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Epigenetics and the Estrogen Receptor

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ABSTRACT: The position effect variegation in *Drosophila* and *Schizosaccharomyces pombe*, and higher-order chromatin structure regulation in yeast, is orchestrated by modifier genes of the Su(var) group, (e.g., histone deacetylases ([HDACs]), protein phosphatases) and enhancer E(Var) group (e.g., ATP [adenosine 5-triphosphate]-dependent nucleosome remodeling proteins). Higher-order chromatin structure is regulated in part by covalent modification of the N-terminal histone tails of chromatin, and histone tails in turn serve as platforms for recruitment of signaling modules that include nonhistone proteins such as heterochromatin protein (HP1) and NuRD. Because the enzymes governing chromatin structure through covalent modifications of histones (acetylation, methylation, phosphorylation, ubiquitination) can also target nonhistone substrates, a mechanism is in place by which epigenetic regulatory processes can affect the function of these alternate substrates. The

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posttranslational modification of histones, through phosphorylation and acetylation at specific residues, alters chromatin structure in an orchestrated manner in response to specific signals and is considered the basis of a “histone code.” In an analogous manner, specific residues within transcription factors form a signaling module within the transcription factor to determine genetic target specificity and cellular fate. The architecture of these signaling cascades in transcription factors (SCITs) are poorly understood. The regulation of estrogen receptor (ER) by enzymes that convey epigenetic signals is carefully orchestrated and is reviewed here.

EPIGENETICS

Epigenomics refers to the study of heritable changes in gene expression that occur without a change in DNA sequence. Through the silencing of tumor suppressor genes, epigenetic gene regulation frequently plays a critical role in the pathogenesis of cancer. Epigenomic modifications include covalent modifications of DNA and histones as well as noncovalent changes regulating nucleosome positioning. The enzymes regulating epigenetic change have been characterized in a number of animal systems including *Drosophila* and transgenic mice. Recent studies have characterized the mammalian enzymes that regulate epigenetic change. Environmental factors including hormones in turn regulate activities of several enzymes, altering DNA methylation and histone acetylation patterns.

Histone Methylation

Several recent studies have demonstrated the importance of histone methylases and histone demethylases in regulating estrogen receptor (ER) activity and expression. Posttranslational modification that does not alter DNA sequence requiring methylation occurs both on DNA and on proteins. Methylation of

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chromatin is often linked to methylation of DNA. A number of histone methylating enzymes directly interact with DNA methylating enzymes (DNA methyltransferases (DNMTs) and methyl-binding proteins).¹ Modification by methylation of DNA is generally targeted to cytosine residues in CpG dinucleotide pairs. Methylation governs genomic stability, retroelement suppression, and gene promoter regulation. Regions of CpG in the mammalian genome include large CpG islands (>500 bp), small CpG islands (200–500 bp) associated with transposons, and nonisland CpGs. DNA methyltransferases are associated with the replication complex in mammalian cells. This finding is consistent with a model in which the signals for methylating sequences in the genome are provided by a pre-existing hemimethylation. Thus, replication of the sequence to daughter chromatids temporarily results in hemimethylated DNA, which is recognized by DNMTs, ensures restoration of the symmetrical methylation of both DNA strands. The other major signal for methylation in mammalian cells is the presence of SINE³⁶ elements.

Methylation of histones occurs on either lysine or alanine residues, resulting in either condensation or relaxation of the chromatin architecture. Methylation likely provides binding sites for regulatory proteins with specialized binding domains. The main sites of methylation of histones occur on either heterochromatin or euchromatin. Heterochromatin is condensed and considered transcriptionally silent, whereas euchromatin is less densely packed and transcriptionally active. Methylated lysine residues are located characteristically within heterochromatin and demarcate subdomains.¹ Methylated histone residues serve as docking sites for repressive proteins, including the polycomb protein (PC) and heterochromatin protein (HP1), which recognize histone H3, K27 or H3, K9, respectively. HP1 and PC recognize methylated lysine residues through their chromo domain. Other proteins recognize methylated lysine

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residues though two other motifs, known as the Tudor domain and the WD40 repeat domain.

