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RUNX1 Repression-Independent Mechanisms of Leukemogenesis by Fusion Genes *CBFB–MYH11* and *AML1–ETO* (*RUNX1–RUNX1T1*)

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ABSTRACT

The core binding factor (CBF) acute myeloid leukemias (AMLs) are a prognostically distinct subgroup that includes patients with the inv(16) and t(8:21) chromosomal rearrangements. Both of these rearrangements result in the formation of fusion proteins, *CBFB–MYH11* and *AML1–ETO*, respectively, that involve members of the CBF family of transcription factors. It has been proposed that both of these fusion proteins function primarily by dominantly repressing normal CBF transcription. However, recent reports have indicted that additional, CBF-repression independent activities may be equally important during leukemogenesis. This article will focus on these recent advances. J. Cell. Biochem. 110: 1039–1045, 2010. Published 2010 Wiley-Liss, Inc.†

KEY WORDS: CBFB; RUNX1; AML1–ETO; CBFB–MYH11; AML

The CBF family is composed of four proteins, the three α subunits, RUNX1 (AML1, Cbfa2), RUNX2 (Cbfα1), and RUNX3 (Cbfα3) [Ogawa et al., 1993b], and the single β subunit, CBFβ [Wang et al., 1993; Ogawa et al., 1993a]. Disruptions of both CBFβ and RUNX1 are associated with acute myeloid leukemia (AML). CBFβ is involved in the recurrent chromosomal abnormality inv(16)(p13q22) as well as the less common t(16;16)(p13q22) translocation, both of which create a fusion between the *CBFB* gene on 16q22, and *MYH11* on 16p13, the gene that encodes smooth muscle myosin heavy chain (SMMHC) [Liu et al., 1993]. The resulting *CBFB–MYH11* fusion gene, which encodes the oncoprotein CBFβ–SMMHC, is found in nearly all patients with French–American–British (FAB) classification subtype M4 with eosinophilia (M4Eo) AML [Le Beau et al., 1983; Liu et al., 1995]. *RUNX1* is involved in the t(8;21) translocation that results in a fusion between *RUNX1* and the gene for an E-box family protein, *ETO* (*RUNX1T1*, *MTG8*), to generate *AML1–ETO* (*RUNX1–RUNX1T1*) [Erickson et al., 1992], which is associated with AML subtype M2 [Rowley, 1973]. Together, the inv(16)(p13q22) and t(8:21) translocations account for approximately 20–25% of adult AML [Speck and Gilliland, 2002], making *RUNX1* and *CBFB* the most commonly targeted genes in human AML. In addition, point mutations in *RUNX1* have been found in families with a familial platelet disorder with predisposition to AML [Minelli et al., 2004; Osato, 2004] and in patients with de novo AML, particularly among those with subtype M0 [Roumier et al., 2003; Osato, 2004]. Gene expression profiling also indicates that *RUNX1* inactivation is associated with a distinct M0 subgroup [Silva et al., 2009; Tang et al., 2009].

CBFβ and RUNX1 form a heterodimer and together they bind to the consensus TGTGGT DNA sequence and regulate gene expression. The RUNX1 protein contains a conserved RUNT homology domain (RHD), which is responsible for binding DNA and CBFβ [Speck and Gilliland, 2002]. CBFβ does not bind DNA directly but stabilizes the RUNX1–DNA interaction allosterically [Tang et al., 2000] and protects RUNX1 from ubiquitination and degradation [Huang et al., 2001]. Both RUNX1 and CBFβ are master regulators of definitive hematopoiesis.

It is thought that both CBFβ–SMMHC and AML1–ETO function by dominantly repressing normal CBFβ/RUNX1 heterodimer activity. Based on this model of dominant repression, the development of new therapies for CBF leukemias has focused on disrupting this activity. However, recent work indicates that these fusion proteins may have gain-of-function activities as well, which could represent additional targets for future drug discovery. In this

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article we will review the relevant literature establishing the dominant negative model, as well as highlight recent reports that challenge this model.

