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Translating the Histone Code Into Leukemia

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Abstract The ''histone code'' is comprised of the covalent modifications of histone tails that function to regulate gene transcription. The post-translational modifications that occur in histones within the regulatory regions of genes include acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and ADP-ribosylation. These modifications serve to alter chromatin structure and accessibility, and to act as docking sites for transcription factors or other histone modifying enzymes. Several of the factors that are disrupted by chromosomal translocations associated with hematological malignancies can alter the histone code in a gene-specific manner. Here, we discuss how the histone code may be disrupted by chromosomal translocations, either directly by altering the activity of histone modifying enzymes, or indirectly by recruitment of this type of enzyme by oncogenic transcription factors. These alterations in the histone code may alter gene expression pattern to set the stage for leukemogenesis. J. Cell. Biochem. 96: 938–950, 2005.

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HISTONE MODIFYING ENZYMES

Mammalian genes are contained in chromatin fibers that are extensive yet dynamic in their structure and organization. The modification of the highly conserved N-terminal tails of the core histones is a key regulatory point in the organization of nucleosomes into chromatin fibers. The conserved histone tails are modified by phosphorylation, acetylation, methylation, ubiquitination, sumoylation, and ADP-ribosylation on over 30 residues (Fig. 1, reviewed in [Spotswood and Turner, 2002; Iizuka and Smith, 2003; Peterson and Laniel, 2004]). The large number of residues involved, and the sequential nature in which the modifications occur, create a complex "histone code" [Strahl and Allis, 2000; Turner, 2000, 2005; Jenuwein and Allis, 2001; Lachner et al., 2003]. These combinations can be further expanded with trans-histone regulation. For instance, in yeast, ubiquitination of H2B lysine 123 (Lys 123) is required for methylation of histone H3 Lys 4 and Lys 79 [Turner, 2002]. Histone modifications are often linked to the DNA methylation state of a given promoter, providing a second level of epigenetic regulation that has been extensively reviewed [Jones and Baylin, 2002].

Because of its complexity, the histone modification "code" has only been broken for the most extreme cases. For example, the inactive X

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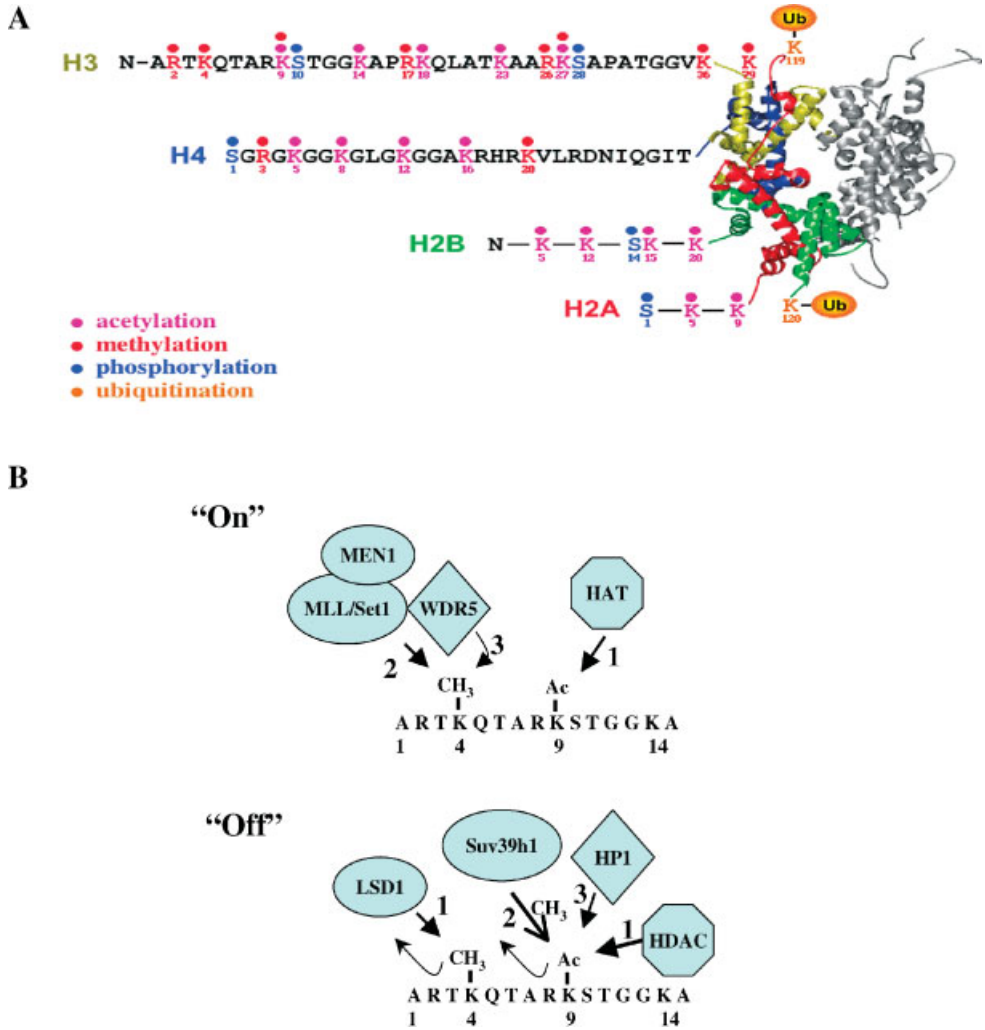

