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RUNX1 and RUNX1-ETO: roles in hematopoiesis and leukemogenesis

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TABLE OF CONTENTS

1. ABSTRACT

RUNX1 is a transcription factor that regulates critical processes in many aspects of hematopoiesis. RUNX1 is also integral in defining the definitive hematopoietic stem cell. In addition, many hematological diseases like myelodysplastic syndrome and myeloproliferative neoplasms have been associated with mutations in *RUNX1*. Located on chromosomal 21, the *RUNX1* gene is involved in many forms of chromosomal translocations in leukemia. t(8,21) is one of the most common chromosomal translocations found in acute myeloid leukemia (AML), where it results in a fusion protein between RUNX1 and ETO. The RUNX1-ETO fusion protein is found in approximately 12% of all AML patients. In this review, we detail the structural features, functions, and models used to study both RUNX1 and RUNX1-ETO in hematopoiesis over the past two decades.

2. INTRODUCTION

RUNX1, also known as AML1, CBFalpha2, and PEBP2alphaB, belongs to the family of Runt-related transcription factors (RUNXs) (1). The Runt protein is encoded by the Drosophila *runt* gene, which is required for normal segmentation, sex determination, and neurogenesis during Drosophila embryogenesis (2-4). Other RUNX family members include RUNX2 and RUNX3. This family of proteins was first described as a component of Moloney murine leukemia virus enhancer core binding factor (CBF) and Polyomavirus enhancer binding protein 2 (PEBP2) (5-7). RUNX1 is also known as acute myeloid leukemia 1 due to the discovery of its gene sequence from human patient with acute myeloid leukemia (8). Over the past 20 years, studies have elucidated many important functions of RUNX1 in hematopoietic development, hematopoietic stem cell homeostasis, and various blood malignancies. In this

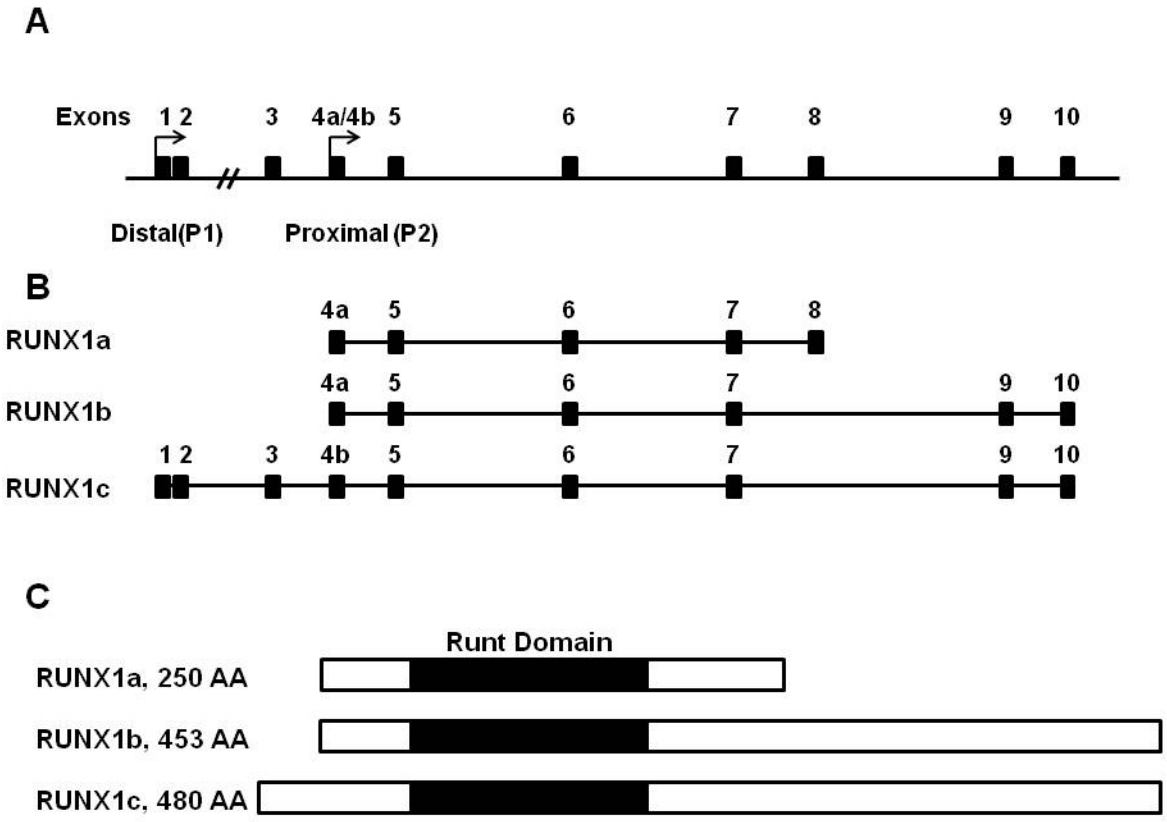

Figure 1. RUNX1 isoforms and genomic locus. (A) The RUNX1 genomic locus on chromosome 21 is shown with the location of the proximal and distal promotors and exons based on the National Center for Biotechnology Information Nucleotide database. (B) The three main transcriptional isoform of RUNX1 are shown. RUNX1a is consists of exons 4a through 8. RUNX1b consists of exons 4a through 10, but excludes exon 8. RUNX1c includes exons 1 through 3 and exons 4b through 10, but also excludes exon 8. (C) The three main RUNX1 isoforms are shown with the Runt homology domain shaded.

review, we will focus on the role that RUNX1 plays in these various biological processes. In addition, we will discuss RUNX1-ETO, a fusion protein resulting from a translocation between chromosomes 8 and 21. The t(8,21)(q22,q22) translocation is one of the most common chromosomal translocations found in patients with AML and especially in those with the French-American-British M2 subtype of AML (9-11). Although RUNX1-ETO cannot by itself induce leukemia in mouse models, the fusion protein provides a critical hit toward leukemogenesis (12,13). The major roles that RUNX1 and RUNX1-ETO play in hematopoiesis and leukemogenesis, respectively, make them highly interesting subjects for further research.

3. RUNX1 STRUCTURE AND REGULATION

3.1. RUNX1 promoters (proximal and distal) and RUNX1 isoforms

RUNX1 was first cloned from DNA obtained from an AML patient with t(8,21)-positive leukemia (8). Although there may be at least 12 different *RUNX1* mRNA isoforms, three main protein isoforms of RUNX1 are primarily discussed (14). These are known as RUNX1a, RUNX1b, and RUNX1c (Figure 1a). These three major isoforms all contain the Runt domain located in the N-terminal region. RUNX1a, consisting of 250 amino acids, and RUNX1b, consisting of 453 amino acids, share the same N-terminal region and are the result of alternative splicing (15). RUNX1a lacks the transcriptional regulatory domains present in the C-terminal region common in the other two RUNX1 isoforms (16). RUNX1c, consisting of 480 amino acids, is the longest of the RUNX1 isoforms and its transcript is transcribed from a distal promoter in the *RUNX1* locus, while *RUNX1a* and *RUNX1b* are transcribed from the proximal promoter (Figure 1b) (15). RUNX1b and RUNX1c have the same C-terminal region.