MECHANISMS OF CBFβ–SMMHC INDUCED LEUKEMOGENESIS

Initial studies of *Cbfb–MYH11* in mice suggest a dominant repression model. Mice heterozygous for a knocked-in *Cbfb–MYH11* fusion allele (*Cbfb*$^{+/MYH11}$) have a nearly identical phenotype [Castilla et al., 1996] as mice null for either *Cbfb* (*Cbfb*$^{-/-}$) or *Runx1* (*Runx1*$^{-/-}$) [Okuda et al., 1996; Sasaki et al., 1996; Wang et al., 1996a,b; Niki et al., 1997; Okada et al., 1998], which includes embryonic lethality from massive hemorrhaging and a complete block in definitive hematopoiesis. Subsequent in vitro studies indicate that the fusion protein CBFβ–SMMHC has a higher affinity for RUNX1 than endogenous CBFβ [Lukasik et al., 2002]. The N-terminus of the fusion protein retains the RUNX1 dimerization residues from CBFβ, but CBFβ–SMMHC also contains a second RUNX1 high-affinity binding domain (HABD) located at the proximal end of SMMHC [Lukasik et al., 2002] (Fig. 1A). As a result, CBFβ–SMMHC binds RUNX1 at two sites and can outcompete CBFβ for RUNX1 binding. After preferentially binding RUNX1, it has been proposed that CBFβ–SMMHC represses RUNX1 transactivation by a number of different mechanisms, including sequestration to the cytoplasm [Adya et al., 1998], and recruitment of transcriptional repressors by the SMMHC tail [Lutterbach et al., 1999].

The HABD is predicted to be important for leukemogenesis by CBFβ–SMMHC if dominant repression of RUNX1/CBFβ is a critical step for leukemia development. To test this hypothesis, we generated knockin mice expressing a mutant *Cbfb–MYH11* allele (*Cbfb–MYH11*$_{d179-221}$, expressing CBFβ–SMMHC$_{d179-221}$, Fig. 1B) in which the HABD (aa 179–221) is deleted [Kamikubo et al., 2010]. As expected, this allele had reduced repression of *Cbfβ/RUNX1* functions as evidenced by in vitro studies as well as partial rescue of the embryonic lethality and definitive hematopoiesis blockage phenotypes in the *Cbfb*$^{+/MYH11}{}_{d179-221}$ embryos. Surprisingly, the decreased repression of Runx1 did not correlate with reduced or delayed leukemogenesis. Mice carrying the *Cbfb–MYH11*$_{d179-221}$ allele developed leukemia faster than those expressing full-length *Cbfb–MYH11*. Furthermore, we found that expression of *Cbfb–MYH11*$_{d179-221}$ induced clonal expansion of human CD34^{+} cells with a similar efficiency as full-length *Cbfb–MYH11*. Taken together, these results indicate that the HABD in CBFβ–SMMHC is not required for leukemogenesis, implying that dominant repression of RUNX1 may not be as central to CBFβ–SMMHC's oncogenic activity as previously believed.

Consistent with these findings is the observation that the so-called type I *CBFB–MYH11* fusion, detected in a small percentage of inv(16) AML patients, produces a CBFβ–SMMHC fusion protein that lacks the HABD and a significant portion of the C-terminal segment of CBFβ (Fig. 1C) [Dissing et al., 1998; Van der Reijden et al., 2001]. Consequently, the type I fusion protein has very low binding affinity for RUNX1 [Kamikubo et al., 2010]. The clinical course and the characteristics of leukemia with the type I fusion are indistinguishable from those with longer forms of the fusion protein, further indicating that dominant repression of RUNX1 is not strictly required for CBFβ–SMMHC to induce leukemia.

A corollary implication of this conclusion is that CBFβ–SMMHC has activities not directly related to RUNX1 repression. In fact, we have recently shown that, in primitive blood cells, which are mostly nucleated erythrocytes that arise from the initial wave of embryonic hematopoiesis, *Cbfb–MYH11* blocks differentiation through a *Cbfb/Runx1*-repression-independent mechanism [Hyde et al., 2009]. Primitive blood cells from *Cbfb*$^{+/MYH11}$ embryos have the histological appearance of more immature precursor cells [Castilla et al., 1996], as well as continued expression of genes associated with early progenitor or stem cells, as detected by microarray analysis [Hyde et al., 2009]. Primitive blood cells from neither *Cbfb*$^{-/-}$ nor *Runx1*$^{-/-}$ embryos showed significant differentiation