Fig. 1. Schematic diagram depicting how the histone code functions. **A**: Diagram of the structure of the core histone octamer and the unstructured N-terminal histone tails with their potential posttranslational modifications. **B**: An example of how the histone code is written, read, and erased. The **upper panel** shows how histone acetyltransferases (HATs) first acetylate H3 Lys 9 (1), which triggers methylation of Lys 4 by a Set1-like complex (2). The methylation creates a binding site for WDR5 (3). The **bottom panel** depicts the action of histone deacetylases and the LSD1 demethylase acting to remove the acetylation and methylation to Lys 9 and Lys 4, respectively (1). This allows Suv39h1 to methylate Lys 9 (2) to create a binding site for HP1 (3). The numbers near the arrows indicate the sequence of events.

chromosome, Xi, is under-acetylated in all four core histones and is deficient in H3 Lys 4 methylation, while it is methylated at H3 Lys 9 and Lys 27 [Lachner et al., 2003; Plath et al., 2003]. In addition, Lys 9-methylated H3 is also found in other regions of heterochromatin such as centromeric regions. These observations have lead to the generalizations that hyperacetylation of histones is associated with gene activation, as is methylation of histone H3 Lys 4 [Iizuka and Smith, 2003; Peterson and Laniel, 2004]. Conversely histone deacetylation is associated with transcriptional repression, and methylation of histone H3 Lys 9 is thought to be a more permanent mark of repression and gene silencing. However, these are the most extreme cases and there are exceptions to this "code," which suggest that these modifications are not a set of strict rules, but patterns of histone modifications that direct chromatin structure to allow or inhibit transcription.

This histone code has been characterized as being "written" by histone modifying enzymes such as kinases, histone methyltransferases and histone acetyltransferases (HATs), while it is "read" by factors that associate with the modified histone (reviewed in [Hake et al., 2004; de la Cruz et al., 2005]). In this light, the factors

that reverse these histone marks such as phosphatases, histone deacetylases (HDACs), and histone demethylases may act as "erasers" to allow new marks to be entered. Figure 1B shows one example for histone H3 in which acetylation of Lys 9 by HATs triggers the methylation of Lys 4 [Hess, 2004; Croce, 2005], which allows the binding of WDR5 for gene activation [Wysocka et al., 2005]. Indeed, WDR5 is a component of a histone methyltransferase complex whose activity is associated with RNA polymerase II initiation and elongation [Tenney and Shilatifard, 2005]. This process can be reversed by demethylation of Lys 4 by LSD1 and deacetylation of Lys 9 [Shi et al., 2004], followed by methylation of H3 Lys 9, which is associated with gene silencing. Adding to this complexity is the observation that Lys residues can be mono, di-, or tri-methylated, which are marks that can be independently read. In terms of tumorigenesis, the normal regulation is often subverted, with the gene expression switch permanently turned on or off.

Acute leukemia has been a rich source of information about the role of histone modifying enzymes in cancer. Many of the chromosomal translocations associated with acute leukemia disrupt genes that either encode histone-modifying factors or express transcription factors that recruit histone-modifying enzymes. Although many of these enzymes show remarkable specificity, histone-modifying enzymes can also modify non-histone proteins, including other transcription factors, making it difficult to pinpoint the action of histone modifying enzymes in leukemogenesis. Indeed, the notion of "histone" modifying enzymes may be a misnomer propagated by the ease with which histones can be used as substrates. Nevertheless, these chromosomal translocations indicate how disruptions of the function of the enzymes that control chromatin structure can cause alterations of the histone code and chromatin structure to alter gene expression at specific loci, eventually causing cellular transformation. Here, rather than an attempt to comprehensively review the molecular mechanisms of multiple chromosomal translocation fusion proteins, we provide an overview of the histone modifying enzymes that are directly and indirectly involved in acute leukemia in select leukemia subtypes. Hopefully, this survey will provide a framework with which to dig deeper into the mechanism of action of the individual translocations that are associated with acute leukemia. We apologize to the many authors whose primary work we are unable to reference due to the restrictions on space, but we hope that by providing the references to several recent reviews for each topic we will aid the readers in finding more information.

DISRUPTIONS OF HISTONE METHYLTRANSFERASES

The action of histone methyltransferases was first recognized in genetic studies in the fruitfly. The *Drosophila* methyltransferase Su(Var)3–9 was identified in a screen for suppressors of variegated gene expression patterns, and it is required for gene silencing [Sinclair et al., 1992; Lachner et al., 2003]. The mammalian homologues of Su(Var)3–9 include the closely related histone methyltransferases known as Suv39h1 and Suv39h2 [Peterson and Laniel, 2004]. These enzymes contain conserved "SET" domains (Su(var)3–9, Enhancer of Zeste, Trithorax) that effect histone methylation. Many histone methyl transferases show remarkable specificity not only for a given lysine residue in one histone, but for a histone with a predetermined set of modifications. For example, Suv39h1, Suv39h2, and G9a recognize deacetylated histone H3 Lys 9 and catalyze the methylation of this residue to create a binding site for heterochromatin protein-1 (HP1), thereby promoting gene silencing [Jenuwein and Allis, 2001; Peterson and Laniel, 2004]. Conversely, methylation of Lys 4 of H3 is associated with activation of gene expression, and mixed lineage leukemia (*MLL*, also known as *ALL1* or *HRX*) is one such enzyme capable of catalyzing this reaction. *MLL* is the target of multiple translocations in infant and therapy related AML and ALL.