Histone methylation by protein arginine methyltransferases represents a relatively prevalent modification of proteins. The protein arginine methyl transferases (PRMTs) consist of two types, differing in the asymmetry of the dimethylarginine, product. The Type 1 PRMT forms monomethylarginine and asymmetric dimethylarginine, whereas Type 2 PRMT forms monomethylarginine and symmetric dimethylarginine. The PRMTs, 1, 2, 3, 4, and 6 are Type 1 PRMTs. The Type 2 PRMT is represented by PRMT5, also known as CARM1 (coactivator-associated arginine methyltransferase). Stallcup and coworkers⁴ demonstrated that CARM1 encodes both an arginine methyltransferase and a nuclear receptor coactivator, linking posttranslational modification by methylation to ER receptor signaling.

The histone lysine methylases share a common SET (Su/var, Enhancer of Zeste, Trithorax) domain. The SET domain conveys the *S*-adenosyl-Lmethionine cofactor to the epsilon amino group of the lysine residues. The histone H3 lysine 9 methyltransferase group catalyzes H3, lysine 9 methylation and includes *Suv39h1*, *Suv39 h2*, *G9a*, *G9a-related protein* and the *SET DB1* gene products.

Enzymatic demethylation of histone was first described in 1973 by Paik and Kim.³⁷ The identification of specific proteins regulating this activity took many years to be identified, however. Recently several lysine specific demethylases have been described, including lysine specific demethylase 1 (LSD1), JHDM1, JHDM2A, and JMJD2.³²

In addition to removal of arginine methylation by lysine demethylases, the methyl group can be removed from the

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arginine by the conversion of the methylarginine residue into citrulline, referred to as deimination. Deimination is conducted by arginine peptidyl arginine deiminase 4 (PADI4), which converts unmodified arginine and monomethylated arginine to citrulline at specific sites on the tails of H3 and H4.⁶ Importantly, for the purpose of this review, PADI4 repressed the estrogen-regulated gene, *pS2*, linking arginine demethylation to ER signaling.

Histone Acetyltransferases and Deacetylases

ER function can be regulated by histone acetylases and histone deacetylases (HDACs). Euchromatic DNA is packaged by histones into nucleosomes composed of 147 base pairs of DNA and the core histone proteins (H2A, H2B, H3, and H4). Dramatic alterations in chromatin structure are modulated through posttranslational modification of lysine tails.⁹ Conserved lysine residues are present in the amino terminal tails of all four core histones. Acetylation of lysine residues is thought to both neutralize the basic charge of histone tails and to serve as epigenetic markers which provide recognition motifs for docking of proteins that recruit transcriptional activators or repressors. The histone acetyl-transferase (HAT) enzymes were historically described as Type A, located in the nucleus, and Type B, located in the cytoplasm. Type B typically have a housekeeping role, acetylating newly synthesized free histones, whereas Type A HAT acetylate nucleosomal histones within chromatin in the nucleus. The Type A nuclear HATs include five families, the Gcn5-related acetyltransferases (GNATs), the MYST (MOZ, Ybf2/Sas3, Sas2, and Tip60)-related HATs, the HATs regulating general transcription (TAFII250), the CBP/p300 cointegrator HATs; and the p160 coactivator HATs, (SRC-1 and SRC3).⁹

The dynamic remodeling of acetylated lysine residues is mediated through two distinct types of HDACs: trichostatin A

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(TSA)-sensitive and NAD-regulated. TSA-sensitive HDACs include the class I HDACs (HDACs 1, 2, 3, and 8) which are related to the *Saccharomyces cerevisiae* transcriptional regulator RPD3. The class II HDACs are related to the yeast HDA1 protein and include HDACs 4, 5, 6, 7, 9, and 10. HDAC 11 is more related to the Type I HDACs and contains a catalytic domain at the N-terminus with HDAC activity.

