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Epigenetic regulation of nuclear steroid receptors

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Abbreviations:
NR, nuclear receptor
AR, androgen receptor
ERα, estrogen receptor
PPARγ, peroxisome proliferator-activated receptor γ
HAT, histone acetyltransferase
HDAC, histone deacetylase
SRC, steroid receptor coactivator
N-CoR, nuclear receptor corepresssor
SMRT, silencing mediator of retinoid and thyroid hormone receptor
TSA, trichostatin A
SIRT1, Sirtuin 1
CBP, CREB binding protein
p/CAF, p300/CBP-associated factor
DNMT, DNA methyltransferase

ABSTRACT

Histone modifier proteins have come to the forefront in the study of gene regulation. It is now known that histone methyltransferases, acetytransferases, kinases, ubiquitinases, deacetylases and demethylases orchestrate expression of target genes by modifying both histone and non-histone proteins. The nuclear receptor (NR) superfamily govern such diverse biological processes as development, physiology and disease, including human cancer. The involvement of NR in complexes with coactivators and corepressors is necessary for regulation of target genes. This review focuses on the newly recognized interactions between the NR and histone modifying enzymes. In addition to regulating histones, the histone modifying proteins directly modify and thereby regulate NR activity. In the same manner that signaling platforms exist within the histone tails that are post-translationally processed by histone modifying proteins, cascades of post-translational modification have been identified within the NR that coordinate their activity. This review focuses on the regulation of the NR estrogen receptor (ERα), androgen receptor (AR) and peroxisome proliferator activated receptor-gamma (PPARγ), given their role in tumor onset and progression.

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ChIP, chromatin immunoprecipitation
LSD1, lysine specific demethylase

1. Introduction

Nuclear receptors (NR) are part of multi-protein complexes that include transcription factors and coactivator proteins that modify chromatin. The BRG/BRM proteins, for example, bind NR and regulate chromatin structure in an ATPase-dependent manner. NR associate with histone modifying proteins that convey transcriptional repression or activation. These histone modifying proteins, including histone acetyltransferases, kinases, ubiquitinases, deacetylases, histone methyltransferases and demethylases, can regulate the activity and/or the expression of NR. Several NR are directly modified by kinases and histone acetylases including the estrogen receptor α (ERα), androgen receptor (AR) and peroxisome proliferator receptor γ (PPARγ). The acetylation of NR occurs at a conserved motif. This motif is observed in most NR and is conserved between species. Acetylation of NR is regulated by physiological stimuli. This review focuses on the regulation of NR by histone modifying proteins and the effects of NR acetylation on their biological function.

2. Epigenomic modification

Epigenomic modifications can be defined as heritable, yet reversible, chromatin alterations that govern the expression of genes. This area has been examined for many years, beginning in 1983 with the discovery of methyltransferases that alter DNA [1]. While examining colorectal cells Feinberg and Vogelstein were the first to note an altered DNA methylation pattern in tumors. It was not until the identification of histone acetyltransferases, or HATs, in 1995 [2], that histone modification involved in cellular differentiation was more thoroughly examined. Since then several main chemical alterations of histones that regulate gene expression have been determined and well-studied. In addition to histone acetylation and DNA methylation, these alterations include histone phosphorylation, ubiquitination and methylation, all of which can either silence or activate gene expression.

In 1999 Holliday and Beck, Olek and Walter [3,4] commented on epigenomic modifications, hinting at the idea of ''deciphering an epigenetic code''. Strahl and Allis [5] proposed an inherited order to post-translational histone modifications; which became known as the 'histone code hypothesis'. Histone acetyltransferases (HATs) (CBP, p300, etc.), deacetylases (HDACs), kinases (Aurora) and methyltransferases (HMTs) have already been shown to play significant roles in cancer. As the epigenetic phenotype may be reversible, enzymes regulating these epigenomic changes may be ideal targets for cancer drug development. In fact, several different histone deacetylase inhibitors are currently in phase I or II clinical trials.

3. Nuclear receptors (NR)

NR, or steroid receptors, share structurally conserved domains and are regulated through steroids, thyroid hormone, retinoic acid, vitamins or other proteins. They function as transcription factors, often in complex with other coregulators, that govern transcription of target genes involved in such varied processes as homeostasis, reproduction, development and metabolism [6]. All NR contain four main conserved domains, the activation function domain (AF), the DNA binding domain (DBD), the hinge region and the ligand-binding domain (LBD). Protein–protein interactions are typically found through an N-terminal domain and the LBD. The DBD binds specific target DNA sequences, while the LBD additionally binds hormones.