MLL was hypothesized to be a histone methyltransferase due to a C-terminal SET domain (Fig. 2), but experimental confirmation of this activity was elusive, likely due to its residue-specific activity. The early clues that MLL functioned in gene expression came from targeted disruption of murine *Mll*, which indicated that Mll, like the *Drosophila* SET domain-containing factor Trithorax, was required for the expression of a number of the Hox genes (e.g., the "a" and "c" clusters) [Daser and Rabbitts, 2004; Hess, 2004]. When *MLL* was re-expressed in *MLL*-null cells, Hoxa9 and

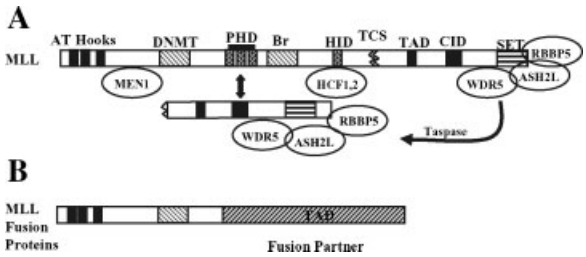

Fig. 2. Schematic diagram of the domains of MLL (**A**) and MLL fusion proteins (**B**). Domains with known structural motifs or homology to other proteins are indicated with shaded boxes. TAD, TAD, transcriptional activation domain; SET, Su(Var)-enhancer of Zeste-Trithorax domain. PHD, plant homology domain zinc finger motifs; AT Hooks, motif that binds cruciform DNA; DNMT, DNA methyltransferase homology domain that binds DNA; Br, Bromodomain; TCS, Taspase cleavage sites; HID, HCF1 and HCF2 interaction domain; CID, C-terminal interaction domain that mediates association with the PHD domain in the N-terminal fragment. The arrow indicates association of the N-terminal and C-terminal fragments after Taspase-mediated cleavage.

Hoxc8 were expressed, indicating that MLL can act to induce Hox gene expression in much the same way that Trithorax maintains the expression of Hox genes by opposing the action of Polycomb group factors [Hess, 2004].

The identification of a specific target gene for MLL-dependent regulation has allowed an extensive analysis of MLL functions at the molecular level. The C-terminal SET domain was required for Hox gene expression, and chromatin immunoprecipitation established that MLL induced the methylation of histone H3 Lys 4 on the *Hoxa9* and *Hoxc8* promoters, which is a mark for gene activation (Fig. 1) [Hess, 2004; Croce, 2005]. In vitro, MLL catalyzed the methylation of histone H3 Lys 4, and acetylated histone H3 was a better substrate for MLL than non-acetylated histone [Hess, 2004; Croce, 2005]. Although not confirmed experimentally, the presence of a bromodomain in MLL (Fig. 2), a motif important in recognition of acetylated histones [de la Cruz et al., 2005], suggests that acetylation of histones at the Hox gene promoters recruits MLL to methylate H3 Lys 4 to maintain *Hox* gene expression.

MLL is proteolytically cleaved by Taspase1, a threonine aspartase, into two fragments that heterodimerize to presumably orient the C-terminal SET domain adjacent to the PHD domain [Daser and Rabbitts, 2004; Hess, 2004]. This observation adds both clarity and complexity to the analysis of the types of chromatin regulating complexes that contain MLL. For example, the uncleaved form of MLL was purified in a very large complex containing components of TFIID, including the TATA binding protein [Croce, 2005] and components of transcriptional repression complexes [Croce, 2005]. By contrast, a smaller complex containing the cleaved MLL fragments contained the multiple endocrine neoplasia type 1 (*MEN1*) tumor suppressor MENIN [Hess, 2004; Yokoyama et al., 2004]. The MENIN/MLL complex also displays histone methyltransferase activity and is more akin to the *Drosophila* Trithorax complex [Smith et al., 2004; Tenney and Shilatifard, 2005]. Mapping studies indicated that MENIN and the HCF family members associate with the N-terminal fragment of MLL, whereas the C-terminal MLL fragment associates with ASH2L, WDR5, and RBBP5. Genetic studies using siRNA indicate that both MLL and MENIN are required for the maintenance of *HOXA9* expression [Yokoyama et al., 2004]. Thus, MLL is one of the methyltransferases that regulates histone H3 Lys 4 to control transcription (Fig. 1B).

The yeast gene with the most homology to MLL is Set1. Set1 is not only required for methylation of H3 Lys 4 in relation to activation of gene expression, but it is found in a complex termed "COMPASS" that is similar in nature to the MLL/Menin and the Trithorax complexes [Tenney and Shilatifard, 2005]. A screen of deletion mutants of non-essential genes in yeast for genes that affect histone methylation identified several factors in addition to the previously identified E2 ubiquitin-conjugating enzyme Rad6. These included the Bre1 E3 ubiquitin ligase, which interacts with Rad6, which is consistent with the role of Rad6 in H2B Lys 123 ubiquitnation as being required for H3 Lys 4 methylation [Tenney and Shilatifard, 2005]. Bre1 was also required for methylation of H3 Lys 79, which is mediated by the Dot1 methyltransferase. This genetic screen also identified several members of the Paf1 complex as being required for H3 Lys 4 and Lys 79 methylation by Set1 and Dot1, respectively. The Paf1 complex is recruited by phosphorylated RNA Polymerase II and may function to stimulate transcriptional elongation. The Paf1 complex appears to regulate Set1 and Dot1 functions by regulating H2B Lys 123 monoubiquitination and by directing COMPASS to actively transcribed chromatin through

physical association [Tenney and Shilatifard, 2005]. By analogy, this predicts that the MLL/Menin complex also alters chromatin structure in association with RNA Polymerase II. Given that histone methylation is a more stable histone code mark than acetylation, it is possible that this is one mechanism by which MLL maintains the expression of Hox cluster genes.