NUCLEAR RECEPTORS

Nuclear receptors function as transcription factors that govern transcription of genes. Their activity can be regulated by steroids, thyroid hormone, retinoic acids, or vitamins, and they coordinate diverse processes such as homeostasis, reproduction, development, metabolism, and disease.⁴⁰ There are four main conserved domains of all nuclear receptors. These domains include the activation function domain (AF), the DNA-binding domain (DBD), the hinge region, and the ligand-binding domain (LBD), the latter being the site of many protein–protein and hormone interactions.

Coregulator proteins, both coactivators and corepressors, work with nuclear receptors in complexes that govern gene expression. Nuclear receptors and the transcriptional apparatus are often linked together through coactivator recruitment of protein complexes. These complexes then can use their histone-modifying abilities to alter the local chromatin structure. Several coactivators that bind to nuclear receptors include steroid receptor coactivator-1 (SRC-1), amplified in breast cancer/ thyroid and RA receptor/SRC-2 (AIB1/ACTR/SRC-2), glucocorticoid receptor interacting protein 1/transcriptional intermediary factor 2/SRC-3 (GRIP1/TIF-2/SRC3), menin, and p300/CBP and p300/CBP-associated factor (p/CAF).^{17,18,51} Corepressors function with unliganded nuclear receptors to silence gene expression. These corepressors include proteins like

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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.^{17,18,51}

THE ESTROGEN RECEPTOR

The last two decades have seen an evolving body of literature providing a compelling case for dynamic regulation of ER function through both posttranslational modification by acetylation and through epigenetic signaling cascades. The nuclear receptor, ER, is activated by, and controls the activity of, the steroid hormone, estrogen. Together estrogen and its receptor are vitally important in normal development, reproduction, and various diseases. ER is distributed at the cellular membrane, the cytoplasm, the nucleus, and the mitochondria. The membrane-bound form of ER regulates nongenomic function through interactions with SHC and caveolin-1, inducing acute activation of PI3 kinase and Akt signaling pathway. The nuclear form provides DNA-dependent regulation of gene expression. Epigenetic regulation has been well characterized for the nuclear located ER. The role of epigenetic signals of ER located in other compartments of the cell remains to be better understood.

ER binds DNA either directly or through other transcription factors (AP-1, Sp1) in order to regulate transcription of target genes. The ER functional activity in the nucleus is mediated through binding of coactivator and corepressors, which encode enzymes with HAT-modulating activity. The cointegrators CBP/p300 (CREB-binding factor) encode intrinsic HAT activity. The binding of HATs to the ER provides a docking function leading to acetylation of local histones with consequent nucleosomal destabilization facilitating transcription factor binding to local DNA sequences at promoter regions of estrogen-

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responsive genes. Some proteins that are already known to bind the ER include members of the p160 family (SRC-1, TIF2/GRIP1/SRC-2, AIB1/ACTR/SRC-3), cyclin D1, menin, and many HATs (CBP, p300, p/CAF).^{21,29,30} The p160 coactivator family (SRC1/amplified in breast cancer/activator of the thyroid and RA receptor/ SRC-2 to [AIB1/ACTR/SRC3]) and the related group 1 (GRIP-1/TIP-2/SRC-2) facilitate the interaction between the p300 coactivator and the nuclear receptor. In addition, nuclear receptor repressors of the N-CoR and SMRT complex physically associate with the ER particularly in the presence of the ER ligand antagonists such as tamoxifen. The ER has been shown to physically associate with several corepressors which encode intrinsic HDAC activities including BRCA1 and MTA1.⁸ BRCA1 binds the ER to repress ligand-induced gene expression. BRCA1 repression of ER is opposed by endogenous cyclin D1 through physical association within local chromatin⁴⁵ (FIG. 1). It is plausible that BRCA1 association with ER may play a role in the recently described finding that transient double-stranded DNA break formation occurs during ER ligand-dependent gene transcription.²⁴