Interestingly, the various RUNX1 isoforms play specific roles in specifying the hematopoietic stem cell (HSC) and regulating embryonic hematopoiesis. A study done by Tsuzuki *et al.* demonstrated that the *RUNX1a* isoform is found relatively more abundantly in the $CD34^+$ progenitor population in human cord blood and that over-expression of RUNX1a compared with RUNX1b in mouse bone marrow progenitor cells can potentiate engraftment ability upon competitive transplantation (17). Hence, manipulating the levels of RUNX1a may be used to drive proliferation of human bone marrow cells for use in transplantation therapy. Another study, however, showed that over-expression of RUNX1a may also lead to the

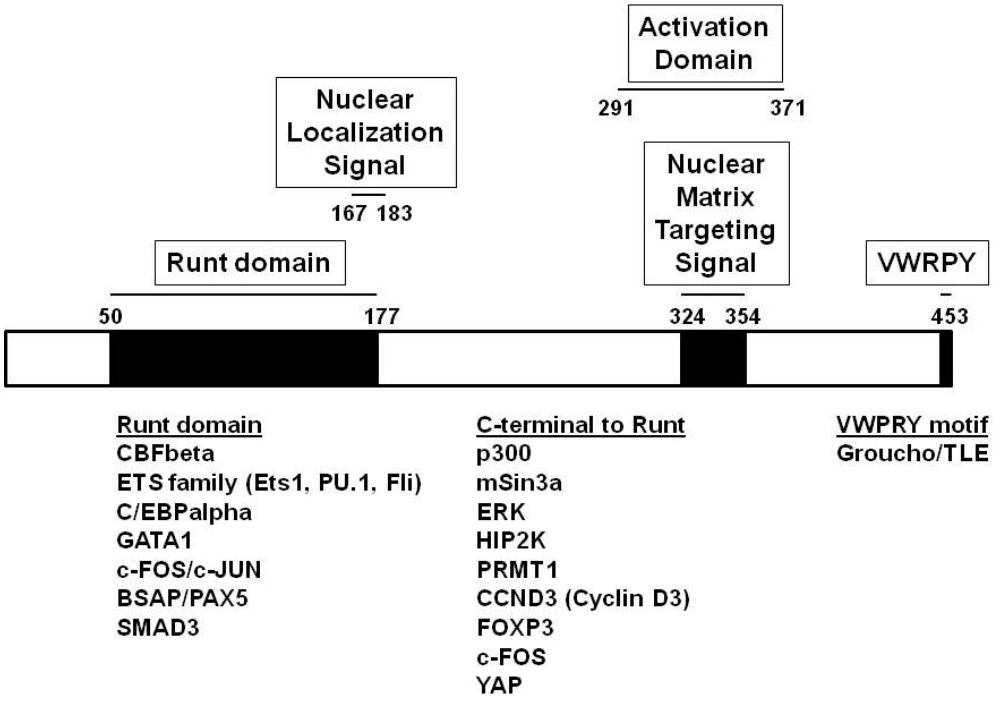

Figure 2. RUNX1 protein domains and interaction partners. The RUNX1b isoform is shown with the major domains listed above and interaction partners listed below.

development of leukemia in a mouse transplantation model (18). These studies suggest that RUNX1a, because it includes the Runt domain but lacks the C-terminal regulatory domains, may act as a dominant-negative regulator of the other RUNX1 isoforms (16,18). As discussed in subsequent sections, the C-terminal domains are necessary for normal RUNX1 function and over-expression of a RUNX1 isoform lacking these domains may lead to abnormal hematopoiesis.

The ability of the RUNX1a isoform to direct a program of self-renewal reflects its importance in embryonic development. Early work using oligonucleotide PCR primers specific for either the proximal or distal transcriptional forms of RUNX1 in T cells showed that the distal form is more prevalent in developing T cells (19). However, when the proximal form, which in this case is RUNX1b, was retrovirally over-expressed in the 32Dcl.3 myeloid progenitor line, significantly more proliferation and neutrophil differentiation was observed when compared to over-expression of the distal RUNX1c isoform (19). In zebrafish, where the transcriptional regulation of *RUNX1* using two promoters is conserved, transgenic lines that express fluorescently labeled RUNX1 isoforms specific for each of the two promoters show that the distal isoform is expressed in areas where erythromyeloid progenitors arise while the proximal isoform originates where definitive HSCs develop (20). More recent studies using mouse knock-in models to label the expression patterns of the *RUNX1* distal and proximal promoters have also shown that the proximal promoter may be important for the initial development of definitive hematopoietic cells from hemogenic endothelium while the distal promoter is active in more mature progenitors (21). Furthermore, mice hypomorphic for the proximal promoter make it term but die perinatally, while mice null for the distal promoter show no overt phenotype (22,23). The aforementioned studies indicate that the two *RUNX1* promoters may have varying yet overlapping functions and interestingly, the proximal RUNX1 isoform has a more involved role in defining the HSC.

The two promoters of *RUNX1* rely on a cis-regulatory element located approximately 23.5 kilobases downstream of the transcriptional start site of the distal promoter (24,25). This promoter contains sites for various essential hematopoietic transcription factors like Gata2, Ets family members, and Lmo2 (24). Furthermore, this element can drive specific expression of genetic markers like lacZ or green fluorescent protein (GFP) in HSCs and the hemogenic endothelium in transgenic mice (25,26). More studies regarding the expression patterns, transcriptional control, and dosage levels of the various RUNX1 isoforms in HSCs and during embryonic hematopoiesis will be needed to further elucidate their ability to regulate HSCs and to potentiate leukemia development.

3.2. RUNX1 protein structure, domains and functions

RUNX1 is defined by its 128 amino acid Runt domain found in the N-terminal region (Figure 2). The Runt domain mediates binding to DNA and interaction with its heterodimerization partner, core-binding factor beta (CBFbeta), which itself does not bind to DNA.

Heterodimerization of RUNX1 and CBFbeta increases the DNA-binding affinity of RUNX1 (27,28). The complex consisting of RUNX1, CBFbeta, and DNA was one of the first gene regulatory complexes where detailed structural investigations have been conducted. NMR spectroscopy studies of the RUNX1/CBFbeta/DNA complex have revealed that it resembles an immunoglobulin fold similar to the DNA-binding domains of NF-kappaB, NFAT1, p53, and the STAT proteins (29,30). Crystal structure studies of this complex have demonstrated that CBFbeta interacts with RUNX1 allosterically to stabilize its ability to bind DNA and that diseases associated with mutations in RUNX1 correspond to sites in its DNA-binding domain (31-33).