Coregulator proteins that bind to NR help to modify target gene expression through protein complex formation. These interactions aid in either corepression or coactivation of gene expression. Coactivators work by recruiting protein complexes to function as a link between the NR and the transcriptional apparatus. They also can use their histone modifying abilities to alter the local chromatin structure. The coactivators that bind NR include steroid receptor coactivator-1 (SRC-1), amplified in breast cancer 1/thyroid and RA receptor/steroid receptor coactivator-2 (AIB1/ACTR/SRC-2), glucocorticoid receptor interacting protein 1/transcriptional intermediary factor 2/steroid receptor coactivator-3 (GRIP1/TIF-2/SRC-2), p300/CBP and p/CAF (p300/CBP-associated factor) [7–9]. NR corepressors typically interact with unliganded NR and recruit histone modifying proteins, like HDACs, to silence target gene expression. Several NR corepressors have been identified and include nuclear receptor corepressor (N-CoR), silencing mediator of retinoid and thyroid hormone receptor (SMRT), Sin3, HDACs, thyroid hormone receptor uncoupling protein (TRUP), BRCA1, NuRD, Suv39h1, DNMT1, pRb2/p130, and E2F4/5.

3.1. *Histone methylation*

Histone methylation is dynamically regulated by HMTs and demethylases, such as Lysine Specific demethylase 1 (LSD1), JHDM1, JHDM2A and JMJD2. Histone methylation regulates chromatin structure, transcription and the epigenetic state of the cell. Methylation occurs at lysine and arginine residues. Lysine residues can be mono-, di- or tri-methylated. Euchromatic histone methylation contributes to both transcriptional repression and activation. Methylation at H3-K4 and H3-K36 is typically linked to transcriptional activation, while H3 Lys 9 is typically a repressive mark.

All of the histone lysine methylases, except Dot, share a SET (Su(var), Enhancer of zeste, Trithorax) domain that is responsible for the addition of the S-adenosyl-L-methionine cofactor. HMTs add methyl groups to the ε-amino group of

lysine residues. The histone H3 Lys 9 methyltransferase group (*Suv39h1, Suv39h2, G9a, G9a-related protein, SETDB1* gene products) catalyze H3-K9 methylation, G9a is a HMT that silences genes in euchromatic regions of DNA and forms homo or heterodimers with the related protein GLP. G9a regulates histone H3-K9 mono- and di-methylation.

The protein arginine methyltransferases (PRMT) consist of two types that differ in the symmetry of the di-methyl arginine product. The Type I PRMT enzyme forms mono-methyl arginine and asymmetric di-methyl arginine, whereas Type II PRMT forms monomethyl arginine and symmetric di-methyl arginine. The PRMT1, 2, 3, 4 and 6 are Type I PRMT and PRMT5 is a type II PRMT. PRMT7 may be a third type of PRMT. PRMT5, known as CARM1 (co-activator associated arginine methyltransferase) was identified as a p160 co-activator of nuclear receptors by Stallcup and co-workers [10], providing a fundamental new mechanism for cross talk between nuclear receptors and this enzyme family, and showing for the first time that these enzymes can function as transcriptional regulators.

The histone lysine demethylases identified to date involve distinct biochemical processes as LSD1 functions via FAD-dependent oxidative reactions (amine oxidase family) [11] and the JHDMs require Fe(II) and α-ketoglutarate as cofactors for oxidative hydroxylation through the JmjC domain (JmjC domain containing family) [12]. LSD1, was identified in a search for new AR-interacting partners [13].

3.2. *The cyclin D1 gene*

The cyclin D1 gene encodes the regulatory subunit of the holoenzyme that phosphorylates the retinoblastoma, pRB, protein and associates with HDACs/HATs to regulate activity of several transcription factors. The cyclin D1 protein is both a coactivator as well as a corepressor of NR. Cyclin D1 is a coactivator of ERα transcription and a corepressor of AR, PPARγ and the Thyroid Receptor (TR). Cyclin D1 can bind directly to ERα and recruits SRCs to the ERα promoter [14]. It acts to upregulate ERα gene expression by antagonizing BRCA1 mediated repression of ERα [15], see Fig. 1A. Cyclin D1 physically associates with the AR, inhibiting AR transactivation by competing with p/CAF binding [16], see Fig. 1B.

