knockout are similar to those observed during the differentiation of stem-like progenitors to hypertrophic chondrocytes in the developing growth plate (Constância et al., 2005). This similarity between the in vivo and in vitro data supports the idea that in vivo imprinting may be regulated by RAR $\alpha$ , and highlights the important roles of specific RARs in regulating epigenetic changes during development. Further exploration of this topic is expected to deepen our understanding of genomic imprinting and to expand the realm of RAR regulated transcription beyond the well-known ligand-induced regulation of gene activity.

## 7.5 Relevance: RA Regulated epigenetic changes in carcinogenesis

Retinoid signaling is often disrupted during carcinogenesis, suggesting that restoration of retinoid signaling may be a viable option for cancer prevention and/or treatment (Mongan and Gudas, 2005; Tang and Gudas, 2011). Synthetic retinoids modify the levels of the various RARs during breast carcinogenesis (Bosch et al., 2012), and RA inhibits the growth



of human osteosarcoma by promoting cell differentiation (Yang et al., 2012). In a glioma animal model, RA also promoted the differentiation of cancer stem cells (Campos et al., 2010). As a result, retinoids are currently being tested and/or used for treatment of many different cancers, including breast, ovarian, renal, head and neck, melanoma, leukemias, and prostate cancers. However, epigenetic changes, such as histone modifications and DNA methylation, and subsequent changes in gene expression are also thought to play major roles in cancer initiation and progression. Therefore, in line with the aforementioned “push-pull” model, combination cancer therapies that include retinoids together with epigenetic therapeutic agents are believed to be more effective in treating different cancers.

Histone deacetylase inhibitors, such as suberoylanilidehydroxamic acid (SAHA) have been extensively studied as potential cancer therapies, and are currently being used to treat multiple cancers, including cutaneous T-cell lymphoma and non-small cell lung cancer (Bantscheff et al., 2011). Chemoproteomics profiling of HDAC inhibitors revealed selective targeting of histone deacetylase (HDAC) complexes as promising cancer therapies (Bantscheff et al., 2011).

It is believed that treatment with HDAC inhibitors together with retinoid therapies may be an even more effective treatment regimen for certain cancers. When combined with HDAC inhibitors such as TSA and valproic acid, RA can re-induce RAR $\beta$  expression in kidney (Touma et al., 2005) or breast (Mongan and Gudas, 2005) cancers, and inhibit cell proliferation in many types of cancers (Cimino et al., 2006; Feng et al., 2012; Kato et al., 2007; Pili et al., 2012; Qi and Ratnam, 2006; Savickiene et al., 2012; Tavares et al., 2008; Trus et al., 2005; Wang et al., 2005). Furthermore, RA synergizes with valproic acid to promote the degradation of the PML-RAR $\alpha$  oncoprotein, destroying the leukemia initiating cells *in vivo* (Leiva et al., 2012). Recently a phase I trial using valproic acid and liposomal RA for patients with solid tumors yielded positive results, suggesting that this therapy maybe used for various solid tumors (David et al., 2010).

Another promising treatment approach is the co-administration of retinoids with DNA methyltransferase inhibitors. Mice treated with a combination of RA and the DNA methyltransferase inhibitor 5-Aza-2'-deoxycytidine (5-Aza) exhibit a decreased incidence of oral cancer after carcinogen treatment (Tang et al., 2009), and valproic acid, 5-Aza, and RA promoted growth arrest and cell differentiation of cultured human head and neck squamous cell carcinoma (HNSCC) cells (Gan et al., 2012). Additionally, a phase II clinical trial for patients with acute myeloid leukemia combining 5-Aza with RA was just completed with promising results (Lübbert et al., 2012).

Finally, other studies have examined the potential efficacy of treatments with RA, HDAC inhibitors, and DNA methyltransferases together. RA treatment in the presence of both valproic acid and 5-Aza promotes the re-expression of RAR $\beta$  and inhibits cell growth in breast cancer cell lines (Mongan and Gudas, 2005). Additionally, promyelocytic leukemia cells exhibit cell growth inhibition and increased granulocyte differentiation after treatment with all three drugs (Savickiene et al., 2012). Overall, these studies indicate that combinations of retinoids and epigenetic modulating drugs are promising treatment options for multiple types of cancer, in part because of their actions in promoting cell differentiation

and the inhibition of cell proliferation. Depicted in Fig. 7.5 are various epigenetic machinery inhibitors that are being intensely studied as possible cancer treatments (Rodríguez-Paredes and Esteller, 2011), and these could potentially be even more effective in combination with RA.