MLL-X Fusion Proteins

The chromosomal translocation fusion proteins that affect MLL appear to act by locking the MLL-dependent transcriptional switch in the "on" position to allow inappropriate activation of gene expression, which ultimately stimulates leukemogenesis. Whether this is due the inability of the MLL-X fusion proteins to contact co-repressors or to the formation of a dominantly acting transactivation complex is not yet clear. The translocation breakpoints in *MLL* lie downstream of the DNA methyltransferase homology domain and the MENIN and HCF binding motifs, suggesting that these sequences are critical for the action of the fusion protein (Fig. 2). This is underscored by the observation that the ability of MLL to associated with DNA via CpG sites is required for the fusion proteins to induce leukemia [Ayton et al., 2004]. Indeed, the MLL-fusion proteins activate *Hoxa9*, and *Hoxa9* is consistently overexpressed in leukemia containing MLL translocations [Armstrong et al., 2003]. Targeted disruption of either *Hoxa9* or *Hoxa7* impaired the ability of MLL fusion proteins to immortalize primary myeloid progenitor cells [Ayton and Cleary, 2003], highlighting the importance of the dys-regulation of Hox gene expression to leukemogenesis induced by MLL fusion proteins.

The diversity of fusion partners makes it difficult to understand how these chromosomal translocations create an unregulated transactivating form of MLL. These various partners appear to have little in common, although many fusion partners contain a dimerization motif and some contain a transcriptional activation domain (Fig. 2). However, these translocation partners are required for both the MLL fusion protein-mediated immortalization of myeloid progenitor cells in vitro and leukemic potential in vivo, which suggests that these are gain-of-function mutations [Daser and Rabbitts, 2004; Hess, 2004; So and Cleary, 2004; Croce, 2005]. In several cases where the fusion partner contains a transactivation domain, this sequence contributes to in vitro cellular immortalization and even leukemogenesis. However, artificial dimerization of the N-terminal domain of MLL was also sufficient to activate the ability of MLL to immortalize myeloid progenitor cells and cause AML in both gene "knock-in" and retroviral expression mouse models [Daser and Rabbitts, 2004; Hess, 2004; So and Cleary, 2004; Croce, 2005]. In addition, to these fusions, partial tandem duplication of the N-terminus of MLL is also observed. It is temping to speculate that forcing the N-terminal domain of MLL into a more stable association with the MENIN complex, either through dimerization or the addition of a transactivation domain, artificially induces histone methylation to maintain the expression of the HOX gene clusters.

The link between the yeast methyltransferase Set1 and transcriptional elongation is also of interest in relation to the t(11;19), which fuses MLL to ELL (Eleven-nineteen lysine rich leukemia). One function of ELL is in transcriptional elongation [Tenney and Shilatifard, 2005], but mouse models suggest that the domain of ELL responsible for this function is not required for MLL-ELL-dependent transformation [DiMartino et al., 2000]. However, it is possible that the domains in ELL that are required to induce cellular immortalization contact components of the elongation machinery. ELL associated protein (EAP) is one possible candidate, as it contains a putative transcriptional activation domain and co-localizes with ELL in Cajal bodies, which are subnuclear sites of RNA transcription and processing [Polak et al., 2003].

Similar to MLL-ELL, the molecular mechanism of the MLL-AF10 fusion protein, may also involve effects on transcriptional elongation. A yeast two-hybrid screen detected an interaction between AF10 and the histone methyltransferase hDOT1 [Okada et al., 2005]. hDOT1 is a non-SET containing methyltransferase that targets histone H3 Lys 79 for gene activation. Not only was the hDOT1L interaction motif in MLL-AF10 required for MLL-AF10-mediated leukemogenesis [DiMartino et al., 2002], but hDOT1L was able to substitute for AF10 in a mouse model of leukemia [Okada et al., 2005]. Moreover, a catalytically inactive MLL-hDOT1L failed to induce leukemia, and

siRNA-mediated knockdown of endogenous hDOT1L impaired MLL-AF10-induced leukemogenesis [Okada et al., 2005]. Thus, the recruitment of this histone methyltransferase was required for disruption of the histone code and the induction of leukemia. In yeast, components of the Paf1 complex that contacts phosphorylated RNA Pol II along with Rad6 and Bre1 are required for Dot1p-dependent methylation. Thus, MLL-AF10 may stimulate Hox gene expression by affecting transcriptional elongation or it may act by subverting the normal regulation of H3 Lys 79 to keep the Hox cluster in an open chromatin context and thereby maintain Hox gene expression.

While the large size of MLL with its numerous functional domains has made pinpointing a simple model for the action of either wild type MLL or the MLL fusion proteins difficult, it is likely that many of the actions of MLL are mediated through its ability to catalyze the methylation of histones to alter chromatin structure. In addition, given the key role that over-expression of Hox genes appear to have in generating MLL-related leukemia, it seems reasonable to speculate that the chromosomal translocations create dominant positive mutations of MLL. That is, these are unregulated transcriptional activators that constitutively activate Hox gene expression. This may seem counterintuitive given that loss of function of *MEN1* (MENIN) is associated with tumorigenesis, but there is little evidence to suggest that loss of MLL function is linked to leukemogenesis. Thus, this may simply indicate that Hox gene expression is critical for the hematopoietic system and leukemogenesis, whereas the loss of expression of other key regulatory genes that are controlled by MLL/MENIN, such as the p16 and p27 cyclin dependent kinase inhibitors [Milne et al., 2005], is more critical in endocrine tumorigenesis. It is clear that only the surface has been scratched in terms of the complexity of MLL-mediated transcriptional control, but these recent advances allow a framework to define the molecular mechanisms of action.