4. Estrogen receptor α

ERα regulates the numerous activities of estrogen, a steroid hormone important in normal development and reproduction as well as diseases such as breast cancer, cardiovascular disease, osteoporosis and Alzheimer's disease. Upon ligand binding, ERα can bind either target DNA sequences directly or through other coactivators/corepressors to regulate transcription of specific genes. Proteins that cooperate with ERα in transcriptional regulation include the p160 family (SRC-1, TIF2/SRC-2/GRIP1, AIB1/ACTR/SRC-3), cyclin D1 and several HATs (CBP, p300 and P/CAF) [17–19].

4.1. *Silencing of the ERα gene through histone deacetylation, DNA methylation and histone modifier protein complexes*

Many genes are silenced through a combination of both DNA methylation and/or histone deacetylation. Importantly, a significant number of breast cancers lose expression of the ERα gene as a result of DNA hypermethylation within the ERα promoter [20,21]. Yang et al. [22] demonstrated that the treatment of ER-negative breast cancer cells with the DNA

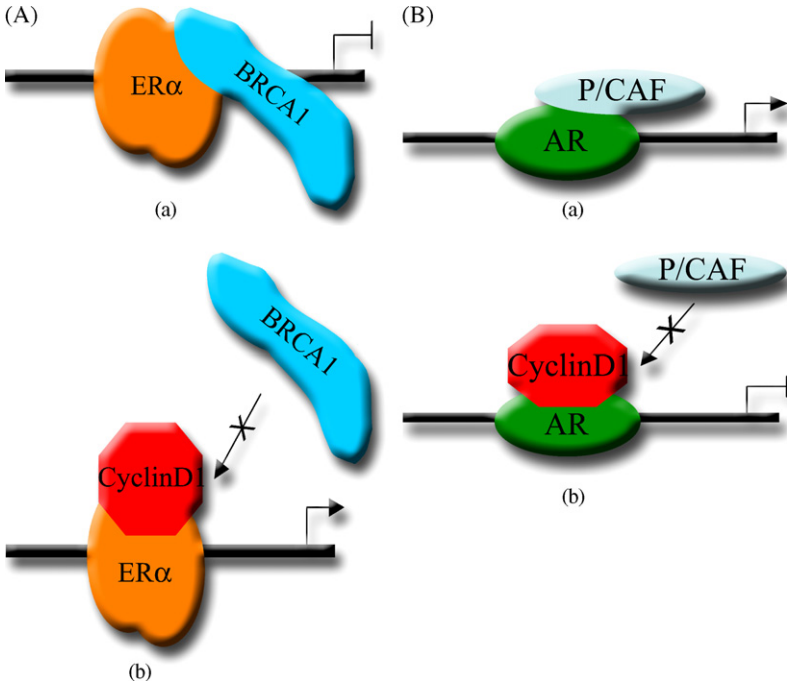

Fig. 1 – Regulation of nuclear receptors by cyclin D1. (A) BRCA1 represses ERα activity. Cyclin D1 upregulates ERα activity by antagonizing BRCA1 repression of ERα activity [15]. (B) Cyclin D1 inhibits AR by competing with p/CAF for binding to the AR [16].

methylation inhibitor, 5-aza-2′-deoxycytidine (5-aza-dC) yielded demethylation of the ERα promoter and reexpression of both ERα mRNA and protein. siRNA mediated reduction in the DNA methyltransferase, DNMT1 induced ERα expression [23,24].

The histone deacetylase, HDAC1, binds ERα and suppresses its transcriptional activity [22]. ER-negative cells that were treated with an inhibitor of HDAC1 activity, Trichostatin A (TSA), like the 5-aza-dC treated cells, had induced ERα mRNA and protein expression [25]. TSA and 5-aza-dC synergistically induce ERα expression in cells considered ER-negative [26]. DNA hypermethylation, histone hypoacetylation, H3-K9 methylation and recruitment of methyl CpG binding proteins (MeCP2, MBD1, MBD2), DNA methyltransferases (DNMT1 and DNMT3b) and HDAC1 all can work together to silence the ER promoter [27]. This was in contrast to ERα positive breast cancer cells, in which H3 and H4 acetylation and H3-K4 methylation increased with little methyl CpG binding protein and DNMT1 association and increased H3-K9 methylation at the ERα promoter. In ChIP assays, complexes containing histone modifiying enzymes and DNMTs (pRb2/p130-E2F4/5-HDAC1-SUV39H1-p300 and pRb2/p130-E2F4/5-HDAC1-SUV39H1-DNMT1 complexes) bound the ERα promoter to silence ERα gene expression [28]. Consistent with this finding, 5-aza-dC and TSA increase ERα expression and release the methyl CpG binding proteins (MeCP2, MBD1 and MBD2), DNMTs (DNMT1 and DNMT3b), methylated H3-K9 and HDAC1 [27].

