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Post-translational modifications of Runx1 regulate its activity in the cell

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Abstract

In this report we review the current knowledge of the interaction of RUNX1(AML1) with serine/threonine kinases, lysine and arginine methyltransferases, lysine acetyltransferases, and histone deacetylases. We also discuss the effect of RUNX1-ETO fusion gene on DNA methylation. RUNX1 post-transcriptional modification can affect its role in influencing differentiation and self-renewal of hematopoietic cells. The goal of these studies is to develop targets for improved leukemia therapy.

Introduction

Transcription factors (TFs) can be modified through phosphorylation, acetylation, ubiquitination, SUMOylation, and methylation, and such post-translational modifications are important for fine-tuning transcriptional regulation of gene expression. These modifications impact on the auto inhibition, dimerization, proteolytic cleavage, cellular localization and ubiquitin mediated degradation of TFs. The DNA binding activity of transcription factors such as p53 can be regulated through acetylation, phosphorylation [1] and deacetylation[2] and so can transcriptional activation or repression. Similar modifications (such as phosphorylation of the CTD tail of the largest subunit of the RNA polymerase II) can affect transcriptional elongation and other processes that affect gene expression.

Our lab has been studying the RUNX1 (AML1) and RUNX1-ETO (AML1-ETO) proteins[3–11]. Both RUNX1 and the leukemia-associated RUNX1-ETO protein can activate or repress gene expression and this dual capability may reflect the ability of these proteins to bind co-activator and co-repressor molecules. Our lab is interested in understanding what controls this switch, and how specific post-translational modifications in RUNX1 and RUNX1-ETO, and the promoter-specific arrangement of regulatory elements and chromatin architecture control gene expression. We have been focused on enzymes that post-translationally modify histones, as we have discovered that several such enzymes also modify transcription factors. Indeed, we believe that DNA sequence specific transcription factor binding may drive the deposition of most chromatin marks, by bringing specific enzymatic activities to the DNA and/or histones. In this review we will cover various post-translational modifications of RUNX1 and speculate as to how these modifications generate a profoundly diverse mode of regulation.

Interactions of RUNX1 with serine/threonine kinases

It is important to note that while the study of post-translational modifications (PTMs) has rapidly expanded, one of the earliest PTMs to be recognized was phosphorylation. RUNX1 has been shown to be phosphorylated by the ERK1/2 serine/threonine kinases[12], which can be activated by hematopoietic cytokines, such as IL-3[12] thrombopoietin[13], and also EGF and other signals[14]. RUNX1 is phosphorylated by ERK1/2 at several sites within its transactivation domain, including Serine-249 and serine-266[12], and ERK2 increases transactivation by RUNX1b, at least in part because phosphorylated RUNX1 preferentially associates with the p300 co-activator protein [15].

Phosphorylation of RUNX1 also leads to its dissociation from the mSIN3 complex[14], even though phosphorylation occurs at a distance from its mSIN3 binding site [16]. While disruption of mSIN3A binding[14] potentiates the transactivation activity of RUNX1[12], phosphorylated RUNX1 is also targeted for proteasome-mediated degradation, due to its release from the nuclear matrix[14,17], establishing a negative feedback loop. Thus, mutation of RUNX1 phosphorylation sites prevents RUNX1 from dissociating from mSIN3A, but also stabilizes RUNX1 following cytokine activation of ERK. RUNX1 is required for megakaryocytic maturation in adult hematopoiesis[18], and sustained ERK1/2 activation is required for cell differentiation into megakaryocytic cells[19–21] implying that RUNX1 phosphorylation by ERK1/2 may be critical for megakaryocytic differentiation.