DISRUPTIONS OF HATs

Histone acetylation is associated with activation of gene expression. Although transcription factors are often disrupted by chromosomal translocations, and these factors recruit HATs or HDACs, the enzymes themselves are only rarely affected by translocations. Although the translocations affecting HATs are rare, they are recurrent and, therefore, informative. For example, a clue to the function of the MLL fusion proteins was provided by the identification of a translocation fusing MLL to the CREB binding protein (CBP) or the closely related p300 HATs [Sobulo et al., 1997]. The MLL-CBP fusion is especially informative, as MLL normally associates with CBP and another histone acetyltransferase, MOF, which targets histone H4 Lys 14 [Daser and Rabbitts, 2004; Hess, 2004; Dou et al., 2005]. The fusion protein contains the bromodomain and HAT domain of CBP, and these domains contribute to MLL-CBP-mediated immortalization of bone marrow in vitro [Lavau et al., 2000], suggesting that the fusion protein acts by acetylating histones. The identification of cooperative histone modifications by MLL and MOF, suggests that fusion of CBP to MLL subverts the normal regulatory mechanisms to constitutively activate transcription.

Chromosomal translocations also disrupt the histone acetyltransferases MOZ (monocytic leukemia zinc finger) and the related MORF (monocytic leukemia zinc finger protein-related factor). These factors are members of the MYST (MOZ, YBF2, SAS2, and TIP60) family of HATs that are recruited by DNA-binding transcription factors to activate transcription [Iyer et al., 2004; Kalkhoven, 2004]. The inv(8) fuses the MYST domain of MOZ to the TIF2 transcription factor [Liang et al., 1998], whereas the t(8;16) fuses the N-terminus of MOZ to nearly all of CBP (Fig. 3) [Borrow et al., 1996]. The fusion of 2 HATs by the t(8;16) and the t(10;16), which fuses MORF to CBP, strongly suggests that these fusion proteins alter histone acetylation in leukemogenesis. Similarly, genetic dissection of the inv(8) fusion protein using a mouse model of leukemogenesis points to regulation of chromatin structure. Deletion of the N-terminal PHD domain had little effect in leukemogenesis assays, whereas the MYST (HAT) domain and the CBP interaction domain of TIF2 (CID, Fig. 3) were indispensable. Unexpectedly, the acetyltransferase activity of the MYST domain was not required if the chromatin association motif was retained [Deguchi et al., 2003]. Thus, the most likely scenario is that the MYST domain of MOZ directs the fusion protein to chromatin where either the intrinsic HAT activity of the fusion protein modifies histones or the TIF2

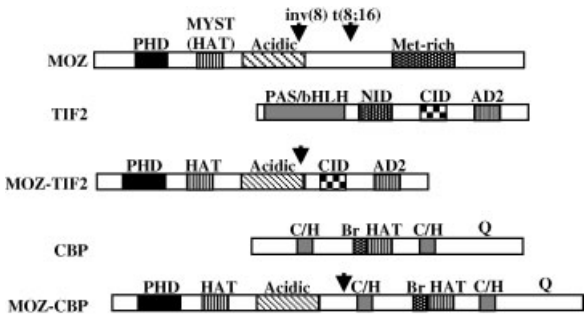

Fig. 3. Schematic diagram of the inv(8) and t(8;16) fusion proteins. Domains with known structural motifs or homology to other proteins are indicated with shaded boxes. PHD, plant homology domain zinc finger motifs; MYST, MOZ, YBF2, SAS2, and TIP60 homology region; HAT, histone acetyltransferase; Met-rich, methionine rich; CID, CREB binding protein interaction domain; PAS/bHLH, Period-ARNT-singleminded/basic helix-loop-helix DNA binding domain; NID, nuclear receptor interaction domain; Br, bromodomain. Q, glutamine rich region.

sequences recruit a HAT, likely CBP or p300, to alter the histone code of regulated genes and induce leukemia. However, specific target genes for regulation are not known, leaving open the possibility that acetylation of transcription factors that recruit these HATs may also contribute to leukemogenesis. In addition, it remains possible that these fusion protein act as dominant inhibitors of HAT functions.

RECRUITMENT OF HISTONE DEACETYLASES

Although chromosomal translocations do not directly disrupt histone deacetylases (HDACs), these enzymes likely play a key role in mediating transcriptional repression by multiple translocation fusion proteins associated with both AML and ALL. These include the translocations that disrupt or over express the DNA binding proteins RUNX1, RARα, PLZF, BCL6, and TAL1/SCL. We will focus on only a subset of these factors and provide specific examples of the chromosomal translocations that target them as examples of how HDACs are involved in leukemia.

RUNX1

Translocations that disrupt the function of the RUNX1 (RUNT-related-1 also known as acute-myeloid-leukemia-1 or AML1) transcription factor complex are common in AML and B-cell ALL [Blyth et al., 2005]. RUNX1 binds to DNA and associates with either HATs or HDACs to act as a switch to both activate and repress transcription. RUNX1 associates with the histone acetyltransferases MOZ, MORF, and p300 [Kitabayashi et al., 1998, 2001; Pelletier et al., 2002; Blyth et al., 2005], raising the possibility that fusion proteins that disrupt these HATs target RUNX1. Indeed, these HATs may even regulate the DNA binding activity of RUNX1 through direct acetylation [Yamaguchi et al., 2004]. Nevertheless, when expressed alone RUNX1 is a poor activator of transcription. However, it cooperates with other DNA-binding transcription factors, such as C/EBPα and ETS family members, to potently activate transcription [de Bruijn and Speck, 2004; Blyth et al., 2005; Yang et al., 2005]. Although RUNX1 and ETS1 can cooperatively bind to DNA [Gu et al., 2000], it is not clear whether the association between RUNX1 and other DNA-binding factors acts to enhance DNA binding to build an "activation complex" or whether association with other DNA binding proteins stimulates the association with HATs [Blyth et al., 2005].