4.2. *ERα acetylation regulates ERα activity*

Acetylation of ERα occurs both *in vitro* and *in vivo* through the HAT, p300 [29]. One acetylation site is located within the hinge/LBD of ERα which regulates transactivation, hormone sensitivity and phosphorylation function [30]. Glutamine or Arginine substitutions at the ERα acetylation site increase ERα's hormone sensitivity. Somatic mutations of the ERα Lys630 occur in breast cancer [31]. In 34% of patients with breast hyperplasia a Lys-to-Arg substitution at residue 303 was reported. The K303R mutation in the ERα resulted in an increased sensitivity to estrogen and resistance to repression by metastasis associated protein 2 (MTA-2) [32] and to BRCA1 [RGP unpublished] and therefore may play a role in breast cancer development.

5. Androgen receptor (AR)

The AR is important in the production of secondary sexual characteristics as well as in prostate cancer. Activity of the AR is regulated by hormones, and by hormone-independent (through EGF, IGF-1, KGF, IL-6 or HER-2/neu signaling [33–36]) mechanisms. Several coactivators enhance AR activity, including the SRC (p160) coactivators, p300/CBP, Ubc9, ARA70, ARA55, TIP60 and others [37–39]. An equally diverse group of corepressors inhibit AR activity through recruitment of HDAC activity or inhibition of HAT activity [37–39]. Post-translational modification by acetylation and phosphorylation regulate AR activity through modifying local chromatin and directly modifying the AR itself.

5.1. *Acetylation of the AR*

The AR is acetylated within its hinge domain by HATs, including p/CAF, p300 and TIP60 [40]. AR acetylation governs distinct properties of the AR including its transcriptional activity, its affinity for p300, and its ability to regulate prostate cancer cell growth. The AR acetylation site determines association with corepressor complexes. Acetylation dead mutants show enhanced binding of corepressors and reduced binding of coactivators in cultured cells. Conversely the AR acetylation mimic mutants have increased p300 and decreased N-CoR/HDAC/Smad3 corepressor binding [41,43,44].

The AR is acetylated in cultured cells, AR acetylation is regulated by physiological stimuli [44] and substitution mutations of the residues acetylated *in vitro* induce ligand-dependent transcriptional activity of the AR [41]. AR point mutations identified in prostate cancer patients at the acetylation site (AR_{K630T}) convey gain of function in human prostate cancer cells when implanted into nude mice. AR acetylation site mutants mimicking acetylation (AR_{K630Q} and AR_{K630T}) increased cellular proliferation and colony formation of prostate cancer cell lines in culture and in nude mice *in vivo*. The AR acetylation site growth properties may be mediated in part through enhanced recruitment to the promoters of a subset of target genes that augment cellular growth, including the *cyclin D1* gene [41–43]. Both cyclin D1 and cyclin E protein levels and activity were increased in prostate cancer cell lines expressing the AR acetylation mimic mutant compared with AR wild type. Furthermore, in ChIP assays, when comparison was made between the wild type AR and AR gain of function acetylation mimic mutants, increased recruitment of the mutant AR was observed at the cyclin D1 promoter. Thus aberrant acetylation of the AR may lead to enhanced growth through activation of cell cycle control genes [41].

The AR acetylation site regulates both cellular proliferation and evasion of apoptosis. Prostate cancer cells expressing the AR acetylation mimic mutants showed significantly reduced apoptosis compared to wild type controls [41]. The evasion of apoptosis was seen primarily through the apoptotic signals mediated by MEKK1 and JNK [43]. The contribution of AR acetylation to evasion of current therapies may become of increasing importance. AR acetylation mimic mutants show reduced response to current AR antagonists used to treat patients with prostate cancer [41,43]. In addition, the mechanisms governing AR acetylation and deacetylation may be an ideal new area for therapeutic intervention. Studies to date have shown the AR function is repressed by TSA-sensitive HDACs. p300 is a key coactivator of the AR, and p300 is deacetylated and repressed by the NAD-dependent HDAC, SIRT1 [45]. It will be of interest to determine whether the AR functions as a direct target for the NAD-dependent HDACs.