The other protein domains of RUNX1 also help to regulate its ability to control transcription of its target genes. Various deletion studies of full-length RUNX1 have shown that the N-terminal and C-terminal regions directly adjacent to the Runt domain inhibit DNA binding (34,35). Binding to CBFbeta relieves this inhibition and allows RUNX1 to bind to DNA at its full potential (34). Furthermore, RUNX1 contains a nuclear matrix targeting signal (NMTS), a 31 amino acid region in the C-terminal region, which aids in transcriptional activation (36). At the very C-terminal end of RUNX1 is a VWRPY motif that is conserved among all Runt family members (37). This motif mediates the Groucho/TLE-dependent transcriptional repressor activities of RUNX1 (37,38). The NMTS and the VWRPY motif have roles in mediating T cell development. By itself, the C-terminal VWPRY motif is not required for developing thymocytes to properly repress CD4 expression (39). When the region containing both the nuclear matrix targeting signal and the VWRPY motif are deleted, however, the thymocytes can no longer repress CD4 (39).

In addition to interacting with CBFbeta, RUNX1 is very versatile in interacting with various other transcription factors and transcriptional co-regulators. For example, RUNX1 and Ets1 interact to coordinate transcriptional activity of the T cell receptor via the Runt domain of RUNX1, including regions just adjacent to the domain (35,40,41). RUNX1 has also been shown to interact with PU.1, C/EBPalpha, p300, mSin3a, GATA1, and Fli1 among many other factors that will not be further discussed in this review (42-47). These studies indicate that RUNX1 function relies heavily on its interaction partners and that these interaction partners may help to regulate its target genes in a tissue-specific manner.

3.3. Post-translational modifications of RUNX1

Aside from being transcriptionally regulated, RUNX1 is regulated by various post-translational mechanisms. RUNX1 was first reported to be phosphorylated by Extracellular Signal-Regulated Kinase (ERK) and phosphorylated forms of RUNX1 have been detected in $CD34^+$ hematopoietic progenitor cells (48,49). Serines 249 and 266 of RUNX1b are ERK-associated phosphorylation sites and these sites are important in regulating the transactivation ability of RUNX1 (48). Further studies have shown that serine 273 and threonine 276 are also phosphorylated by the ERK pathway (50). Serines 249 and 266 are additionally phosphorylated by cyclin-dependent kinases (CDKs), which aids in regulating RUNX1 degradation by the anaphase-promoting complex (APC) (51). In addition, phosphorylation of RUNX1 by CDKs affects not only stability of RUNX1, but the DNA-binding affinity of RUNX1 as demonstrated by mutating three serine residues to aspartic acid which led to decreased DNA-binding affinity (52).

The effect of phosphorylation of RUNX1 is further revealed by studies looking into some of its interaction partners. For example, homeodomain-interacting protein kinase-2 (HIPK2) phosphorylates RUNX1 and subsequently triggers phosphorylation of p300, a transcriptional co-activator that interacts with RUNX1, which may explain how RUNX1 activates transcription of its target genes (53). Furthermore, CBFbeta has been shown to help recruitment of HIPK2 to phosphorylate RUNX1 (54). Phosphorylation of RUNX1 reduces its interaction with histone deacetylases and the transcriptional co-repressor mSin3A (45,55). Hence, phosphorylation of RUNX1 complements its ability to activate transcription by reducing its interactions with transcriptional repressors.

The ubiquitin-proteasome system has been well characterized and is known to regulate transcription (56). RUNX1 is a target of this system as evidenced by its enhanced stability in cells that have been treated with proteasome-specific inhibitors and when the lysine residues of RUNX1 are mutated to arginines (57). Ubiquitin-mediated degradation of RUNX1 is regulated both by heterodimerzation with CBFbeta, which enhances its stability, and by CDK phosphorylation, which promotes degradation by the APC (51,57).

Another post-translational modification that was recently described is methylation of RUNX1. PRMT1, an arginine methyltransferase, was shown to methylate RUNX1 at an area just C-terminal to the Runt domain (58). Methylation of RUNX1 inhibited its interaction with the co-repressor SIN3A which enhanced its transcriptional activity on known target genes like *CD41* and *PU.1* (58).

As evidenced by the aforementioned studies, RUNX1 is a protein that is heavily regulated by post-translational measures. However, the majority of these studies utilized *in vitro* analyses to demonstrate these post-translational effects. A more recent study involved the use of mice carrying knock-in alleles of *Runx1* that had either both serine 249/serine 266 or both serine 249/serine 276 mutated to alanines, resulting in elimination of these sites for phosphorylation (59). Despite previous studies showing the importance of these serines for RUNX1 regulation, these mice exhibited no overt hematopoietic phenotype (59). Although this study serves as an example of how *in vitro* analyses may not necessarily predict *in vivo* functions, the role of phosphorylation and other post-translational modifications of RUNX1 may still be important during specific stages of hematopoiesis. Furthermore, other potential sites of phosphorylation that have yet to be examined may play more essential roles or allow for

functional redundancy with serines 249, 266, and/or 276. Additional investigation will be needed to elucidate where these sites are located and whether these sites participate in RUNX1 regulation.

4. RUNX1 AS A MASTER REGULATOR OF HEMATOPOIESIS

4.1. The role of RUNX1 in the specification and development of the definitive hematopoietic stem cell using mouse models

RUNX1 plays an essential role in specifying the definitive hematopoietic stem cell (HSC). Two waves of hematopoiesis occur during embryonic development. The first wave is known as primitive hematopoiesis, which describes the differentiation of primitive macrophages and early erythrocytes from progenitors in the yolk sac to aid in the rapid development of the embryo (60). After this initial wave, definitive hematopoiesis, which describes the process of generating the various lineages of mature blood cell types from a common definitive HSC, takes place. One of the first sites that HSCs are detected in mammals is the aorta-gonad-mesonephros region at 10.5 days post conception (dpc) in the mouse embryo, where they bud off from the ventral aspect of the dorsal aorta and eventually colonize the fetal liver (61,62). *Runx1* was detected in both locations during embryogenesis, indicating that expression of *Runx1* marks the earliest hematopoietic precursor cells (63). One of the most important pieces of evidence implicating the role of RUNX1 in specifying the HSC was the generation of *Runx1*-null mice (64,65). While heterozygous mice are healthy and fertile, the homozygous knockout mice die between 12.5 to 13.5 dpc with severe hemorrhaging along the central nervous system. Such extensive hemorrhage is most likely due to defects in angiogenesis caused by a lack of angiopoietin-1 expression in these knockout mice (66). Furthermore, although these mice have nucleated primitive erythrocytes, indicating that there was no major defect in primitive hematopoiesis, they lacked definitive hematopoiesis. Cells from embryonic hematopoietic tissues, such as the yolk sac and liver, do not show colony forming units when cultured *in vitro*, and chimeric mice made from Runx1-deficient embryonic stem (ES) cells and wild type mouse blastocysts do not show any Runx1-deficient ES cell contribution to adult hematopoietic cells. More recent studies have further established the essential role that RUNX1 plays in derivation of HSCs from the hemogenic endothelium (67,68). Interestingly, however, once the HSC is defined, RUNX1 is no longer essential for hematopoiesis (68). These results indicate that RUNX1 plays a fundamental role in the establishment of definitive HSCs.