## 7.6 The Future: RA action and epigenetics, cell differentiation and cancer

Further studies are needed to determine the roles and specificities of various KATs and KDMs with respect to RA transcriptional activation and to develop a better understanding of how RAR $\alpha$  (and possibly other RARs) plays a direct role in maintaining gene expression by keeping specific promoters in a hypomethylated state. Many different epigenetic changes must take place for stem cells to differentiate properly, and when these changes do not proceed normally, increased tumorigenesis can result. Aberrant expression of the polycomb protein EZH2, a core component of PRC2, has been found in human breast, prostate, bladder, and colon cancers, and this overexpression is correlated with a poor prognosis (Mills, 2010; Raman et al., 2005). Overexpression of EZH2 in hematopoietic stem cells (HSCs) eliminates the exhaustion of the long-term repopulation potential of these stem cells during multiple, sequential transplantations (Kamminga et al., 2006). EZH2 also enhances leukemogenesis by enhancing the differentiation block in acute myeloid leukemia (Neff et al., 2012; Tanaka et al., 2012). Thus, these epigenetic modifications by EZH2 have profound consequences in terms of reducing the ability of HSCs to differentiate and enhancing tumorigenesis. Likewise, in prostate cancer EZH2 can block differentiation by affecting transcriptional regulation by the androgen receptor (Crea et al., 2011). The recent development of EZH2 inhibitors for treatment of lymphomas shows the power of manipulating epigenetic modifications for cancer treatment (Béguelin et al., 2013).

DZNep, an S-adenosylhomocysteine (SAH) hydrolase inhibitor, can eradicate tumor initiating cells in hepatocellular carcinoma cells and induce apoptosis in acute myeloid leukemia (Chiba et al., 2012; Fiskus et al., 2009; Zhou et al., 2011). DZNep can also inhibit tumorigenicity and progression in prostate cancer (Crea et al., 2011). The inhibition of SAH hydrolase causes an increase in SAH, resulting in inhibition of S-adenosyl-L-methionine dependent methyltransferases such as EZH2. We recently showed that human colon cancer cells, when exposed to RA, DZNep, or to a genetic knockdown of the PRC2 core protein SUZ12, exhibited enhanced PTEN mediated apoptosis, whereas the survival of embryonic stem cells was unaffected (Benoit et al., 2013b). The apoptotic effects of RA, DZNep, or SUZ12 depletion were further enhanced by combination with the TRAIL death receptor (Benoit et al., 2013a). The synergy between TRAIL and RA was confirmed by another report in which the authors demonstrate that treatment with retinyl acetate (another vitamin A metabolite) in combination with TRAIL not only induced apoptosis specifically in intestinal polyps, but also inhibited tumor growth and prolonged survival in a murine model of human colon cancer (Zhang et al., 2010). These results suggest that one mechanism by which RA enhances TRAIL associated apoptosis is via removing PRC2 complexes from various genes involved in differentiation and/or apoptosis (Fig. 7.6).

Research in this field will be enhanced by the recent development of more specific EZH2 inhibitors (Knutson et al., 2012; McCabe et al., 2012), and by the evaluation of new drug

combinations that more efficiently target specific epigenetic regulators. The fact that so many different types of cancer exhibit altered epigenetic profiles and/or mutations in proteins that modify the epigenome indicates that this will be a fruitful area of research that will provide major benefits to cancer patients in terms of new combination therapies.

The APC gene product, which controls intestinal cell fate, can, together with RA, downregulate demethylase components; this promotes DNA methylation of key target genes and increases the commitment of progenitors to differentiation (Rai et al., 2010). We recently showed that, when combined with the TNF-related apoptosis-inducing ligand (TRAIL), RA exhibited enhanced apoptotic effects on human colorectal cancer cells (Benoit et al., 2013a). This additive effect of RA could be substituted by either inhibition or functional depletion of the PRC2 complex by 3-Deazaneplanocin A (DZNep) or knockdown of SUZ12, respectively (Benoit et al., 2013a), which suggests that the observed synergy with TRAIL is a result of the RA induced dissociation of SUZ12 (described in section 7.2).