5.2. *AR regulation by G9a*

Recently Lee et al. [46] have demonstrated that the histone methylase G9a can either silence or activate target transcription factors including the AR and ER in reporter gene assays. G9a, CARM1, GRIP1 and p300 cooperate to activate the expression of both AR and ER, linking histone arginine and

lysine methylation. They further demonstrate that the histone modifications normally associated with transcriptional activation of proteins like the HMT and HAT activity of CARM1 and p300 work to inhibit the G9a's HMT activity, consistent with the histone code hypothesis.

5.3. *The histone demethylase, LSD1, regulates AR function*

Metzger et al. [13] demonstrated that AR and LSD1 proteins interact both *in vitro* and *in vivo* in normal prostate and prostate tumors. The loss of LSD1 protein, through either siRNA knockdown, mutations or specific LSD1 inhibitors, blocked androgen-induced transcriptional activation of reporter genes and LSD1 knockdown inhibited androgen-induced LNCaP cellular proliferation. JHMD2A also directly binds the AR and is recruited in a hormone-dependent manner [12]. Differentially methylated lysine residues are thought to serve as docking sites for platforms of chromatin remodeling proteins, thus the role of the AR acetylation site in the activity of these demethylases will be of considerable interest.

5.4. *Promoter methylation of the AR*

DNA methylation of the AR promoter is an example of epigenetic silencing and is regulated in prostate cancer. In 2000, Nakayama et al. [47] examined the methylation status of prostate cancer cell lines and hormone-refractory patient samples. In 20% of the primary cell lines and 28% of the hormone-refractory patient samples they found aberrant DNA methylation. They further examined AR expression after combination treatments with 5-aza-dC and TSA in DU145 prostate cancer cells. They demonstrated, as was the case with the ERα, that AR expression increased upon treatment with these epigenetic modification inhibitors. Increased AR activity present in patients with premature puberty is also a result of aberrant AR receptor promoter methylation [48] suggesting methylation-dependent AR expression plays a role in diverse disease processes.

6. Peroxisome proliferator-activated receptor γ (PPARγ)

PPARγ is a NR that mediates adipocyte differentiation, insulin sensitivity and can inhibit cellular proliferation [49–52]. Importantly, PPARγ ligands have been shown to inhibit growth of many cancer cell types, including colon, breast, gastric, adenocarcinoma and prostate cancers [53–60]. PPARγ can bind to DNA directly through heterodimerization with the retinoic X receptor (RXR). Upon ligand stimulation, PPARγ binds several coactivators (CBP, p300, SRC-1 family members, PPAR interacting protein (PRIP) and Med220 (PBP, TRAP220 or DRIP205) [61–63]), and in the absence of ligand, forms complexes with the coactivator PGC-1 [64] or corepressors N-CoR, SMRT or HDAC3 [65,66].

6.1. *PPARγ regulation by cyclin D1 and HDACs*

PPARγ inhibits the expression of cyclin D1, a key gene governing the cell cycle during cellular proliferation and adipocyte differentiation. With the addition of ligand, PPARγ competes with p300 for a c-Fos binding site and inhibits expression of cyclin D1 [52], see Fig. 2A. Furthermore, cyclin D1 can regulate ligand-induced PPARγ transactivation, expression and promoter activity independent of cyclin dependent kinase- and Rb protein-binding [67], as seen in Fig. 2B. It was recently determined that cyclin D1 could mediate PPARγ regulation by binding to HDACs 1, 2, 3 and 5 and increasing their activity [68]. It was also shown that cyclin D1 enhanced recruitment of other histone modifying enzymes, like the HMT Suv39H1, to the PPARγ promoter and concurrently led to decreased acetylation of Histone H3 [68]; see Fig. 2. Consistent with these findings Wang et al. [69], demonstrated that HDAC1 overexpression in transgenic mice reduced PPARγ protein levels. p300 is a rate limiting activator in PPARγ transactivation and p300 is repressed by cyclin D1 [70]. ChIP assays have demonstrated that cyclin D1 binds to p300 and represses its activity at the PPARγ-responsive element of the lipoprotein lipase promoter. Together these results suggest that cyclin D1, through the