Although the role of RUNX1 in programming the HSC from embryonic development is well established from the investigations discussed above, one remaining question is whether RUNX1 is important in adult HSC function. Sun *et al.* described how mice that were haploinsufficient for *Runx1* displayed a higher number of HSCs as defined by the cell surface marker phenotype, lineage$^-$sca1$^+$ckit$^+$ (LSK), but contrastingly has lower number of functional long-term HSCs as assayed by limiting dilution analyses (69). To delve into this issue further, several groups have utilized a conditional *Runx1* knockout model with the *Mx1-Cre* transgenic mouse line (70-72). The *Mx1* gene is an interferon-inducible gene and its promoter allows expression of *Cre* to occur in HSCs when exposed to interferon (IFN) or other IFN-inducing agents like the synthetic double-stranded RNA polyinosinic/polycytidylic acid (polyIC) (73). The most striking phenotypes of conditional *Runx1* knockout mice are a significant expansion of the putative HSC population (LSK cells) and myeloid progenitors, thrombocytopenia, and lymphopenia (70,71). Analysis of the spleen and thymus revealed a myeloproliferative phenotype (71,72). Further study of the stem cell compartment in these mice also revealed an expansion not only in LSK cells but in the long-term HSCs as indicated by additional labeling with CD34$^-$Flt3$^-$ (74,75). Although Runx1-deficient cells showed enhanced proliferative ability, they are functionally impaired in their ability to engraft upon competitive transplantation with wild type hematopoietic cells into an irradiated host (71,75). Jacob at el. go further and state that lack of Runx1 causes stem cell exhaustion and use a retroviral insertional mutagenesis screen to identify *Evi5* as a gene that can ameliorate this exhaustion phenotype when over-expressed. Moreover, they attribute the phenotype to a defect in the interaction between HSCs and the niche. *Cxcr4*, a gene important in stem cell homing and niche interactions, is a direct target of Runx1. This transcript is down-regulated in the absence of Runx1 but levels are rescued when *Evi5* is over-expressed (75). Hence, absence of Runx1 may disrupt the HSC-niche interaction leading to aberrant hematopoiesis characterized as a stem cell exhaustion phenotype observed in the *Runx1* knockout mice (75).

Although Jacob *et al.* provide one possible mechanism for the contrast in phenotype between *Runx1* knockout embryos and adults, more studies will need to be conducted to describe exactly what roles and what target genes RUNX1 may additionally regulate in HSCs and hematopoiesis. Is RUNX1 only needed for specification but not maintenance of the HSC? Is RUNX1 required only to define the epigenetic environment conducive for HSC identity but is no longer needed once other hematopoietic-specific transcription factors are in place (76)? More investigation will be required to thoroughly answer these questions and further explain how RUNX1 functions as a master regulator of hematopoiesis.

4.2. RUNX1 target genes as they relate to hematopoiesis

In addition to the mouse models described earlier, numerous studies have focused on RUNX1 as a DNA-binding transcription factor and on the genes that it regulates. The Runt domain of RUNX1 mediates binding to the TG^T/cGGT consensus sequence (77). In various adult blood types, target genes of RUNX1 have been fairly well characterized. In the myeloid lineage, RUNX1 directly binds and regulates the promoter activities of genes related to myeloid growth factor signaling such as *IL-3*, *GM-CSF*, the *M-CSF receptor*, and *c-Mpl* (78-81), and to the function of myeloid cells such as *myeloperoxidase*, *neutrophil elastase*, and *mast cell protease 6* (82,83). In the T cell lineage, RUNX1 targets promoters and enhancers

of *T cell receptors* and the *CD11a* promoter (84-86). In the B cell lineage, RUNX1 targets a B cell specific src family tyrosine kinase known as *blk*, *Igα* promoters, and the immunoglobulin antigen receptor enhancers (87-89).

Although the role of RUNX1 in regulating these genes are well described in adult blood cells, more recent studies have focused on RUNX1 target genes that are important in the regulation of hematopoiesis at the stages of stem cells and early progenitors. For example, RUNX1 has been demonstrated to play an important role in the differentiation and function of regulatory T cells by targeting and interacting with FoxP3 (90,91). RUNX1 also has an essential role in regulating *PU.1*, considered to be a critical transcription factor in myeloid progenitors and other mature myeloid cells (92). In this study, an upstream regulatory element located 14 kilobases upstream relative to the *Pu.1* locus was found to have binding sites for Runx1, and mice harboring mutations in these binding sites exhibit a phenotype similar to the *Runx1* conditional knockout phenotype described earlier (92). In addition, RUNX1 regulates *miR-27a*, which is involved in a feedback loop by binding to sites on the 3' UTR of RUNX1, thereby mediating megakaryopoiesis (93). Since RUNX1 encompasses such a large role in hematopoiesis, more work is needed on the identification of its target genes, especially as they relate to HSCs or early progenitors.

4.3. Mutations in RUNX1 lead to aberrant hematopoiesis

The importance of RUNX1 in hematopoiesis is further exemplified by the mutations found in *RUNX1* in patients with various hematological diseases. In one study, eight out of 160 patients with AML were found to have various mutations in the *RUNX1* gene (94). Interestingly, these mutations were located in the Runt domain and further molecular analyses indicated that some of these mutations resulted in abnormal DNA binding and altered transactivation of the *M-CSF* promoter, a gene known to be regulated by RUNX1 (94).

In another study, six pedigrees of patients with familial platelet disorder with predisposition to acute myeloid leukemia (FPD/AML) were analyzed and found to be linked to a region of chromosome 21 encoding *RUNX1* and concluded that haploinsufficiency of *RUNX1* may be one cause of FPD/AML (95). While haploinsufficiency of *RUNX1* may predispose patients to FPD/AML, biallelic mutations in *RUNX1* resulting from a second hit may lead to full on leukemia (96).

Cases of myelodysplastic syndrome (MDS) were found to have mutations in *RUNX1* (97). These mutations were found to be associated with the Runt domain of RUNX1 and the authors suggest that they result in dominant-negative forms of RUNX1 (97). Although additional studies looking into FPD/AML patients with mutations in *RUNX1* have proposed a similar dominant-negative mechanism towards progression to disease, other case studies have found mutations in the C-terminal portion of RUNX1, which may suggest another mechanism toward disease progression (98-100).

Patients with myeloproliferative neoplasms (MPN) were also found to have point mutations in *RUNX1* (101). Additional analyses of one of these mutated forms being transduced into human $CD34^+$ MPN cells demonstrated that it promoted proliferation, which as the authors suggested, may allow for leukemic transformation in patients with MPN (101). Another study showed that patients with MPN have higher levels of *RUNX1* mRNA transcript which up-regulate the target gene *NF-E2*, a gene that regulates erythropoiesis (102).

More recently patients of chronic myelomonocytic leukemia (CMML) have been found to harbor mutations in *RUNX1* (103,104). Although most mutations were in the Runt homology domain, some patients had mutations in the C-terminal region after the Runt domain. Interestingly these patients progressed to full blown AML much faster when compared to patients without any mutations (104). The prevalence of *RUNX1* mutations in the various hematological diseases described above highlights the important role that RUNX1 plays in normal hematopoiesis and what may happen when normal RUNX1 function goes awry.