The ability of RUNX family members to repress transcription appears to be linked to recruitment of HDACs and transcriptional co-repressors that recruit HDACs (reviewed in [Durst and Hiebert, 2004]). mSin3A, a co-repressor that associates with HDAC1 and HDAC2, interacts with a region adjacent to the RUNX1 DNA binding domain and this domain is necessary for RUNX1-mediated repression. RUNX1 also interacts with the Groucho/TLE co-repressor through the five-carboxyl terminal amino acids, VWRPY. However, deletion of the Groucho binding motif affects the expression of only a small subset of RUNX1-regulated genes, suggesting that Groucho is a promoter-specific co-repressor for RUNX1 [Durst and Hiebert, 2004]. Although both mSin3 and Groucho contact HDACs, RUNX1 co-purified with HDACs 1, 3, and 9, and to a lesser extent HDAC 2, 5, and 6 [Durst et al., 2003]. Given that mSin3 and Groucho are not known to bind to these HDACs, it is likely that RUNX1 directly binds to these enzymes to direct histone deacetylation (or deacetylation of other transcription factors) and transcriptional repression. Consistent with this interpretation, RUNX1-dependent repression is alleviated, at least partially, in the presence of HDAC inhibitors[Durst and Hiebert, 2004]. RUNX1 and RUNX3 are also required for silencing of *CD4* during T cell development in vivo. The availability of a target gene will allow the assessment of how RUNX1 family members affect the histone code.

t(8:21)

The t(8;21), present in up to 15% of AML, fuses sequences encoding the amino terminus of RUNX1, including the DNA binding domain, to nearly all of *ETO* (eight-twenty-one, also known as myeloid translocation gene on chromosome 8 or *MTG8*; Fig. 4) [Peterson and Zhang, 2004; Blyth et al., 2005]. *ETO* sequences are required for transcriptional repression [Amann et al., 2001], and ETO interacts with the nuclear hormone co-repressor N-CoR (and its homologue, SMRT), mSin3A, mSin3B and HDAC-1, -2, and -3 [Amann et al., 2001; Peterson and Zhang, 2004; Blyth et al., 2005]. ETO binds to each of these co-repressors through two distinct domains to form stable complexes [Amann et al., 2001]. ETO is also capable of dimerization. Although dimerization is dispensable for RUNX1-ETO-mediated repression, oligomerization may allow the fusion protein to form a more stable association with these corepressors. However, the oligomerization motif is also one contact point for mSin3A [Amann et al., 2001], further suggesting that co-repressor association is critical to ETO functions. Thus, the fusion protein may become an un-regulated transcriptional repressor by directing histone deacetylation.

Although the involvement of RUNX1 by multiple chromosomal translocations points to disruption of RUNX1 function as a critical event in leukemogenesis, expression of RUNX1-ETO has effects not observed with the simple loss of *RUNX1*. This implies that the fusion protein may also affect the normal functions of ETO and its related family members. Indeed, in transcription assays, RUNX1-ETO impaired repression mediated by PLZF [Melnick et al., 2000]. Therefore, a better understanding of the biological activity of the ETO/MTG family of co-repressors is needed to understand this leukemia.

The identification of the $p14^{ARF}$ and *NF1* tumor suppressors and *IL-3* and the *M-CSF* receptor as direct transcriptional targets of RUNX1-ETO will eventually permit the elucidation of the mechanism of repression at the gene level [Linggi et al., 2002; Follows et al., 2003; Yang et al., 2005]. In terms of histone modifications, only *M-CSFR* has been analyzed in detail. In Kasumi-1 cells that express high levels of RUNX1-ETO, histones at the regulatory sequences of *M-CSFR* were deacetylated with a concomitant increase of histone H3 Lys 9 methylation [Follows et al., 2003], confirming that RUNX1-ETO acts by altering the histone modifications in the regulatory regions of target genes. The only caveat to these studies is that Kasumi-1 cells, which harbor the t(8;21), were compared to a second cell line, rather than using a single cell line with or without ectopically