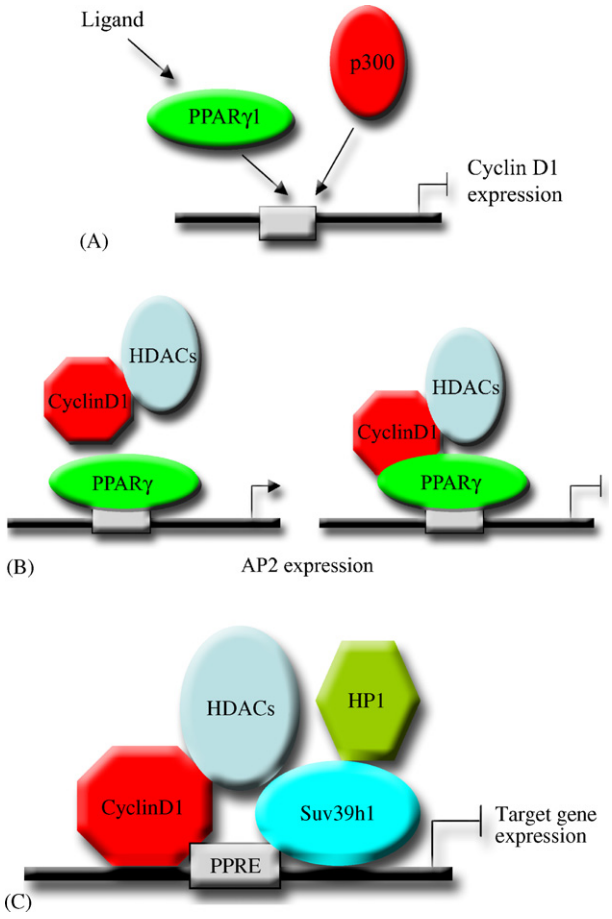

Fig. 2 – Interactions between histone modifying proteins, cyclin D1 and PPARγ. (A) PPARγ inhibits expression of the cyclin D1 promoter by competing with p300 at the AP-1 site of the cyclin D1 promoter [52] (B) Cyclin D1 recruits HDACs to inhibit PPARγ transactivation [67,68]. (C) Cyclin D1 also recruits histone modifying proteins to the local chromatin at PPARγ Response Elements (PPRE) where repression of target genes is seen.

regulation of epigenetic modifiers, plays a very important role in regulating the activity of PPARγ and therefore adipocyte differentiation.

6.2. *SIRT1 regulation of PPARγ*

The epigenomic modifier, mammalian NAD-dependent protein deacetylase, SIRT1, is a protein that is known to be important in fat mobilization in adipocytes upon food scarcity [71,45]. Picard et al., [72] have demonstrated that this regulation is due to SIRT1's ability to regulate PPARγ. In fasting mice, it was demonstrated that SIRT1 bound to and repressed genes normally controlled by PPARγ. It was also seen that SIRT1 bound to the PPARγ corepressors NCoR and SMRT and together these proteins repressed PPARγ. They further hypothesize a novel connection between calorie restriction and extending mammalian lifespan through SIRT1-mediated repression of PPARγ.

7. Conclusions

Histone modifying enzymes (HATs (p300, p/CAF), HDACs (HDACs, SIRTs), HMTs (G9a, Suv39h1) and demethylases (LSD1)) can regulate NR expression and activity. The labile growth factor- and oncogene-inducible factor, cyclin D1, regulates histone acetylation at the promoters of target gene sites within the chromatin at which NR reside. The deacetylation of histone H3 Lys 9 at PPARγ binding sites and recruitment of HDACs and methylases to NR binding sites by cyclin D1 provides a mechanism by which growth factor signals may coordinate epigenetic changes at NR binding sites. NR are acetylated and the acetylation sites regulate cellular growth. Single amino acid substitutions at the acetylated residues of the AR are sufficient to induce contact independent growth of human prostate cancer cells. NR are modified by phosphorylation, acetylation and sumoylation and these modifications coordinate NR function. Finally, cyclin D1 interacts with NRs and histone modifying enzymes suggesting a mechanism by which cyclin D1 may regulate tumor development and other human diseases. This knowledge will undoubtedly lead to new therapies and treatments to combat disease initiation and progression.

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