5. RUNX1 IS A COMMONLY FOUND CONSTITUENT IN CHROMOSOMAL TRANSLOCATIONS ASSOCIATED WITH CANCER

As discussed in the previous section, RUNX1 is commonly found to be mutated in diseases associated with disrupted hematopoiesis. Many of the patients suffering from these diseases are one step away from developing full blown leukemia. Hence it is not surprising that *RUNX1* is the most common target of chromosomal translocations found in acute leukemia. The three most common chromosomal translocations involving *RUNX1* are: t(8,21), t(12,21), and t(3,21) (105). *RUNX1-ETO* (also known as *AML1-ETO*, *RUNX1-MTG8*, and *RUNX1-RUNX1T1*) is a result from t(8,21) and is found in about 12% of AML and 40% of the M2 subtype of AML (11). *TEL-RUNX1* is a result of t(12,21) and was originally cloned from two patients with pediatric precursor B-cell acute lymphoblastic leukemia (ALL) (106). The translocation is present in about 25% of patients with childhood pre-B cell ALL and produces a fusion with the N-terminal HLH domain of the TEL protein and almost the entire RUNX1 protein, including its Runt and transactivation domains (106,107). The third most common translocation involving *RUNX1* is t(3,21), which was first discovered in patients in the blast crisis phase of chronic myelogenous leukemia and later in approximately 3% of therapy-related MDS and AML (108,109). Cloning of fusion transcripts from patient tissue samples revealed that the N-terminal portion of RUNX1, including its Runt domain, was fused with either one of three genes on chromosome three including *EVI*, *MDS1*, or *EAP* (110). Less common chromosomal translocations involving *RUNX1* have also been described in patients with a variety of other leukemias and hematological neoplasms (111-113). The prevalence of RUNX1 in these diseases

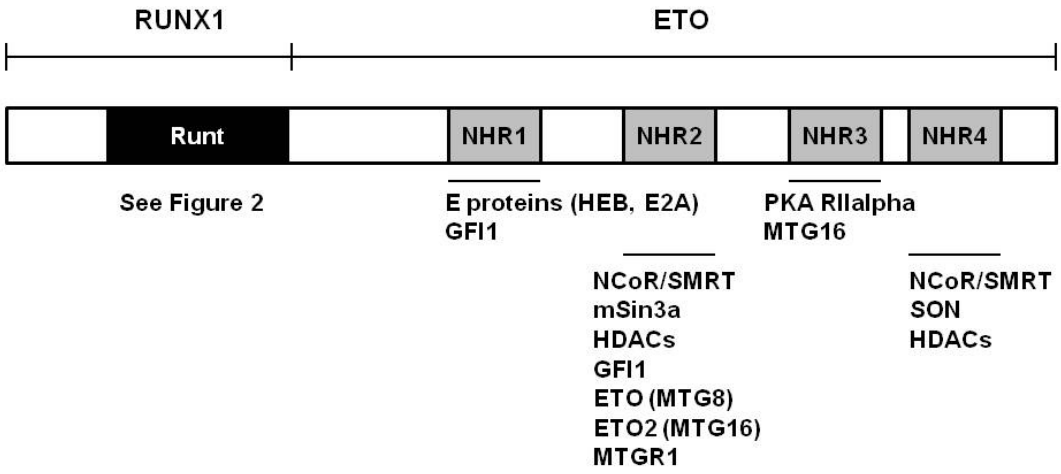

Figure 3. RUNX1-ETO protein domains and interaction partners. The full length RUNX1-ETO is shown with the major domains listed above and interaction partners listed below.

obviously substantiates its role in normal blood development. Moreover, the variety of leukemias found in these patients, which have shown to include those of both myeloid and lymphoid origin, suggests that RUNX1 acts relatively upstream in the hematopoietic lineage tree to regulate the activity of HSCs.

6. RUNX1-ETO STRUCTURE AND REGULATION

6.1. RUNX1-ETO protein domains and interactions

As mentioned earlier, t(8,21) is one of the most prevalent chromosomal translocations in AML, and will be the primary topic for the remainder of this review. The translocation places together the *RUNX1* locus on chromosome 21 and *ETO* locus on chromosome 8. Both the fusion transcript and the *ETO* gene were first cloned in the 1990s (114,115). The breakpoints occur in intron 5 of the *RUNX1* locus and in either intron 1a or 1b of the *ETO* locus (116-118). The RUNX1-ETO protein consists of 752 amino acids involving almost the entire ETO protein and the N-terminal portion of the RUNX1 protein containing the Runt homology domain (Figure 3) (115).

As discussed earlier, RUNX1 has various roles in the hematopoietic system, but the Runt domain in particular functions to bind DNA and interact with other transcription factors. ETO, on the other hand, has mostly been discussed in the context of the RUNX1-ETO fusion protein in hematopoiesis. The *ETO-RUNX1* transcript is not detected in t(8,21) patients, suggesting that ETO gene expression is relatively low in hematopoietic tissues (119). Mice with *Eto* knocked out displayed no hematopoietic abnormalities and was not found to be expressed in the hematopoietic compartment (120). Mice homozygous for the deletion die postnatally due to improper development of the gut, where it is found to be highly expressed (120). The high expression of ETO in the brain also suggests that it may play an important role in the central nervous system (121,122).

The ETO locus consists of 13 exons and spans over 87 kilobases (123). ETO was thought to be a putative transcription factor when it was first cloned based on the fact that in contained two zinc chelating domains (124). However, a screen using ETO protein revealed that it was not able to bind specific DNA sequences and hence does not seem to have any DNA-binding ability (125). ETO is mainly characterized by its four Nervy homology regions (NHRs), which are numbered one through four and share homology with the Drosophila Nervy protein (126). In Drosophila, *nervy* is expressed primarily in central nervous system regions and plays a role in regulating axon growth (126,127). Briefly, NHR1 has sequence similarities with TAF110 and other TAF proteins (124). NHR2 can mediate both alpha-helical tetramer formation, and homo- and hetero-oligomerization of ETO and its other family members, including ETO2 (MTG16) and MTGR1. NHR3 contains a coiled-coil motif, which shares structural homology with PKA anchoring proteins and binds to a PKA regulatory subunit (PKA RIIalpha) (128). A NHR3 point mutation that disrupts its interaction with PKA RIIalpha does not disrupt RUNX1-ETO's ability to transform primary mouse bone marrow cells *in vitro* (129). NHR4 is also known as a myeloid-Nevry-DEAF1 (MYND) domain and has a zinc chelating motif (130).