Epigenetic Modifiers Silence the ER Gene

In breast cancer ER gene expression is an important prognostic factor.⁵² Altered expression and function of ER coregulators with epigenetic function have been demonstrated to play a role in hormone signaling. The AIB1 protein is frequently amplified in breast cancer correlating with the ER-positive status.² The expression levels of TIP2, CBP, and ER are strongly correlated in intraductal breast carcinomas.³¹ Furthermore, tamoxifen-resistant tumors show a shorter relapse of survival in samples with low expression of NCoR.²⁰

Unfortunately though, 30% of breast cancers are ER-negative

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upon diagnosis²² and many breast cancers can lose ER expression during progression of the cancer. In many patients epigenetic modification plays a role in the loss of ER gene expression. Yang *et al.*⁵⁴ demonstrate reduced ER expression due to increased DNA methylation and/or histone deacetylation.⁵⁴ The demethylase PADI4 has been linked to the repression of the estrogen-responsive gene *pS2*.⁶ HDACs bind ER and HDAC overexpression silences expression of the ER gene.²⁵ siRNA-mediated reduction of DNMT1 induced ER expression.^{39,53} Two commonly used inhibitors of this silencing mechanism are the DNMT inhibitor, 5-aza-2-deoxycytidine (5-aza-dC), and the HDAC inhibitor, TSA. Treatment with either inhibitor individually enhanced ER gene expression.^{25,54,55} Use of both 5-aza-dC and TSA enhanced re-expression of both ER mRNA and protein in human breast cancer cells. The re-expressed ER was functional and it induced expression of known ER target genes. Collectively these studies suggest how inhibitors of HDACs and DNMTs may be used to reinduce ER expression and thereby restore therapeutic response to ER antagonists.

Macaluso *et al.*³⁴ examined further the mechanisms silencing the ER gene and identified several multimolecular ER repression complexes. pRb2/p130E2F4/5-HDAC1-SUV39H1-p300 or pRB2/p130-E3F4/5-HDAC1-DNMT1SUV39H1 proteins were found on the ER promoter. These complexes included HDACs, DNMTs, the histone methyltransferase, SUV39H1, and the cell cycle regulatory protein, pRb2/p130. It was hypothesized that protein complexes recruited by pRb2/p130 modulate acetylation or methylation to the ER promoter, regulating its expression by altering the local chromatin structure through the histone methyltransferases, like SUV39H1. Sharma *et al.*⁴² examined the multimolecular protein complexes upon treatment of cells with 5-aza-dC and TSA. Cells with silenced ER demonstrated DNA

hypermethylation, histone hypoacetylation, H3K9 methylation, and an increased abundance of methyl-binding proteins, DNMTs, and HDACs. Treatment of these cells with the HDAC and DNMT inhibitors led to ER re-expression, release of the repressor complexes, enrichment of histone acetylation, and decreased H3K9 methylation.

The ER Works in Regulating Target Gene Transcription with Coregulators

In addition to the HATs and HDAC-containing complexes, ER forms protein complexes with other coregulatory proteins to govern expression of genes.

A component of a histone methyltransferase complex regulates the ER to further regulate target genes.⁷ Menin is the protein product of the multiple endocrine neoplasia 1 (MEN1) tumor suppressor gene. It is a component of the MLL1/MLL2 H3K4 histone methyltransferase complex, which is typically linked to gene activation. Menin physically associates with the ER and works as a coactivator to activate the expression of the ER target gene, pS2 (TFF1).⁷ Importantly, MEN1 mutations associated with disease disrupted any ER-menin interaction and the ER activity.

Acetylation of the ER Regulates its Activity

In the first studies demonstrating direct acetylation, the ER was shown to be acetylated in MCF7 human breast cancer cells by immunoprecipitation Western blotting.⁴⁴ GST-fusion proteins of the ER were then shown to serve as *in vitro* substrates for acetylation in the presence of p300, produced in baculovirus.