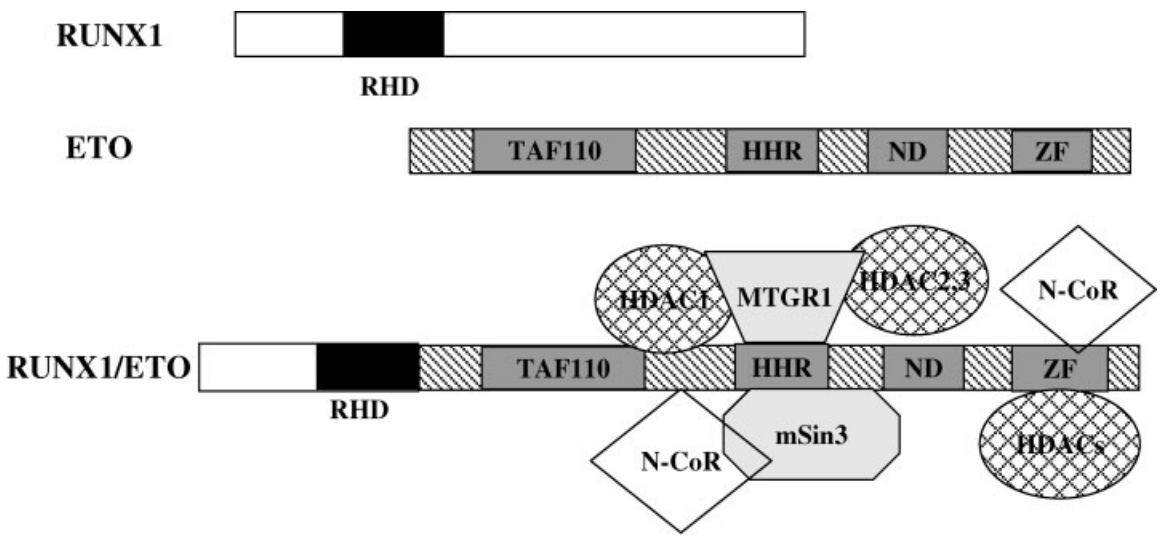

Fig. 4. Schematic diagram of the primary structure of RUNX1, ETO, and the RUNX1-ETO fusion protein. Shaded boxes indicate domains with homology to other proteins. Contact sites for the transcriptional co-repressors mSin3 and N-CoR, and histone deacetylases are indicated. ZF, zinc finger motif; ND, nervy homology domain; TAF110, domain with homology to the *Drosophila* TAF110 co-activator; HHR, hydrophobic heptad repeat which mediates homodimerization and heterodimerization with other family members such as MTGR1.

expressed RUNX1-ETO. In addition, the expression of IL-3 is affected by histone deacetylase inhibitors in Kasumi-1 cells [Klisovic et al., 2003]. However, the expression of IL-3 was extremely low, such that non-quantitative nested RT-PCR was required for its detection.

One major issue that has limited the analysis of RUNX1-ETO regulation of the histone code is that this fusion protein is very difficult to express, even in transformed cell lines, due to slowing of the cell cycle. In fact, many of the fusion proteins that recruit histone deacetylases share this problem. This difficulty is exacerbated because transcriptional repressors of endogenous genes often have only a 3- to 5-fold effect, whereas transcriptional activators can have 20- to 100-fold effects. For example, even compete silencing of a gene in 80% of the cells, translates into only a fourfold reduction in the levels of that mRNA in the culture. This problem is magnified at the level of the histone code, because the antibodies used in chromatin immunoprecipitation assays detect the modified form of the targeted peptide. For example, the use of anti-acetyl lysine antibodies would detect the loss of a signal rather than the gain of a signal. Nevertheless, the identification of direct targets will be very useful for dissecting the effects of these fusion proteins on the histone code.

Murine models of leukemia involving RUNX1 offer one method of gaining indirect information about how histone deacetylases contribute to the action of the t(8;21) fusion protein. The phenotypes observed upon expression of RUNX1-ETO and other chromosomal translocation fusion proteins such as the inv(16) and the t(12;21) fusion proteins are consistent with the role of these factors as dominant repressors of RUNX1-regulated genes, rather than RUNX1 dominant negative proteins. Although embryonic deletion of *RUNX1* or expression of RUNX1-ETO causes mid-gestational lethality, apparently due to impaired hematopoietic stem cell (HSC) function, conditional deletion of *RUNX1* in adult mice does not affect HSC functions [de Bruijn and Speck, 2004]. Rather than loss of the HSC, the most immature progenitors were modestly expanded, with a block in lymphopoiesis and impaired megakaryocytic differentiation. This latter phenotype is consistent with the established role of RUNX1 in a familial platelet disorder [Ichikawa et al., 2004]. The impairment of lymphopoiesis with an expansion of HSC is consistent with expression of RUNX1-ETO, although RUNX1-ETO-expressing mice displayed a much more dramatic expansion of HSC and impaired myeloid differentiation [Peterson and Zhang, 2004]. Thus, the fusion proteins that disrupt RUNX1 are gain-of-function alleles that have dominant effects over the endogenously expressed RUNX1.

BCL6 and TAL1/SCL

BCL6 is a DNA sequence-specific transcriptional repressor. Although it is less frequently targeted in acute leukemia, it is over-expressed due to chromosomal translocations in non-Hodgkin's lymphoma, particularly diffuse large B-cell lymphomas [Baron et al., 1993, 1995]. The co-repressors SMRT [Dhordain et al., 1997; Wong and Privalsky, 1998], N-CoR [Huynh and Bardwell, 1998], and BCoR (for *B*CL6-interacting corepressor) interact with the BTB/POZ domain of BCL6 in a mutually exclusive fashion [Huynh et al., 2000]. mSin3A contacts an adjacent motif [Dhordain et al., 1997] and only to a small extent the BTB/POZ domain [Dhordain et al., 1997; David et al., 1998; Wong and Privalsky, 1998]. HDAC1 is recruited by SMRT, although it may be able to interact with BCL6 directly [Dhordain et al., 1997; Wong and Privalsky, 1998]. The co-repressor BCoR recruits both class I and class II HDACs to BCL6 [Huynh et al., 2000], which is also capable of interacting directly with HDAC4, HDAC5, and HDAC7 through its zinc finger domain [Lemercier et al., 2002]. Like the related PLZF, BCL6 also associates with ETO/MTG8 [Chevallier et al., 2004]. The recurring involvement of ETO/MTG8 in translocations and its association with oncoproteins highlights the special role of the MTG family of co-repressors in transcriptional regulation during hematopoiesis.

The importance of these co-repressor complexes for BCL6 transcriptional functions was demonstrated by the enhancement of BCL6-mediated repression upon its co-expression with SMRT [Dhordain et al., 1997], BCoR [Huynh et al., 2000], or ETO [Chevallier et al., 2004] and the relief of BCL6-directed repression by the HDAC inhibitors trichostatin A and sodium butyrate [Dhordain et al., 1998]. These broad spectrum HDAC inhibitors are ideal for defining the role of HDACs in BCL6 function as BCL6 can directly or indirectly associate with

both class I and class II HDACs. Although numerous potential targets for repression by BCL6 have been identified [Shaffer et al., 2000], histone modifications have yet to be analyzed at the molecular level.