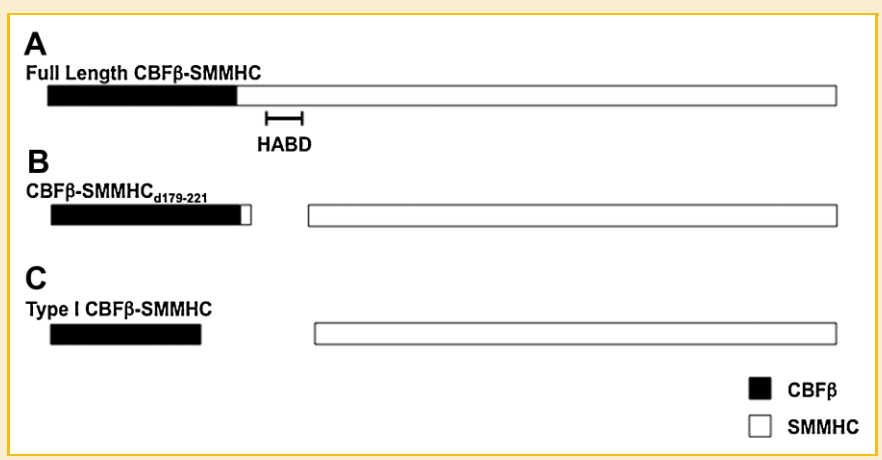

Fig. 1. Diagrammatic representation of CBFβ–SMMHC variants. Schematic of (A) full-length CBFβ–SMMHC, (B) the CBFβ–SMMHC$_{d179-221}$ deletion mutant, and (C) the type I CBFβ–SMMHC fusion. The CBFβ and SMMHC are represented as black and white boxes, respectively. The high-affinity binding domain (HABD) is indicated.

defects, indicating that loss of *Cbfb/Runx1* activity is not responsible for the *Cbfb–MYH11*-induced block in differentiation. Therefore, the fusion gene must have additional, gain-of-function activities.

Interestingly, many of the genes whose expression was deregulated in the $Cbfb^{+/MYH11}$ embryos via this novel activity were also found expressed in leukemic cells from mice and humans. In the case of the mouse leukemias, this gene set was expressed equally in cells from mice with the full-length *Cbfb–MYH11* allele or the $Cbfb\text{–}MYH11_{d179\text{–}221}$ deletion mutant (R.K.H., Y.K., P.P.L., unpublished results). This finding implies that the *Cbfb/Runx1* repression-independent activity described during primitive hematopoiesis is likely involved in *Cbfb–MYH11*-induced leukemogenesis as well.

The mechanism for this novel activity can only be speculated at present. One hypothesis is that CBFβ–SMMHC binds RUNX1 but does not repress its activity. Rather, perhaps through the recruitment of co-factors by the SMMHC tail, the fusion protein changes RUNX1 target gene specificity or transactivation ability. A second possibility is that CBFβ–SMMHC has activities that are completely independent of RUNX1 association, probably mediated by the SMMHC tail. Little is known about the interactions of the SMMHC tail in vivo, and it is conceivable that as yet unknown factors interact with CBFβ–SMMHC and contribute to leukemogenesis.

While the above-described observation indicate that CBFβ–SMMHC has important oncogenic activities independent of RUNX1 repression, it should not be concluded that inactivation of the CBFβ/RUNX1 heterodimer does not also contribute to leukemogenesis. Mice with one *Cbfb–MYH11* knockin allele, and one *Cbfb* null allele ($Cbfb^{-/MYH11}$) show accelerated development of leukemia as compared to $Cbfb^{+/MYH11}$ mice [Heilman et al., 2006]. On the other hand, *Cbfb–MYH11* knockin mice with *Runx1* mutations developed leukemia at rates inversely correlating with the severity of *Runx1* loss (L.Z., PPL, unpublished results). A possible interpretation of these findings is that CBFβ–SMMHC competes with CBFβ for leukemogenesis while partial inhibition of RUNX1 is more leukemogenic than complete RUNX1 inhibition. Of note PU.1 contribution to leukemogenesis is similarly dose dependent; mice carrying hypomorphic alleles of *Pu.1* with reduced expression (20% of normal) developed AML rapidly and efficiently, while mice with homo- or heterozygous deletion of *Pu.1* did not develop leukemia [Rosenbauer et al., 2004]. At present, it is not possible to weigh the relative importance of the CBF-repression-dependent and -independent activities. It seems likely that both pathways contribute substantially to the oncogenic effects of CBFβ–SMMHC, and consequently, could be important targets for the development of new treatments for $inv(16)^{+}$ leukemia.