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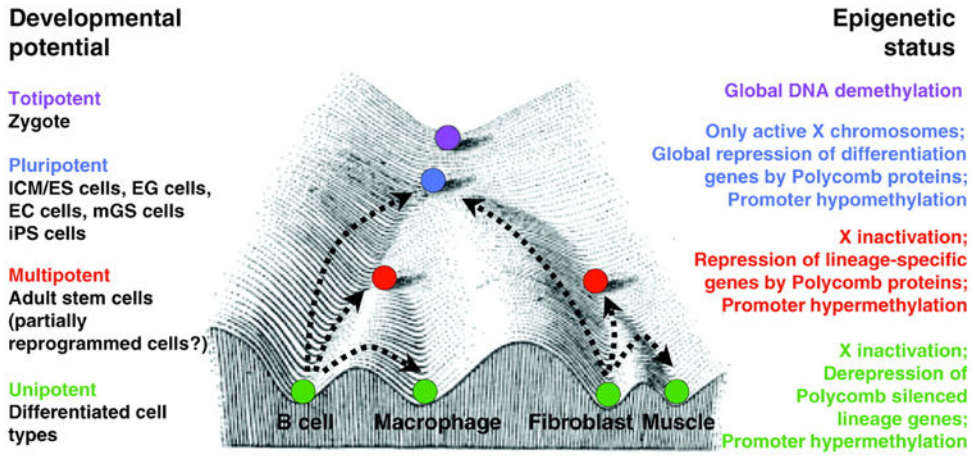


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## Abbreviations

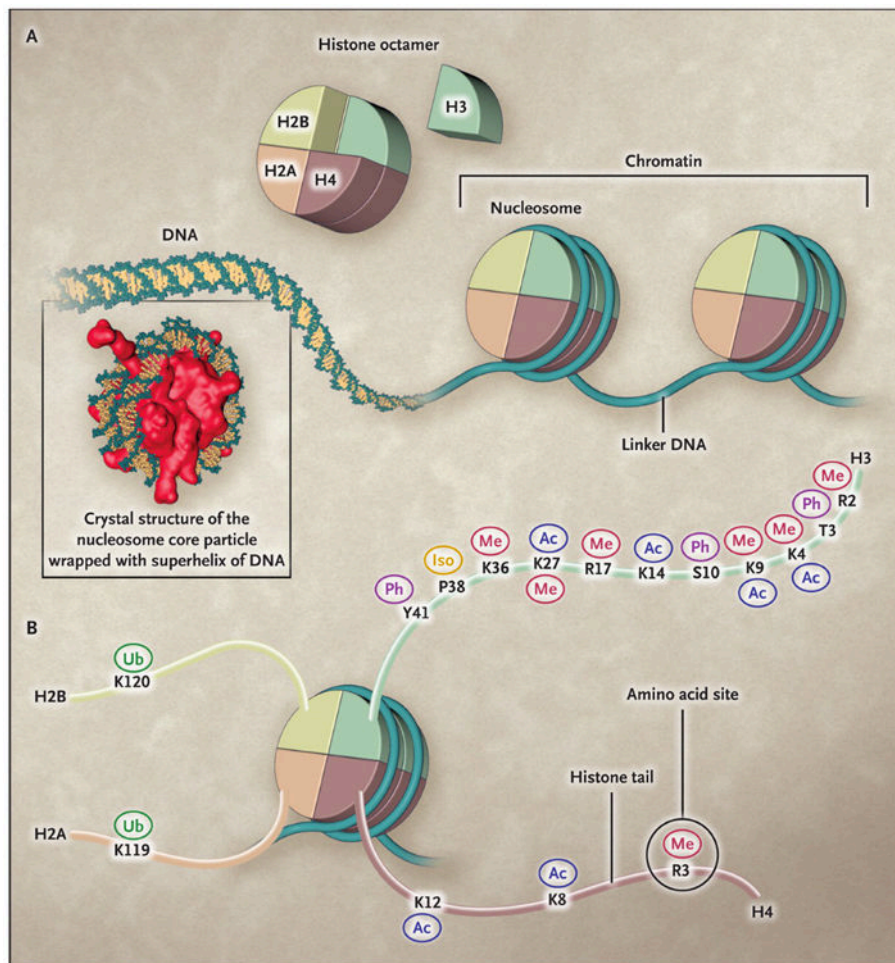
<b>SAH</b>	S-adenosylhomocysteine
<b>SAHA</b>	suberoylanilidehydroxamic acid
<b>KMT/PRMT</b>	lysine/arginine methyltransferase
<b>KDM/PRDM</b>	lysine/arginine demethylase
<b>ES</b>	embryonic stem cells
<b>iPS</b>	induced pluripotent stem cells
<b>HAT</b>	histone acetyltransferase proteins
<b>HDAC</b>	histone deacetylase
<b>CpG</b>	phosphodiester-bonded cytosine-guanine dinucleotide
<b>MECP</b>	methyl-CpG-binding domain proteins
<b>DNMT</b>	DNA methyltransferase
<b>RAR</b>	retinoic acid
<b>RA</b>	<i>all-trans</i> retinoic acid
<b>Hox</b>	homeobox
<b>ChIP</b>	chromatin immunoprecipitation
<b>RXR</b>	retinoid X receptor
<b>RARE</b>	retinoic acid responsive DNA element