Like BCL6, *TAL1* (also known as *SCL*) is over-expressed as a result of chromosomal translocation. *TAL1* encodes a transcription factor of the basic-helix-loop-helix (bHLH) family and it is deregulated in up to 60% of pediatric patients with T-cell acute lymphoblastic leukemia. Similar to many bHLH-containing factors, TAL1 functions through association with other bHLH family members and with other DNA-binding proteins such as GATA1 [Osada et al., 1995]. TAL1 also interacts with the transcriptional co-repressor mSin3A and HDAC1 via its bHLH domain, and TAL1 co-purified with histone deacetylase enzymatic activity [Huang and Brandt, 2000]. Although HDAC1 may be able to contact TAL1 directly, it requires mSin3A for interaction in vivo, and transient transfection analysis indicated that TAL1-directed repression is an active process requiring HDAC function [Huang and Brandt, 2000]. Recent work suggests that TAL1 can also recruit the ETO/MTG8-related proteins mETO2 and Mtgr1 (S.J.B. and S.W.H., unpublished data).

In activating transcription, TAL1 can associate with other DNA binding transactivators as well as with the histone acetyltransferases p300 [Huang et al., 1999] and P/CAF [Huang et al., 2000]. Co-expression of these co-activators significantly augments TAL1-directed transcriptional activation, and, like mSin3A, they interact with TAL1 through its bHLH domain [Huang et al., 1999, 2000]. Thus, TAL1 appears to act as a molecular switch whose activity is differentially regulated by the recruitment of co-activators versus co-repressors.

Because the bHLH domain mediates DNA binding and protein:protein interactions, the recruitment of HDACs or co-repressors versus co-activators has not been dissected in murine models of T cell ALL [Larson et al., 1996]. However, gene expression profiling of pre-leukemic thymocytes indicated that expression of TAL1 mediated the repression of many more genes than were activated [O'Neil et al., 2004]. In addition, ChIP was used to confirm that TAL1 recruited mSin3A to the *CD4* promoter. Although correlative, these data are consistent with a role for TAL1-mediated repression in T-ALL development. Although transcriptional targets of TAL1 are beginning to emerge, the histone code has not been analyzed in detail for these genes. This will be important in dissecting the mechanism underlying TAL1 action.

SUMMARY AND PERSPECTIVES

We have framed this discussion in terms of the histone code and gene expression, because the simplest view of how these chromosomal translocations work is by altering patterns of gene expression through effects on histone modifications. Inhibitors of these histone-modifying enzymes are being tested for therapeutic benefit in multiple types of leukemia and solid tumors. Histone deacetylase inhibitors were the first to be developed and provide a model for how these compounds might be used. In fact, some of these compounds were first identified based on their ability to affect gene expression and induce differentiation [Marks et al., 2004]. Those oncoproteins that recruit histone deacetylases are prime targets for histone deacetylase inhibitors (HDI), which are predicted to impair the action of many of these factors to block transcriptional repression. However, it is important to note that HDIs only inhibit the deacetylases, which will not automatically reactivate gene expression without an activation signal (e.g., recruitment of HATs to the gene of interest, Fig. 1). Nevertheless, the potential targeting of oncoproteins, coupled with the known effects of HDIs on hematopoietic cell differentiation [Marks et al., 2004], provides the rationale for the clinical use of HDIs in several subtypes of acute leukemia.

Although it is well established that HDACs regulate chromatin structure and gene expression, HDIs also affect acetylation of non-histone proteins that alter the cell cycle, possibly triggering cell cycle checkpoints. In addition, constitutive histone acetylation also impairs chromosomal condensation during mitosis, as histones must be deacetylated for compaction of the chromatin. If cells lack the M phase checkpoint, mitotic catastrophe and apoptosis may result, which has been observed in certain cell lines treated with HDIs (recently reviewed [Johnstone and Licht, 2003]). This scenario is underscored by genetic data in which targeted deletion of *mSds3*, an mSin3-associated factor, led to mitotic catastrophe from a failure to form pericentric heterochromatin [David et al., 2003].

As more specific HDAC inhibitors are developed it will be critical to test the potential efficacy of these therapies in genetic studies in mice. For instance, mouse models can be used to determine whether the action of a given translocation protein requires the presence of a specific HDAC in leukemogenesis. Currently the only genetic models available for the function of HDACs are knockouts of *Hdac1*, *Hdac5*, and *Hdac9* [Lagger et al., 2002; Chang et al., 2004]. Deletion of *Hdac1* caused early embryonic lethality due to reduced cellular proliferation from activation of cyclin-dependent kinase inhibitors. While emphasizing the key role that Hdac1 plays not only in development, but also in cell cycle progression [Lagger et al., 2002], these mice would not be useful in determining whether Hdac1 recruitment is required for leukemogenesis. In contrast, mice lacking *Hdac9* are normal until 8 months of age when they develop cardiac hypertrophy [Chang et al., 2004]. Conditional knockouts of the HDACs will ultimately provide a more realistic test of the requirement of a specific HDAC in leukemogenesis by allowing the removal of the targeted HDAC after development of leukemia. This will define whether inhibition of a specific HDAC would be a useful therapeutic approach. These experiments will also directly test whether HDAC recruitment by chromosomal translocation proteins is a defining event in leukemogenesis. Finally, such studies could address the issue of whether HDIs are specific for their intended target(s). At this point, the clinical trials have outdistanced the basic science, and more studies are therefore needed to bridge this gap in knowledge.

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