MECHANISMS OF AML1–ETO-INDUCED LEUKEMOGENESIS

The fusion protein resulting from the t(8;21) translocation, AML1–ETO, contains the N-terminal region of RUNX1 which includes the DNA and CBFβ binding RHD, joined to nearly the entire ETO protein (Fig. 2A). ETO is a member of the E-box family of transcriptional factors and contains four conserved Nervy homology regions (NHR). The ETO NHR domains have been shown to interact with a number of transcriptional repressors, including N-CoR, SMRT, Sin3A, and HDAC1-3 [Peterson and Zhang, 2004]. Based on the structure of the AML1–ETO protein, it has been proposed that it functions through repression of RUNX1 target genes. Because the fusion protein retains the intact RHD, it was originally presumed to share many of the same target genes as the endogenous RUNX1. However, due to the NHR domains of the ETO portion, AML1–ETO has been considered a transcriptional repressor rather than an activator. Consistent with this model, it has been shown that AML1–ETO represses expression of the tumor suppressor *p14ARF*, which is normally activated by RUNX1 [Linggi et al., 2002]. Through recruitment of chromatin

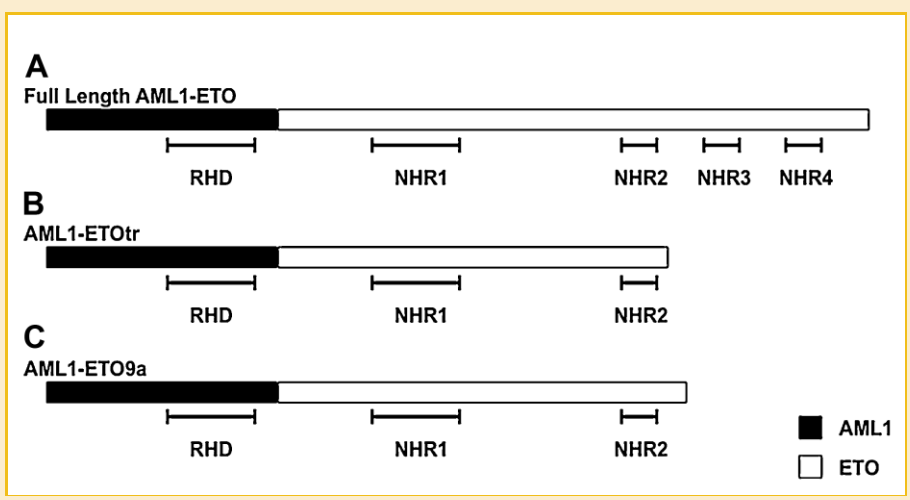

Fig. 2. Diagrammatic representation of AML1–ETO constructs. Schematic of (A) full-length AML1–ETO, (B) the AML1–ETO truncation mutant, and (C) the naturally occurring AML1–ETO9a isoform. The RUNX1 and ETO domains are represented as black and white boxes, respectively. The RUNT homology domain (RHD) and Nervy homology regions (NHR) are indicated.

remodeling proteins, AML1–ETO has also been shown to repress expression of the microRNA miR-223, a potential effector of the AML1–ETO-induced block in differentiation [Fazi et al., 2007]. In addition, as in the case of CBFβ–SMMHC, mice expressing a knockin allele of AML1–ETO [Yergeau et al., 1997; Okuda et al., 1998] have the same phenotype of embryonic lethality and block in definitive hematopoiesis as the *Runx1*$^{-/-}$ and *Cbfb*$^{-/-}$ mice [Okuda et al., 1996; Sasaki et al., 1996; Wang et al., 1996a,b; Niki et al., 1997; Okada et al., 1998], which is consistent with *RUNX1–ETO* having dominant repressor activities.

Despite the attractiveness of this model, there is increasing evidence that AML–ETO-mediated leukemogenesis is more complex than simple repression of RUNX1 target genes. AML1–ETO has also been shown to effect activation of some target genes, such as *p21* [Peterson et al., 2007b], *BCL-2* [Klampfer et al., 1996], and the differentiation blocking microRNA, miR-24 [Zaidi et al., 2009], as well as regulate genes that are not targets of endogenous CBFβ/RUNX1 [Shimada et al., 2000; Gardini et al., 2008]. Consistent with these findings, it has been demonstrated that AML1–ETO, but not RUNX1, preferentially binds promoters with duplicated RUNX1 consensus sites [Okumura et al., 2008]. In addition, immunofluorescent staining of Kasumi-1 cells, a cell line derived from an AML1–ETO^{+} AML patient, shows that RUNX1 and AML1–ETO are associated with different chromosomal regions [Bakshi et al., 2008], implying that AML1–ETO regulates different target genes than RUNX1.