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Deletion analysis identified a minimal region of ER acetylated by p300. Edman degradation assays and MALDI-TOF mass spectrometry identified the acetylated residues as preferentially lysine K302 and K303 with some minimal acetylation of lysine 299. A minimal ER peptide was acetylated by p300 with similar efficiency as histone H3. Point mutations of the lysine residues in ER resulted in ER mutants that were activated at lower concentrations of E2 than the wild-type ER. Glutamine or alanine substitutions of the acetylated lysine residues enhanced transactivation; in particular, ER activation by p300 was enhanced. The alteration in transactivation by the acetylation site was distinct and did not affect activation by other kinases, including mitogen-activated protein kinase or activation by the p68 RNA helicase A.⁴⁴ The ER K303R mutation conferred enhanced activation of ER activity at low subphysiological levels of hormone. The ER lysine motif that was acetylated directly by p300 was shown to be conserved across species and the motif was identified in many other phylogenetically related receptors, including the androgen receptor and PPAR⁴⁴ (FIG. 2). Analysis of the related lysine motif in the AR demonstrated a similar biochemical function and growth-regulatory properties.^{11,14-16}

Independently of these findings another laboratory had identified a point mutation of the ER at K303 occurring as a somatic mutation in human breast cancer.¹⁹ The mutation of K303 occurred with high frequency (30%) of early breast cancer lesions, referred to as ductal carcinoma *in situ*. The ERK303R mutant conveyed a growth advantage to breast carcinoma cells in culture.¹⁹ Growth assays demonstrated that the acetylation site ERK303R mutation enhanced cellular proliferation in response to low concentrations of estradiol, suggesting the ER K303R mutation provides a “gain of function” mutation in human breast cancer.

Analysis of other nonhistone substrates for acetylation, such as

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p53, provides evidence that the acetylation of transcription factors may in turn regulate their phosphorylation²⁸ by distinct kinases.²⁸ Thus, acetylation of lysine 320 prevents phosphorylation of serine in the NH₂-terminal region of p53. The ER is phosphorylated by protein kinase A at residue 305. The generation of a kinase-active mutant of the ER through the introduction of an aspartic acid residue blocked acetylation of the ER.⁵ Studies by Cui *et al.* demonstrating ER acetylation is linked to phosphorylation of the ER at residue serine 305 may be of importance to therapy resistance in breast cancer, as PKA activation of ER has been linked to tamoxifen resistance.³⁵ These studies suggesting that acetylation and phosphorylation within the ER are coupled are consistent with prior studies coupling acetylation and phosphorylation of the androgen receptor.^{11,50}

p53 is known to be acetylated at distinct sites coordinating distinct signaling pathways.²⁸ It is known that p300/CBP acetylates carboxyl-terminal lysine residues of p53 (lysines 372, 373, 382). P/CAF acetylates a residue within the flexible linker region of p53 (lysine 320). In response to genotoxic stress, DNA damage induces acetylation of lysine 320 and lysine 373 with distinguishable kinetics. These two distinct acetylation sites regulate distinct clusters of genes with high-affinity p53-binding sites, promoting cell survival.²⁸ In contrast K373 regulates interactions with affinity DNA-binding sites found in proapoptotic genes leading to cell death. In keeping with the finding of multiple modular acetylation sites in p53, additional acetylation sites were identified in the ER at lysine K266 and K268.²⁷ Contrary to K302/303, which were acetylated directly by p300,⁴⁴ K266/268 was acetylated in an assay using p300/p160 (SRC1) and the ligand estradiol.