RUNX1 can stimulate G1 cell cycle progression, and RUNX1 protein levels change as cells progress from G1 to S phase[22], which implies a close interaction between RUNX1 and the cell cycle machinery. RUNX1 is a substrate of Cdk 1/2/6[17,23], which phosphorylate RUNX1 at 4 major sites: serine 276, 293, 300 and 303. In a similar mode of regulation, this phosphorylation promotes the APC (anaphase promoting complex) mediated degradation of RUNX1[17]. Recently, Zhang et al. demonstrated that cdk1 and cdk/6 phosphorylate RUNX1 at serines 303 and also 48 and 424[23]. Triple mutant forms of RUNX1, in which all three residues were mutated to alanine (TripleA) or aspartic acid (TripleD), were analyzed and the TripleA mutant was found to have a longer half-life than the TripleD mutant protein. Yet, the TripleD protein had enhanced transactivation potency in a promoter-based reporter assay, and more effectively rescued the inhibitory effects of CBFβ-SMMHC then did wild type RUNX1.

RUNX1 has also been shown to interact with cyclin D3[24] and Cdk6[25], which negatively affect its DNA binding activity. Importantly, down regulation of Cdk6, and release of RUNX1 from cdk6 inhibition, are required for promoting granulocytic differentiation. Taken together, these studies reveal a complex interaction between RUNX1 and its kinases, which may initially potentiate its function but then trigger its degradation. Modification of DNA binding, transactivation and protein-protein interactions, the key features of all TFs, are subject to regulation by phosphorylation.

Interactions of RUNX1 with lysine methyltransferases

Histone marks are associated with transcriptional activation or repression, and with distinct types of chromatin: euchromatin, facultative heterochromatin and constitutive heterochromatin. Whereas histone acetylation is generally an additive process, histone methylation has site-specific effects. Histone H3K9 methylation marks repressed chromatin, while histone H3K4 methylation generally marks sites of active transcription[26]. SUV39H1 is one of the SET domain-containing histone methyltransferases responsible for histone H3K9 methylation. RUNX1 has been shown to interact with SUV39H1 through its runt domain, and this interaction disrupts the binding of RUNX1 to DNA[27]. Lysine methylated RUNX1 can be detected in 293T cells following overexpression of Flag-RUNX1b and SUV39H1. However, it remains to be determined whether RUNX1 is lysine methylated in hematopoietic cells, and

what effect that PTM plays in transcriptional regulation by RUNX1. Thus far, it has not been shown whether recruitment of the SUV39H1 gene by RUNX1 contributes to H3K9 methylation or to gene repression by RUNX1.

Interactions with arginine methyltransferases

Arginine methyltransferases are a group of enzymes that transfer methyl groups from S-Adenosylmethionine (SAM) to the guanidino-side chain of arginine residues, resulting in ω-NG-monomethyl arginine, ω-NG,NG-asymmetric dimethyl arginine, or ω-NG,NG symmetric dimethyl arginine in proteins[28]. Arginine methyltransferases exist as dimers or oligomers, and often shuttle between the nucleus and the cytoplasm. PRMT1 is the most abundant arginine methyltransferase in the cell and knock out of PRMT1 in mice results in embryonic lethality [29]. PRMT1 has been demonstrated to be a co activator for many transcription factors including p53, the nuclear hormone receptors, and several FoxO family members[30]. PRMT1, through methylation of histone H4R3, enhances p300 mediated acetylation and thus transcriptional activation[31].

We have found that PRMT1 expression is upregulated when CD34+ cells are stimulated to differentiate into myeloid cells in *in vitro* cultures. Along with PRMT1 upregulation, RUNX1 and methylated RUNX1 levels are also upregulated. However, when the CD34+ cells differentiate towards the erythroid lineage, PRMT1 is not expressed and methyl RUNX1 is not detectable. RUNX1 is directly methylated on several arginine residues by PRMT1; one region just C terminal to the RUNX1 runt domain that is methylated (RTAMR motif) is not the consensus PRMT1 “RGG” sequence defined in earlier studies (see review of Gary and Clarke, 1998)[28]. Nevertheless, the RTAMR region is flanked by positive amino acids (arginine and histidine), much like the RGG sequence. Methylation of the RTAMR site results in the dissociation of RUNX1 from the mSIN3a complex[32]. Changes in PRMT1 levels affect the ability of RUNX1 to activate its endogenous target genes, such as CD41 and PU.1[32]. Both RUNX1 and PRMT1 bind to the URE that regulate PU.1 expression and as predicted PRMT1 binds to a broader region of the PU.1 gene than RUNX1. In addition to RUNX1 methylation, the chromatin structure of the PU.1 promoter may be modified by arginine methylation by PRMT1.