These NHRs define the domains of ETO that mediate interactions with other proteins. For example, NHR1 mediates interactions with E proteins and inhibits the ability for E proteins to recruit co-activator molecules like p300/CBP (131). NHR2 can interact with co-repressors like NCoR/SMRT, mSin3a and HDACs (132-134). NHR4 also interacts with co-repressors NCoR/SMRT (133,135,136). Hence, earlier works on RUNX1-ETO have focused primarily on its function as a transcriptional repressor based on ETO's interaction with these co-repressors (133,135,136). The results suggest that RUNX1-ETO binds to DNA using the Runt homology domain contained in its N-terminus to repress the transcription of RUNX1 target genes. Another recent study discovered SON as an RUNX1-ETO-interacting protein through the NHR4 domain. A point mutation in the NHR4 domain that disrupts RUNX1-ETO's ability to interact with

Table 1. Various animal models used to study effect of RUNX1-ETO expression

Species	Descritpion	Hematopoietic phenotype	References
Drosophila melanogaster	Expression of RUNX1-ETO in eye	n/a	(179)
Drosophila melanogaster	Expression of RUNX1-ETO in blood cells	Increased number of blood progenitors	(146,180)
Danio rerio	Inducible expression of RUNX1-ETO by *Hsp70* promoter	Increased number of immature blasts	(147,148)
Mus musculus	RUNX1-ETO knockin at *Runx1* locus	Embryonic lethality due to hemorrhaging and block in hematopoiesis	(149,150)
Mus musculus	Inducible transgenic expression of RUNX1-ETO	Normal hematopoiesis	(12)
Mus musculus	Inducible Cre/loxP-mediated translocation of Runx1-Eto by *Nestin* Cre	Normal hematopoiesis	(139)
Mus musculus	Transgenic expression of RUNX1-ETO under *MRP8* promoter	Normal hematopoiesis, treatment with ENU causes leukemia	(13)
Mus musculus	Inducible Cre/loxP RUNX1-ETO knockin expression by *Mx1* Cre	Normal hematopoiesis, treatment with ENU causes leukemia	(140)
Mus musculus	Bone marrow transduction/transplantation with RUNX1-ETO	Abnormal myelopoiesis, no leukemia	(153)
Mus musculus	Bone marrow transduction/transplantation of ICSBP-deficient cells with RUNX1-ETO	Myeloid leukemia	(154)
Mus musculus	Bone marrow transduction/transplantation with RUNX1-ETO and TEL-PDGFbetaR	Myeloid leukemia	(155)
Mus musculus	RUNX1-ETO knockin at *Sca1* locus	Myeloproliferative disease	(152)
Mus musculus	Bone marrow transduction/transplantation with RUNX1-ETO and FLT3 length mutation	Myeloid leukemia	(156)
Mus musculus	Bone marrow transduction/transplantation of human CD34+ cells with RUNX1-ETO in NOD/SCID mice	Abnormal myelopoiesis, no leukemia	(158)
Mus musculus	Bone marrow transduction/transplantation with WT1-overexpressing cells transduced with RUNX1-ETO	Myeloid leukemia	(181)
Mus musculus	Bone marrow transduction/transplantation with RUNX1-ETO9a	Myeloid leukemia	(142)
Mus musculus	Inducible Cre/loxP RUNX1-ETO knockin and HIP1-PDGFbetaR knockin expression by *Mx1* Cre	Myeloid leukemia	(151)

SON and NCoR/SMRT induces leukemia in a mouse transplantation model (137).

6.2. RUNX1-ETO splice isoforms and effects on leukemogenesis

The discussion of RUNX1-ETO's various conserved domains and structure provides a natural transition to a discussion on its transcriptional isoforms. Several early reports revealed that multiple isoforms of *RUNX1-ETO* transcripts are detectable in t(8,21) AML patient samples (116,138). However, most RUNX1-ETO related studies have focused on the full length 752 amino acid form of RUNX1-ETO. Several models used to study the effect of RUNX1-ETO expression in mice have shown that without the presence of major additional mutations, RUNX1-ETO cannot induce leukemia on its own (12,139,140). Interestingly in one study, one mouse transplanted with RUNX1-ETO-transduced bone marrow cells was found to have developed AML rapidly (141). The leukemia cells in this mouse harbored a mutated *RUNX1-ETO* cDNA, which coded for a C-terminal truncated form of RUNX1-ETO that only had 552 amino acids (141). One extra exon, exon 9a, of ETO was previously reported (123). An RUNX1-ETO fusion transcript that included this exon 9a has been widely detected in t(8,21) AML. Such a transcript leads to the production of a C-terminal truncated RUNX1-ETO protein, termed RUNX1-ETO9a, which has 572 amino acids, and was almost identical to the leukemogenic 552 amino acid RUNX1-ETO protein just mentioned (142). Indeed, this RUNX1-ETO9a splice isoform rapidly induces leukemia in this mouse model (142).

The truncated version of RUNX1-ETO and RUNX1-ETO9a both lack the NHR3 and NHR4 domains. Notably, the NHR3 and NHR4 domains have been described to interact with transcriptional repressors like NCoR and SMRT (133,135,136). The ability of these truncated versions of RUNX1-ETO to rapidly induce leukemia have called into question how important these C-terminal NHR domains really are in this process. Deletion of the entire NHR4 domain or one amino acid point mutation that disrupts the zinc-chelating structure of RUNX1-ETO is sufficient to make RUNX1-ETO leukemogenic (137). This discovery demonstrates that the C-terminal portion of RUNX1-ETO does not participate in promoting leukemia. On the contrary, the C-terminal portion of full length RUNX1-ETO acts as an inhibitor to leukemogenesis. Therefore, any conditions or mutations that interfere with the function of NHR4 or its downstream signaling pathways may enhance t(8,21)-involved leukemia development. Currently, no NHR4 DNA sequence mutation has been identified in t(8,21)-positive AML (143). Further study on the biochemical function of this domain is important since it may provide valuable insights into the mechanisms of leukemogenic transformation.

6.3. Post translational modifications of RUNX1-ETO

Post translational modifications of RUNX1-ETO or ETO have not been as well described as those for RUNX1. Phosphorylated forms of ETO protein have been reported in human $CD34^+$ hematopoietic cells (49). One study has looked into the role of the ubiquitin-proteasome pathway in the degradation of RUNX1-ETO, which demonstrated that RUNX1-ETO was ubiquitinylated and interacted with the E2-conjugase, UbcH8, and the E3-ligase, SIAH-1 (144). In addition to ubiquitin-mediated degradation, two non-classical aspartate residues (amino acids 188 and 368) of RUNX1-ETO are target sites of caspase-3 (145). They are responsible for caspase-3-mediated cleavage of RUNX1-ETO during apoptotic conditions (145).

Since RUNX1-ETO still retains the Runt homology domain, it is considered to share some of the

same modifications as observed for RUNX1 and lack of the regulatory elements on the C-terminal portion of RUNX1 may contribute to RUNX1-ETO's characteristics. For example, the arginine residues that are present just C-terminal to the Runt domain in RUNX1 are absent in the fusion protein. The lack of this particular region has been suggested to play a role in why RUNX1-ETO may have a stronger interaction with the co-repressor SIN3A than RUNX1 and therefore acts as a dominant-negative inhibitor of RUNX1 (58). Overall, however, post translational modifications of RUNX1-ETO have not been as well characterized as that of RUNX1 and offers an intriguing opportunity for further investigation.