<b>PRC</b>	Polycomb repressive complex
<b>5hmU</b>	5-hydroxymethyluracil
<b>5-Aza</b>	5-Aza-2'-deoxycytidine
<b>DZNep</b>	3-deazaneplanocin A
<b>HSCs</b>	hematopoietic stem cells
<b>TRAIL</b>	TNF-related apoptosis-inducing ligand
<b>WT</b>	Wild type



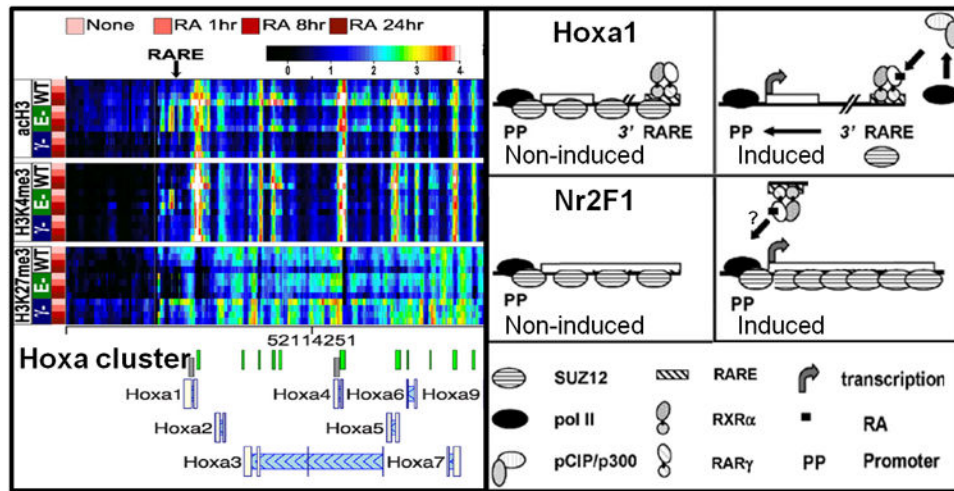
**Figure 7.1. The Epigenetic Landscape**

Conrad Waddington's Epigenetic Landscape illustrates the causal mechanisms by which the genotype brings about phenotypic effects. The progressive cellular differentiation (represented by purple, blue, red, and green) into specific cell types (illustrated by the different valleys) is associated with distinct epigenetic modifications. Polycomb repression and promoter hypermethylation are key mechanisms in the regulation of cellular differentiation. The corresponding epigenetic status of the various differentiation states is depicted on the right. (Hochedlinger and Plath, 2009).