Although the distinguishable biological functions of the ER

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lysine residues remains to be determined, the acetylated residues in the androgen receptor (K302/303) promote DNA synthesis and antiapoptotic signals.^{11,14-16} The AR acetylated residues regulate access to DNA in chromatin immune precipitation assays¹¹ and promote binding to promoters of target genes that induce DNA synthesis such as cyclin D1.^{11,14-16} The p53 acetylation site also alters access within the local chromatin structure to enhance DNA binding.²⁸ In electrophoretic mobility shift assays, the ER acetylation (K266, and K268) was shown to regulate DNA binding, whereas acetylation of the ERK303 site does not affect binding. The application of an unbiased proteomic approach to examine ER residues acetylated *in vivo* under physiological conditions and in tumors will be of interest (FIG. 3).

Cyclin D1 Regulates ER Activity

The cyclin D1 gene product is overexpressed in 30–40% of cancers and is associated with poor prognosis in ER-positive breast cancer patients.^{26,38} Initially cloned as part of a breakpoint rearrangement in parathyroid adenoma, the cyclin D1 protein has subsequently been shown to bind HDACs, the pRB protein, and several transcription factors.¹³ Cyclin D1 contributes a catalytic subunit function of a kinase that phosphorylates pRb and NRF1.^{41,47} Phosphorylation of pRB in the nucleus regulates DNA synthesis, while phosphorylation of NRF1 regulates mitochondrial biosynthesis.^{41,47} At the membrane, cyclin D1 promotes cellular migration, and angiogenesis through induction of cytokines and vascular growth regulatory proteins (VEGF, TSP1).³³

The role of cyclin D1 in regulating the nuclear receptor is complex. *In vivo*, cyclin D1 knockout mice develop a phenotype of enhanced PPAR activity with hepatic steatosis due to the

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inhibition of PPAR activity by cyclin D1.⁴⁸ Cyclin D1 has been shown to associate with the ER and enhance ER activity, in part through recruiting the SRC1 p160 coactivator in a ligand-independent manner,⁵⁶ and also through antagonizing the ER corepressor BRCA1.⁴³ Cyclin D1 functions in regulating both ER and BRCA1 occupancy in the context of local chromatin at an estrogen response element of the *pS2* gene.⁴³ The finding that cyclin D1 regulates local chromatin occupancy is consistent with the findings that cyclin D1 binds SUV39, HP1 and HDACs and that the abundance of cyclin D1 determines the local acetylation of histones, including histone H3 Lys9.^{10,23,46} It is known that mammary-targeted expression of cyclin D1 induces mammary tumorigenesis in transgenic mice. Therefore, it will be of interest to determine the relative importance of these kinase-independent functions of cyclin D1 in inducing mammary tumorigenesis and estrogen responsiveness.

CONCLUSIONS

Over the last decade histone-modifying enzymes have been successfully cloned and characterized. These posttranslational modifications within histone tails provide the basis of signaling modules within the local chromatin, known as the “histone code.” Recent studies have demonstrated the presence of similar enzyme complexes associated with the ER. Similar to histone tails, transcription factors, such as p53 and the nuclear receptors, ER and the AR, encode signaling modules within the protein, in which one posttranslational modification leads to a sequential commitment to other types of posttranslational modifications. Cascades of acetylation, phosphorylation, and ubiquitination modify the receptors’ function, genetic response, and consequent cell fate decisions. Although these signaling cascades in transcription factor or SCITs are poorly understood at this time, the residues for posttranslational modification appear to be well conserved across species. In the same manner that cytoplasmic

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signaling cascades in the last decades defined kinase modules, so too will the next decade provide important insights into intratranscription factor signaling cascades that may ultimately contribute to the biological specificity of nuclear receptor signaling.