7. RUNX1-ETO AND INDUCTION OF LEUKEMIA

7.1. Effects on hematopoiesis through the use of animal models

With t(8,21) being so prevalent in AML, there have been numerous models used to study the effects of RUNX1-ETO on hematopoiesis (Table 1). For example in *Drosophila*, expression of RUNX1-ETO causes an expansion of blood cell lineage progenitors (146). This model was further used to elucidate calpainB, which is a calcium-dependent protease conserved in *Drosophila*, as a modulator of RUNX1-ETO function (146). In zebrafish, which have a similar blood system to mammals, inducible expression of RUNX1-ETO recapitulates some of the phenotypes seen in AML patients like disruption of normal hematopoiesis and accumulation of immature blasts (147). Some of the unique qualities of zebrafish like its relatively short generation time and the large numbers of offspring per mating have allowed this model to become a useful method to screen for chemical modulators of RUNX1-ETO activity (148). The majority of models, however, that have been used to study RUNX1-ETO function center around the mouse.

The first mouse models utilized a knock-in strategy to insert the *RUNX1-ETO* fusion gene into the *Runx1* locus and revealed that heterozygous *RUNX1-ETO* mice die during embryogenesis (149,150). The embryonic lethality is similar to that exhibited by *Runx1* knockout mice, but slight differences were observed. Populations of yolk sac and fetal liver cells from the heterozygous embryos were able to differentiate into macrophages and dysplastic myeloid cells, respectively, whereas the homozygous knockout lacked definitive hematopoiesis entirely (149,150). These initial mouse studies demonstrated that although RUNX1-ETO primarily acted as a dominant-negative regulator of RUNX1, it may have other properties that may be important in its ability to promote leukemogenesis.

The next wave of models consisted of transgenic mice with *RUNX1-ETO* under the control of various promoters like a tetracycline-responsive element and *MRP8*, a calcium binding protein expressed in myeloid progenitors and mature neutrophils/monocytes (12,13). Although heterozygous *RUNX1-ETO* mice die embryonically, these transgenic mouse appear to display normal hematopoiesis and have a healthy lifespan (12,13). Only in the presence of additional mutations induced by treating these mice with N-ethyl-N-nitrosourea do they develop overt AML (13). Another group that used the *Mx1-Cre* inducible system and conditional *RUNX1-ETO* knock-in mice observed similar results (140). The presence of another oncogene like HIP1-PDFGbetaR together with RUNX1-ETO is also sufficient to create a myeloproliferative phenotype in mice (151). Interestingly, a knock-in mouse model with *RUNX1-ETO* being expressed from the *Sca1* gene locus, which is active in HSCs, displayed myeloproliferative disease although spontaneous AML was still not observed (152).

The general lack of a leukemia phenotype from the models above led to additional studies utilizing cell transduction with RUNX1-ETO-expressing retrovirus followed by bone marrow transplantation of these cells into lethally irradiated hosts. This model system allowed for the selection of HSCs that expressed RUNX1-ETO because they were labeled with GFP or other markers (153,154). Interestingly, these models showed a slightly more severe phenotype in the hematopoietic system as illustrated by the appearance of immature blasts and inhibition of myeloid differentiation (153,154). Survival times, however, remained relatively normal and spontaneous AML was not observed (153,154). Furthermore, in agreement with previous results, the presence of additional perturbations such as deficiency of ICSBP, presence of Flt3 length mutation, or presence of TEL-PDGFbetaR fusion protein cooperated with RUNX1-ETO to induce AML (154-156). Based on the models described so far, RUNX1-ETO has the ability to induce changes in adult hematopoiesis, especially if expressed in the HSC compartment, and establishes an environment that is conducive for leukemogenesis with the presence of additional mutations.

In contrast to the mouse systems described so far, one recent discovery has identified that truncated isoforms of RUNX1-ETO transcript are capable to inducing full blown leukemia by themselves. The RUNX1-ETO9a is one such splice isoform, that when transduced and transplanted into mice, rapidly induces leukemia (142). Further studies are warranted into why full length RUNX1-ETO requires additional mutations to generate leukemia while shorter isoforms which are missing pieces of the C-terminal domain are capable of generating leukemia on their own.

A great deal of knowledge has been elucidated using the mouse models above. Xenografts using human $CD34^+$ cells transduced with RUNX1-ETO and transplanted into NOD/SCID mice have revealed similar results—that RUNX1-ETO alone cannot induce leukemia development (157,158). What remains to be seen is whether this xenograph model utilizing the shorter RUNX1-ETO9a isoform can induce leukemia on its own.

7.2. RUNX1-ETO target genes

Since both RUNX1 and RUNX1-ETO contain the Runt homology domain, which is responsible for their DNA-binding properties, RUNX1-ETO has generally been assumed to share the same target genes as RUNX1. However, these two DNA-binding proteins may have

varying preferences for target genes because RUNX1-ETO has an affinity for binding DNA segments that contain duplicated RUNX1 consensus sites (125). Hence, presence of the fusion protein may involve a gain-of-function in some circumstances.

Most of the target gene studies conducted on RUNX1-ETO have specifically focused on its role as an inducer of leukemia. One of the first target gene studies demonstrated that RUNX1-ETO directly regulated repression of the $p14^{Arf}$ tumor suppressor gene, which is a mediator of p53 (159). Another study delved into the role that nerve growth factor (NGF) plays in t(8,21)-mediated leukemogenesis by observing that expression of RUNX1-ETO in human $CD34^+$ hematopoietic cells up-regulates *TRKA* mRNA, which subsequently allows these cells to respond to NGF (160). Yet another study implicated the negative cell cycle regulator $p21^{WAF1}$ as a transcriptional target of RUNX1-ETO and that in the absence of $p21^{WAF1}$, expression of RUNX1-ETO was able to induce leukemia (161). These works have given clues to which single genes, when mutated or differentially expressed, may help RUNX1-ETO to promote leukemia development.

With the advent of more high-throughput methods like differential gene expression microarrays, a variety of studies have been conducted on a genome-wide scale to characterize the transcriptional environment that RUNX1-ETO may promote. In one study using cell lines that expressed RUNX1-ETO, the authors discovered that DNA repair genes were generally inhibited while genes that promote stem cell self-renewal, like those in the Notch pathway, were generally up-regulated (162). Another study looked at 285 samples from patients with various types of AML and were able to place t(8,21)-positive patients into a unique cluster based on their gene profiles alone (163). A similar investigation using samples from cases of pediatric AML also concluded that t(8,21)-positive patients carry a unique but common transcriptional signature (164). This transcriptional signature also includes those that are part of the endogenous microRNA (miRNA) system. Genome-wide analyses of 52 AML patient samples revealed that patients with t(8,21)-positive leukemia have a distinct miRNA expression pattern from AML patients with other chromosomal translocations (165). Recent work has already characterized specific miRNAs regulated by RUNX1-ETO like *miR-126* and *miR-223* (165,166).