### Figure 7.2. The Nucleosome and the Histone Tails

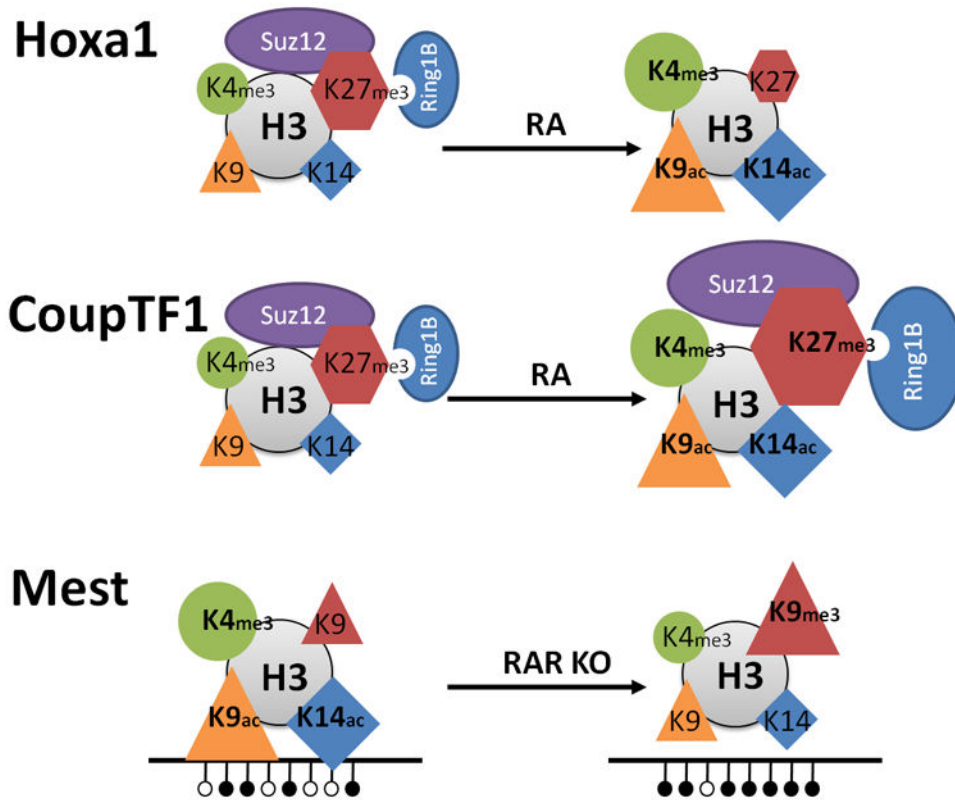
The basic functional unit of chromatin is the nucleosome (Panel A), which is composed of a histone octamer around which DNA is wrapped. Octamers are separated by linker DNA. The histone octamer is assembled from a histone H3:H4 tetramer and two H2A:H2B dimers. The histone tails of all four core histones are subject to a variety of post-translational modifications (Panel B). These include methylation (Me), acetylation (Ac), phosphorylation (Ph), ubiquitylation (Ub), and proline isomerization (Iso), all of which occur at the site of a specific amino acid, such as K4 and K9 on the histone H3 tail. The same histone amino acid may be subject to different post-translational modifications, which may facilitate different biologic outcomes. (Dawson et al., 2012).