Arginine methylation of RUNX1 occurs in a lineage-dependent manner, and may influence the position of differentiation. The BTG family of proteins can enhance PRMT1 enzymatic activity and both PRMT1 levels and BTG1 expression is upregulated when cells commit to the erythroid lineage[33]. Yet BTG1 expression is low in megakaryocytic cells lineage even though RUNX1 is abundantly expressed. Because PRMT1 is minimally expressed in megakaryocytes, we hypothesize that RUNX1 can repress PU.1 expression in these cells by binding the SIN3a complex. Indeed, ChIP assays of the PU.1 gene in megakaryocytic cells confirm this hypothesis by showing that PU.1 is repressed when RUNX1 and the SIN3 complex are found in the PU.1 URE region. Though RUNX1 participates in forming a repressor complex in the URE region[9], it is important to note that RUNX1 may not be the sole transcription factor involved in repressing PU.1 gene expression. Nonetheless, we found that overexpression of RUNX1 in the context of reduced PRMT1 levels can switch wild type RUNX1 from an activator to a repressor. We believe that PRMT1, by interacting with master regulators of hematopoietic cell differentiation such as RUNX1, is able to facilitate differentiation by establishing the correct chromatin structure for the expression of lineage specific genes.

Interactions of RUNX1 with lysine acetyltransferases

The acetylation of histones, transcription factors, and other proteins can alter nucleosome conformation, regulate gene transcription, and thereby affect diverse biological events. The lysine acetyltransferases p300 and CREB-binding protein (CBP) function as transcriptional

co-activators and play distinct roles in normal hematopoiesis[34–36]. Furthermore, genetically engineered mice that lack p300 and/or CBP develop hematological malignancies[36–39]. p300/CBP and the MYST family of lysine acetyltransferases [monocytic leukemia zinc-finger protein (MOZ) and monocytic leukemia zinc finger protein-related factor (MORF)] are the targets of chromosome translocations associated with acute myeloid leukemia and myelodysplastic syndromes[40,41]. Based on their involvement in chromosomal rearrangements, and their role in normal stem cell biology, it is likely that aberrant lysine acetylation is implicated in leukemogenesis.

RUNX1 can regulate local histone acetylation and the transcription of its target genes by recruiting lysine acetyltransferases including p300, CBP, MOZ and MORF[42]. The region between amino acid 178 and the C-terminus in RUNX1 is responsible for its interaction with p300[43], and phosphorylation of RUNX1 may be a critical step in its binding p300[44]. Formation of RUNX1/ CBFβ heterodimers and their binding to DNA leads to the phosphorylation of RUNX1, which in turn leads to the phosphorylation of p300[45]. Homeodomain interacting kinase 2 (HIPK2) forms a complex with RUNX1 and p300, and it can phosphorylate RUNX1 and p300 both *in vitro* and *in vivo*. Thus, HIPK2 stimulates transcriptional activation by RUNX1 and also stimulates the acetyltransferase activity of p300 [44,45]. The sites of the HIPK2-mediated phosphorylation in AML are Ser-249, Ser-273 and Thr-276; p300 specifically acetylates RUNX1 at two conserved lysine residues (Lys-24 and Lys-43), significantly augmenting its DNA binding activity[46]. Mutation of these two lysines severely impairs the DNA binding of RUNX1 and reduces its transcriptional activity and transforming potential[46]. Overexpression of p300 stimulates RUNX1-dependent transcription and induces the differentiation of myeloid cells[43]. RUNX1 and p300 can be recruited into PML nuclear bodies by PML1, a specific isoform of the promyelocytic leukemia (PML) protein, leading to activation of RUNX1-dependent transcription and myeloid cell differentiation[15].