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FIGURES

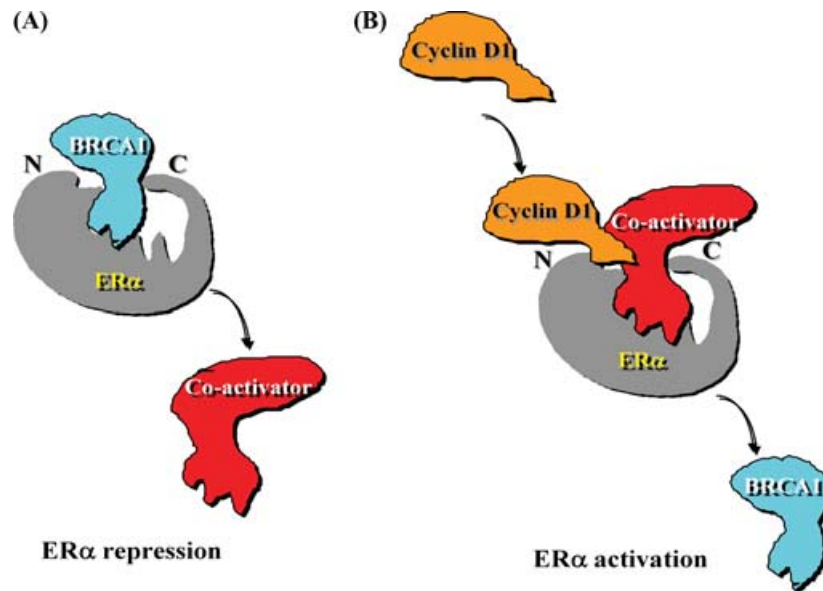


FIGURE 1. Hypothetical model by which cyclin D1 augments ER signaling. The ER activity is repressed by BRCA1,⁸ Endogenous cyclin D1 opposes the action of BRCA1 at an ERE. Cyclin D1 associates directly with the ER. The prevailing view in cancer biology is that two general classes of cancer genes exist, those that regulate genomic stability including antimutators and DNA repair genes, and a second class of genes regulating cell cycle control. Cyclin D1 shares both properties, promoting DNA synthesis and regulating the function of BRCA1.⁴³ Estradiol induces BRCA1 to the pS2⁴³ ERE in ChIP assays, an effect antagonized by cyclin D1,⁴³ and E2 induces transient double-strand breaks with the recruitment of DNA repair complexes to the ERE of the pS2 gene. Collectively these studies support a model in which cyclin D1

regulates both cell proliferation and DNA repair function.