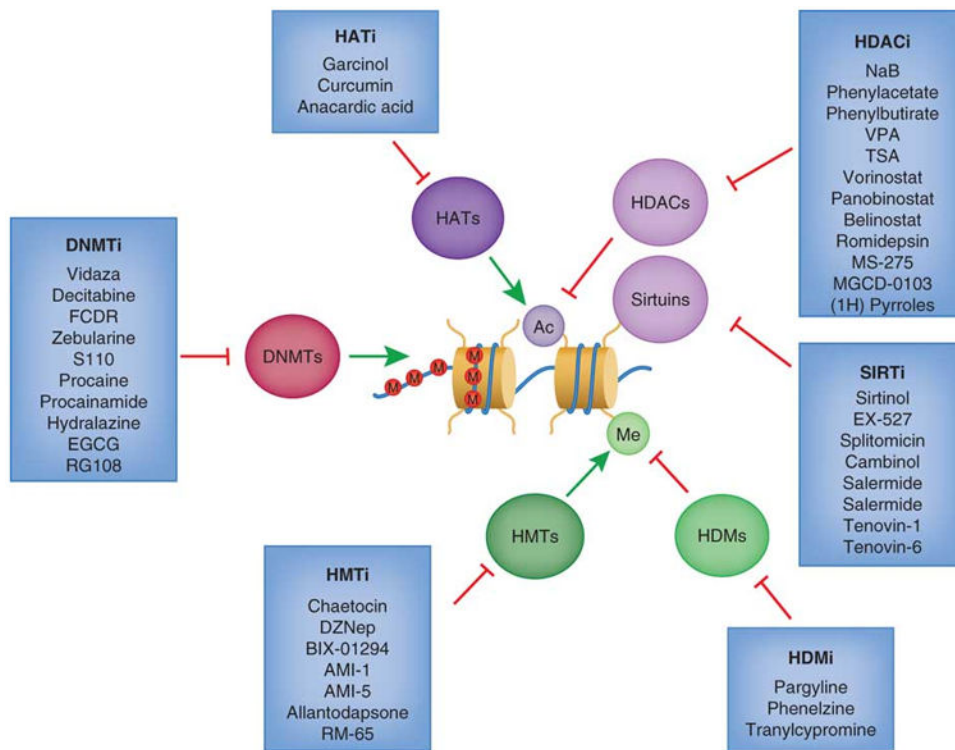
**Figure 7.3. Epigenetic changes induced along the Hoxa cluster in response to RA**

The epigenetic changes of the RA responsive Hoxa gene cluster are shown, with the locations of the Hoxa1 proximal promoter (PP) and RA responsive element (RARE) indicated by arrows. The levels of acH3, H3K4me3, and H3K27me3 determined by ChIP-chip are presented as heatmaps, with rows representing individual timepoints for each genotype, and columns indicating specific genomic regions. The genotypes of the stem cell lines are as follows: WT, RARE-KO (E-), and RAR $\gamma$ -KO ( $\gamma$ -). The cells were cultured in RA for 1, 8, and 24 h, as indicated. The color scale representing log<sub>2</sub>-transformed ChIP enrichment is indicated at the top of the figure. Note the reduced levels of H3ac and H3K4me3 at Hoxa1 PP and RARE in the RAR $\gamma$ -KO cell line

Models for RA mediated transcription of RA target genes Hoxa1 and Nr2F1. In the absence of RA, RAR $\gamma$ -RXR $\alpha$  heterodimers associated with Hoxa1 RAREs presumably associate with co-repressors, thereby generating a SUZ12-rich environment which represses transcription. Binding of the RA ligand causes a conformational change in the RAR $\gamma$ -RXR $\alpha$  heterodimer bound to the Hoxa1 RARE. This results in the recruitment of pCIP/p300, which generates an euchromatic environment, presumably by acetylating the histone tails. This allows pol II to initiate transcription of Hoxa1. The Nr2F1 promoter region (PP) is bound by SUZ12 in the absence of RA. Upon exposure to RA the increase in activating marks is initially counteracted by a concomitant increase in SUZ12, which attenuates the transcription of Nr2F1. Eventually, the SUZ12 levels decline, allowing the increased transcriptional activation of Nr2F1. (modified from Kashyap et al., 2011 and Gillespie and Gudas, 2007b).