MOZ and CBP also can acetylate RUNX1 in vitro[47]. MOZ is a component of the RUNX1 complex in normal hematopoietic cells, and it too functions as a transcriptional co-activator, potentiating RUNX1-dependent gene expression. However, stimulation of RUNX1- mediated transcription by MOZ is independent of its HAT activity, which is contained in its MYST domain. Instead, a potent transactivation domain within MOZ appears to be necessary for its stimulatory effects on RUNX1-mediated transcription, suggesting a cooperative model of activation. The amount of RUNX1-MOZ complex increases during the differentiation of M1 myeloid cells into monocytes/macrophages, suggesting the interaction of RUNX1 with MOZ might play a role in promoting monocytic differentiation[47].

Thus, lysine acetyltransferases associate with RUNX1, increase its DNA binding activity, and assist in promoting gene expression. They change the nearby nucleosomal environment by acetylating lysine residues in histones and upregulate the expression of genes by recruiting the basal transcription machinery. RUNX1 not only activates but also represses gene transcription, raising a question as to how the acetyltransferases selectively bind to RUNX1 on certain gene loci, or whether the enzymatic activity of the RUNX1/acetyltransferase complex can be inactivated under certain conditions. Acetylation of RUNX1 may also drive differentiation down distinct paths.

Interactions of RUNX1-ETO with Histone Deacetylases

In the leukemia-associated RUNX1-ETO protein, the N-terminal 177 amino acids of RUNX1, which include its DNA binding domain, are fused to an almost complete ETO protein (amino acids 30 to 604). RUNX1-ETO functions as an inhibitor of RUNX1 function during fetal development and it has been shown that the co repressor and HDAC binding domains within

ETO contribute strongly to the repressive qualities of RUNX1-ETO. Though there are numerous co repressor binding domains throughout ETO, the NHR2 and NHR4 domains appear to be of particular importance for the interactions of ETO with histone deacetylases (HDACs). Sin3 directly binds ETO at the NHR2 domain and SIN3 has been co purified with NuRD complexes and with HDAC 1 and 2. N-CoR, typically complexed with SMRT, can bind ETO at the NHR4 site and also bind HDAC3[48]. In addition to co-repressor mediated HDAC binding, direct interactions of ETO with HDAC 1, 2, and 3, which are independent of co repressor binding have been documented[49].

Regardless of the means of interaction, however, once assembled, HDAC complexes become critical effectors of RUNX1-ETO-mediated repression. The introduction of single amino acid mutations that impair the ability of RUNX1-ETO to interact with N-CoR leads to loss of repression of a RUNX1 target promoter NP-3[50]. The ability of RUNX1-ETO to repress the $p21^{Waf1/Cip1}$ promoter, was inhibited by adding the HDAC inhibitor Trichostatin A (TSA)[7]. Another HDAC inhibitor, Valproic acid (VPA), which selectively targets HDAC2 for proteasomal degradation was found by Marcucci *et al* to disrupt the physical interaction of RUNX1-ETO with HDAC1, and to induce the re-localization of RUNX1-ETO (and HDAC1) from the nuclear to the perinuclear region. This re-localization correlated with the hyperacetylation of histones H3 and H4 and the release of RUNX1-ETO and HDAC1 from the IL-3 promoter[51]. In a similar study, Santini *et al* showed release of RUNX1-ETO and HDAC1 from the IL-3 promoter in Kasumi-1 cells, after treatment with the HDAC inhibitor ITF2357[19].