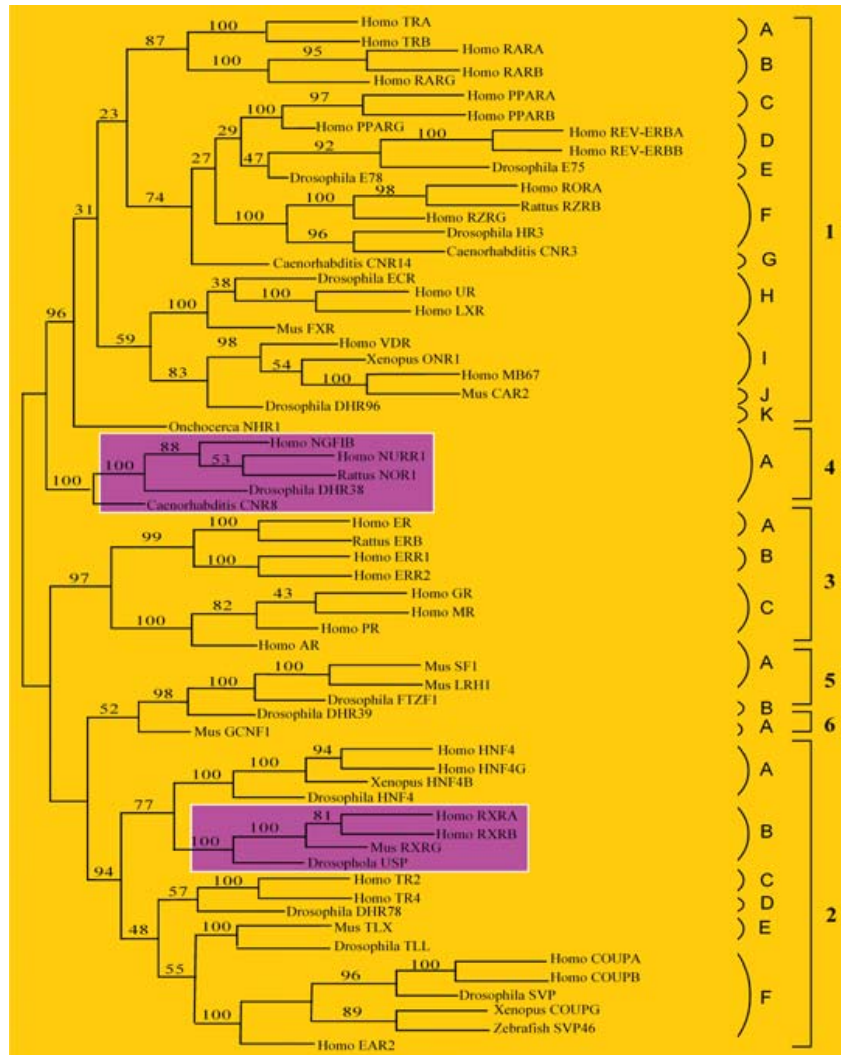


FIGURE 2. Phylogenetic conservation of the acetylation motif. The phylogenetic tree linking nuclear receptors in vertebrate arthropods and nematodes is shown. Nuclear receptors containing the acetylation motif are shown in yellow, whereas

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nuclear receptors lacking the motif are shown in pink.⁴⁹

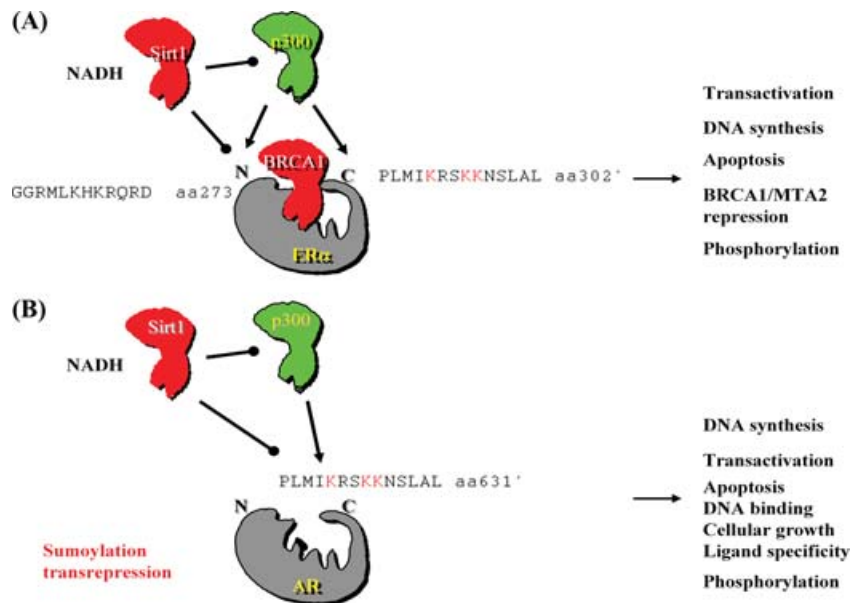


FIGURE 3. The ER is regulated by TSA and NAD-dependent HDACs. The ER and the AR are acetylated at conserved lysine residues. Although NAD regulates ER activity, p300, an essential coactivator of ER, is also repressed by Sirt1.³ The AR has been shown to be directly regulated by acetylation in response to physiological stimuli and to be repressed by SIRT1 in a catalytic domain-dependent manner.¹² An acetylated AR substrate functions as an excellent substrate for SIRT1. The nuclear receptor acetylation site governs nuclear receptor function, as indicated to the right, including transactivation DNA synthesis and apoptosis. In the case of the AR, the transrepression and sumoylation of the AR are unaffected by acetylation.