The genome-wide microarray studies have undoubtedly revealed significant differences in gene expression with the presence of RUNX1-ETO. Advances in coupling chromatin immunoprecipitation with microarrays have provided yet another approach to analyze target genes for DNA-binding proteins like RUNX1-ETO. In a recent study utilizing this technology, Gardini *et al.* were able to describe thousands of potential target genes and an important interaction between RUNX1-ETO, RUNX1, and HEB in potentially regulating these target genes (167). The common theme among these single target gene and genome-wide studies reflects what has been observed in the various mouse models described in the previous section—that although RUNX1-ETO cannot induce leukemia by itself, it does promote an environment for additional mutations to occur which may eventually lead to leukemia.

7.3. Regulation of chromatin structure

RUNX1-ETO has primarily been recognized as a dominant-negative regulator of RUNX1 due to the similarities between heterozygous *RUNX1-ETO* knock-in mice and *Runx1* knockout mice. Many reports have shown that RUNX1-ETO is a repressor of transcription. ETO and RUNX1-ETO both interact with transcriptional co-repressors and HDACs through yeast two-hybrid assays and co-immunoprecipitation studies (133,135,136). Specifically, ETO was shown to interact with NCoR and mSin3a, which themselves were shown to interact with HDACs (168). Additional studies by the same group demonstrated that RUNX1-ETO interacts with HDAC-1, HDAC-2, and HDAC-3 and that treatment with trichostatin A, an HDAC inhibitor, blocks the ability of RUNX1-ETO to suppress myeloid differentiation (169). A recent example of this mechanism was described by Fazi *et al.* where they detailed how RUNX1-ETO was able to recruit HDACs to the *miR-223* gene locus, a myelopoiesis regulator, to silence its transcriptional activity (166). Valproic acid, another HDAC inhibitor, has also been shown to promote the differentiation of Kasumi cells, a t(8,21)-positive AML cell line (170). Valproic acid may disrupt the interaction between RUNX1-ETO and HDAC-1 and is able to reactivate transcription of RUNX1 target genes like *IL-3 (171)*. These studies point toward the ability of RUNX1-ETO to recruit HDACs as a major mechanism that may give it its leukemogenic properties, and suggest that the use of HDAC inhibitors may be a potentially useful therapy for patients suffering from t(8,21)-positive AML. It is also worth to note that HDAC inhibitors directly promote the degradation of RUNX1-ETO (144,172).

In addition to recruiting HDACs, RUNX1-ETO has been shown to promote methylation of DNA by recruitment of DNA methyltransferases (DNMTs). The first clue that led to further characterization into whether RUNX1-ETO may be involved in recruiting DNMTs was that the combination of HDAC and DNMT inhibitors were shown to have synergistic effects on histone acetylation and release of target gene repression (173). In a follow-up study, RUNX1-ETO was shown to interact with DNMT1 by co-immunoprecipitation and that both were able to synergistically repress transcription of *IL-3* when transfected together into 293T cells (174). Subsequent investigations have characterized numerous gene loci where RUNX1-ETO may promote hypermethylation (175,176). DNA methylation is not specific for RUNX1-ETO, as other cancers have also been associated with DNA hypermethylation. Interestingly, however, a large scale study of the DNA methylation patterns of patients with various AMLs revealed that the t(8,21) AML-specific gene loci may offer a unique oncologic signature that distinguishes it from other types of AML (177). Further characterization of these specific loci may offer new genes for drug targeting and additional insights into the mechanisms of t(8,21)-positive leukemogenesis.

8. PERSPECTIVES

RUNX1 is undoubtedly a master regulator of hematopoiesis. Its attribute as a critical factor in defining the identity of HSCs make RUNX1 a very attractive molecule to study for potential applications in bone marrow therapy. At the same time, the prevalence of RUNX1-ETO in AML and other types of leukemia have made the fusion protein very highly studied. Although many target gene studies have been conducted on RUNX1, a paucity of data exists pertaining to the AML target genes in HSCs or immature progenitors. A recent study has tried to tackle this problem by utilizing massively parallel sequencing coupled with chromatin-immunoprecipitation (ChIP-seq) on a murine HSC-like cell line and has presented a genome-wide map of RUNX1 chromatin occupancy along with maps of other HSC-specific transcription factors (178). Though no analyses on specific genes were conducted, the study does provide a valuable resource of the RUNX1-targeted genes that may have essential functions in HSC identification, maintenance, or differentiation.

At the time of writing this review, no ChIP-seq studies have been conducted on RUNX1-ETO. Such a study on the genome-wide occupancy status of RUNX1-ETO will be a valuable resource, especially if it could be reasonably compared to RUNX1 occupancy, which would describe how RUNX1-ETO perturbs endogenous RUNX1 function on a more global scale. As technology continues to advance, we may even see ChIP-seq done on RUNX1 and/or RUNX1-ETO occupancy using sorted HSC or progenitor populations.

Another interesting avenue for further work arises from the alternatively spliced isoform of the fusion transcript, RUNX1-ETO9a, that is capable of inducing leukemia on its own. The lack of leukemia-inducing ability of full length RUNX1-ETO suggests that the C-terminal portion of the fusion protein may not be important or may even inhibit leukemia development. The mechanisms of how RUNX1-ETO9a induces leukemia and the role of the C-terminal portion that is missing in RUNX1-ETO9a will further elucidate the pathogenesis of this very common fusion protein.

RUNX1 and RUNX1-ETO have been fixtures in hematological and leukemia research for over two decades. Despite the numerous studies on these two molecules, much remains to be discovered. The critical nature of RUNX1 in hematopoiesis and the prevalence of RUNX1-ETO in leukemia continue to make these proteins highly studied in biomedical research, and breakthroughs in this field will offer much benefit to patients suffering from RUNX1- and RUNX1-ETO-related diseases and neoplasms.

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Abbreviations: AML: acute myeloid leukemia, RUNX: Runt-related transcription factor, PCR: polyermase chain reaction, GFP: green fluorescent protein, CBF: core binding factor, NMTS: nuclear matrix targeting signal, ERK: extracellular signal-regulated kinase, CDK: cyclin-dependent kinase, APC: anaphase-promoting complex, HIPK2: homeodomain-interacting protein kinase-2, HSC: hematopoietic stem cell, dpc: days post conception, ES: embryonic stem, LSK: lineage-negative, sca1-positive, ckit-positive, IFN: interferon, polyIC: polyinosinic/polycytidylic acid, FDP/AML: familial platelet disorder with predisposition to acute myeloid leukemia, MDS: myelodysplastic syndrome, MPN: myeloproliferative neoplasm, CMML: chronic myelomonocytic leukemia, ALL: acute lymphoblastic leukemia, HLH: helix-loop-helix, NHR: Nervy homology domain, PKA: protein kinase A, MYND: myeloid-Nervy-DEAF1, HDAC: histone deacetylase, NCoR: nuclear corepressor, SMRT: silencing mediator of retinoid and thyroid hormone receptor, DNMT: DNA methyltransferase

Key Words: RUNX1, AML1, CBFA2, RUNX1-ETO, AML1-ETO, RUNX1-RUNX1T1, Hematopoiesis, Hematopoietic Stem Cells, Leukemia, AML, Leukemogenesis, Mouse Models, Review

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