Some HDAC inhibitors not only alleviate RUNX1-ETO repression, but also lead to the degradation of the chimeric protein. TSA has been shown to dramatically reduce the levels of RUNX1-ETO in a dose dependent manner prior to inducing apoptosis in Kasumi-1 cells[20]. A similar result was obtained using the HDAC inhibitor Depsipeptide, and by using an HSP90 inhibitor 17-allylamino-geldanamycin (17-AAG), Hiebert *et al.* were able to show that RUNX1-ETO associates with normal, acetylated HSP90 and that HDAC inhibitors trigger its release, as an unfolded or partially folded protein that is rapidly degraded[20]. Additional mechanisms underlying the apoptosis that HDAC inhibitors induce in t(8;21) AML cell lines is also being studied. Treatment of Kasumi-1 cells with depsipeptide or suberoylanilide hydroxamic acid (SAHA) leads to increased annexin A1 (ANXA1) expression, and reducing of ANXA1 activity abrogates HDAC inhibitor-induced apoptosis[21]. Treatment of Kasumi cells with TSA and cytokines promotes their in vitro differentiation into dendritic cells, suggesting that HDAC inhibitors may play additional roles when examined for therapeutic efficacy in AML[52].

The novel association of RUNX1-ETO with both HSP90 inhibitors and with histone deacetylases is being increasingly investigated as a potential target for therapy in AML. Both types of agents are being investigated in clinical trials.

Effects of RUNX1-ETO on DNA methylation

Methylation of DNA at cytosine residues within CpG islands represents a key mechanism of gene inactivation in mammalian cells[53]. DNA methylation is catalyzed by a small family of DNA methyltransferases, including DNMT1, DNMT3a and DNMT3b[54]. The net effect of this modification is to induce a closed chromatin configuration, resulting in stable gene silencing.

In addition to recruiting HDACs, RUNX1-ETO is able to recruit DNA methyltransferases to the regulator regions of its target genes. RUNX1-ETO has been shown to physically interact with DNMT1 in Kasumi-1 cells[55] and DNMT1 is recruited to the RUNX1-target gene IL-3 promoter in Kasumi-1 cells and in RUNX1-ETO-positive patient samples. The IL-3 promoter

is hypermethylated and transcription of the IL-3 gene repressed in Kasumi cells, however, treatment with the DNA methyltransferase inhibitor, decitabine, significantly reverses the repression of IL-3. In addition to IL-3, repression of other RUNX1-target genes by RUNX1-ETO, such as lysozyme, Meis1 and the tumor suppressor p15INK4b may relate to DNMT recruitment and promoter hypermethylation. The work by Berg et al.[56] showed the enhanced sensitivity of Kasumi-1 cells to DAC compared to other RUNX1-ETO-negative cell lines such as KG-1 and KG-1a. The p15/INK4b promoter is heavily methylated in all three cells lines, but only in Kasumi-1 cells was there a significant induction of p15/INK4b protein expression after DAC treatment. However, the role that RUNX1-ETO plays in the sensitization of cells to DNMT inhibition is unclear. However, it was recently shown that RUNX1-ETO silences expression of the mir223 through direct recruitment of DNMT1[57] over expression of mir223 can restore the differentiation potential of cells that still express RUNX1-ETO.

Repression of target genes by RUNX1-ETO may occur independent of DNA methylation initially, but could later draw the DNMTs into the repressed gene promoters. In fact, both lysine methylation and arginine methylation have been shown to trigger subsequent DNA methylation [58,59]. Thus, direct recruitment of DNMTs by RUNX1-ETO may not be needed for DNA methylation to occur.

Summary

In the coming years, it will be important to study how RUNX1 post-transcriptional modifications during hematopoiesis affect its role in regulating differentiation and self-renewal. Much insight needs to be gained into the cross talk between phosphorylation and acetylation, methylation and acetylation, and phosphorylation and methylation. We are interested in understanding how acetylation and methylation of histones and transcription factors play a role in normal and leukemic hematopoiesis. Understanding basic RUNX1-dependent transcriptional mechanisms will hopefully lead us to new leukemia therapies.

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