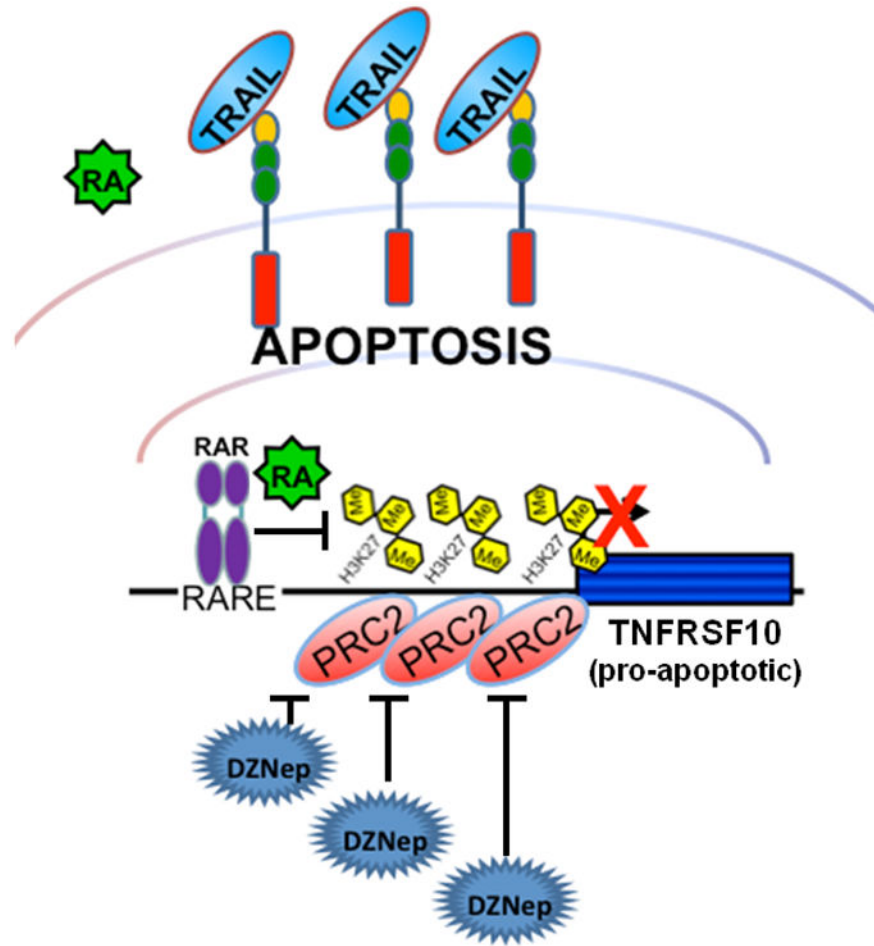


**Figure 7.4. Epigenetic Signatures Associated with RA and RAR regulated transcription**  
 Hoxa1 represents a group of direct target genes induced by RA (upper panel). The induction is characterized by dissociation of PRCs (ovals) and depletion of the H3K27me3 repressive mark (hexagons), and by increased levels of transcriptionally permissive marks, H3K4me3 (circle), H3K9ac (triangle), and H3K14ac (diamond). CoupTF1 represents a group of target genes with delayed transcriptional induction by RA (middle panel). The induction is characterized by an initial increase of PRCs (ovals) and of the H3K27me3 repressive mark (hexagons), concurrent with increased levels of transcriptionally permissive marks; H3K4me3, H3K9ac, and H3K14ac. The imprinted gene Mest is transcribed in the presence of RAR $\alpha$ , but is silenced by DNA methylation upon knockout of RAR $\alpha$  (lower panel). The transcriptional silencing of Mest is associated with increased DNA methylation, increased levels of the H3K9me3 repressive mark, and with decreased levels of transcriptionally permissive marks; H3K4me3, H3K9ac, and H3K14ac. Note that for Hoxa1 and CoupTF1 the transcriptionally active state is shown to the right, whereas for Mest the transcriptionally active state is shown to the left. Published in parts (CoupTF1) in *Nucleic Acid Research* (modified from Laursen, et al., 2013).



**Figure. 7.5. Current drugs targeting various epigenetic machinery**

Histone acetyltransferase inhibitors (HATi) may be effective treatments since HAT proteins are sometimes considered to be tumor promoters. Histone deacetylase inhibitors (HDACi) such as vorinostat (SAHA) and romidepsin have been FDA approved for treatments of certain hematological cancers. SIRT inhibitors (SIRTi) specifically target the SIRT family of histone deacetylases. Histone demethyltransferase inhibitors (HDMi) and histone methyltransferase (HMTi) have importantly become more selective towards specific marks, since methylation can act as both a repressive and an activating mark. DNA methyltransferase inhibitors (DNMTi) may serve as promising drugs for re-sensitizing certain cancer cells to chemotherapy, and two (vidaza and decitabine) are FDA approved for cancer treatment. (Rodríguez-Paredes et al., 2011)



**Figure. 7.6. Representation of RA and DZNep effects on apoptosis regulation in human colon cancer cells**

Retinoic acid treatment promotes TRAIL-related apoptosis in RAR $\beta$ /RAR $\gamma$ -positive HT29 cells, but not in SW480 cells which express only low levels of RAR $\beta$ /RAR $\gamma$ . The functional depletion of PRC2 by either inhibition with DZNep or by knockdown of SUZ12 increases TRAIL-mediated apoptosis in both HT29 and SW480 cell lines. In this scenario the PRC2-mediated repression is alleviated, thereby activating TNFRSF10 even in the absence of RAR $\beta$ /RAR $\gamma$ .



**Table 7.1**  
**Groups of Epigenetic Modifiers and their functions**

“Writers” are a group of enzymes that mediate the addition of epigenetic modifications (marks). “Erasers” are proteins with enzymatic activity that mediate the removal of these marks. “Readers” are proteins, generally with no enzymatic activity, that recognize and bind to posttranslational modifications to mediate downstream effects.

-	Writer	Eraser	Reader
<b>Histone Marks</b>	-	-	-
<b>Lysine methylation</b>	KMT	KDM	CBX proteins
<b>Arginine methylation</b>	PRMT		14-3-3 –proteins
<b>Lysine acetylation</b>	KAT	HDAC	
<b>Serine/Threonine phosphorylation</b>	S/T Kinase	Phosphatase	
<b>DNA methylation</b>	-	-	-
CpG (5meC)	DNMT	DNA demethylase	MeCPMBD1-4

**Key:** Lysine Methyltransferase (KMT); Protein Arginine Methyltransferase (PRMT); Lysine Acetyltransferase (KAT); Serine/Threonine Kinases (S/T Kinase); Lysine Demethylase (KDM); Histone Deacetylase (HDAC); DNA Methyltransferase (DNMT); Methyl-CpG-binding domain proteins (MeCP); Chromobox Homolog (CBX)

**Table 7.2**  
**Selected Histone Modifications and Their Enzymatic Regulators**

Specific histone marks involved in transcriptional regulation, and the enzymes that modify these marks. Activating marks are modifications that generally favor transcription (Activation), whereas repressive marks are modifications that favor transcriptional silencing (Repression). Examples are given of specific histone modifications (Histone marks), and of the specific enzymes depositing (“writers”) and removing (“erasers”) these marks. This is not a comprehensive list, but rather a list of the most well understood regulators of epigenetic changes.

-	Histone mark	Writer	Eraser
Repression	H3K27me3	EZH2, NSD3	KDM6A/B (JMJD3)
	H3K9me3	SETDB1/2 SUV39H1/2	Lysine specific demethylase 4A/B/C/D
Activation	H3K4me1	SETD7	KDM1A KDM5B
	H3K4me2	NSD3	KDM5A/D KDM1A KDM5B
	H3K4me3	MLL MLL3/4 PRDM9 SETD1A/B SET AND MYND domain-containing protein 3	Lysine specific demethylase 4A/B/C/D KDM5B
	H3K36me3	SETD2 NSD2	Lysine specific demethylase 4A Lysine specific demethylase NO66
	H3K14Ac	PCAF MYST3	HDAC3
	H3K9Ac	PCAF KAT13B (pCIP) KAT6A (Moz)	SIRT1 SIRT6
	H3K27Ac	KAT3A/B (P300/CBP)	-
	H3S28Ph	MAPKKK-MLT MSK1/2 STK5	-

**Key:** enhancer of zeste homolog 2 (EZH2); Nuclear SET domain-containing protein (NSD3); lysine (K)-specific demethylase (KDM); SET domain, bifurcated (SETDB); methyltransferase variant (SUV39H12); SET domain (SETD); mixed-lineage leukemia (MLL); Positive Regulatory Domain (PRDM); p300/CBP associated factor (PCAF); histone deacetylase (HDAC); Lysine acetyltransferase (KAT); MAP-kinase-kinase-kinase; mitogen- and stress-activated protein kinase (MSK); Aurora kinase (STK).