Given these findings, it is perhaps not surprising that multiple studies have shown that DNA binding by AML1–ETO is required for leukemogenesis [Kwok et al., 2009; Roudaia et al., 2009; Yan et al., 2009]. However, whether interaction with CBFβ is also required has yet to be resolved. Using in vitro techniques, Matheny et al. [2007] identified point mutations in AML1–ETO (Y113A and T161A) that specifically disrupted CBFβ binding without affecting DNA binding. These point mutants were combined, expressed in mouse bone marrow (BM) cells, and transplanted into recipient mice. Unlike the wild-type AML1–ETO, the mutant AML1–ETO (Y113A/T161A) did not induce leukemia in cooperation with TEL-PDGFβR [Roudaia et al., 2009], indicating that Cbfβ binding is required for leukemogenesis.

In contrast, Kwok et al. [2009] tested two different point mutants of AML1–ETO (M106V and A107T) that by immunoprecipitation and Western blot showed severely reduced CBFβ binding. These constructs, when expressed in mouse hematopoietic cells, retained serial replating ability, similar to the wild-type AML1–ETO. In addition, they found that knockdown of *Cbfb* by short hairpin RNA (shRNA) did not effect AML–ETO's serial replating ability. From these results, the authors concluded that interaction with Cbfβ is dispensable for AML–ETO's leukemogenic activity.

One possible explanation for these contradictory results is that serial replating ability may not precisely correlate with leukemogenic potential. Cbfβ binding may not be required for the former, but still required for the latter. In addition, it may be that AML–ETO can function properly with a very minimal amount of Cbfβ binding. The M106V and A107T mutants [Kwok et al., 2009] may weakly associate with Cbfβ, such that it was barely detectable by immunoprecipitation [Fig. 1D, in Kwok et al., 2009], but would be enough to stabilize AML1–ETO's DNA binding. Follow-up studies by Park et al. [2009] are consistent with this possibility. Similarly, shRNA knockdown of Cbfβ may not have been complete, and the remaining Cbfβ contributed to the serial replating activity. Further experimentation will be needed to clarify the role of Cbfβ in leukemia induction by AML1–ETO. Because this interaction has been proposed as a target for the development of new therapies, resolution of this issue could have important consequences.

AML1–ETO's repression of RUNX1 target gene expression has also been questioned by recent findings indicating that recruitment of co-repressors by the ETO domain may not be required for leukemogenesis. Deletion mutants of the ETO co-repressor binding NHR domains have shown that NHR1, 3, and 4 are dispensable for leukemogenesis [Kwok et al., 2009; Yan et al., 2009]. In addition, it has been shown that loss of NHR3 and 4 either in a truncation mutation (Fig. 2B) [Yan et al., 2004] or in a naturally occurring splice isoform (AML1–ETO9a) (Fig. 2C) [Yan et al., 2006] results in accelerated leukemogenesis. These findings indicate that, rather than contributing to leukemogenesis, NHR3 and 4 actually inhibit the oncogenic activity of AML–ETO.

These findings raise interesting questions as to the relevance of the multiple other AML–ETO isoforms expressed in patient samples. In addition to the AML1–ETO9a isoform described above, nine other isoforms have been described in patients or cell lines [Peterson et al., 2007a]. Often, multiple isoforms are found in a single sample. It will be interesting to determine the relative leukemic potential of the various isoforms, and if their differential expression has any correlation with prognosis.

MECHANISTIC HINTS FROM POINT MUTATIONS IN RUNX1

To date, much of the research on CBF leukemias has centered on the assumption that RUNX1 directly binds the promoters of target genes in order to regulate their expression. However, there is increasing evidence that RUNX1 has DNA-binding-independent activities. In some instances, RUNX1 may be recruited to the promoters of target genes through protein–protein interactions with other transcription factors [Pabst et al., 2001; Wheeler et al., 2002]. Recently, Cammenga et al. [2007] reported that point mutations in the RHD of RUNX1 found in patients with AML subtype M0 led to a gain-of-function activity for the RUNX1 protein. When these RHD mutants, which are not capable of binding DNA, were expressed in murine BM cells, they led to an increase in serial replating efficiency and the accumulation of cells with a blast-like morphology, similar to that seen with AML1–ETO. Interestingly, it was found that CBFβ interaction was not required for this activity. Although loss of *RUNX1* had similar effects on serial replating as expression of the RHD mutants, it did not readily lead to immortalization of BM cells, indicating that the RHD mutants have a gain-of-function activity through a DNA-binding-independent mechanism. From these observations, the authors argue that normal hematopoiesis requires a balance between RUNX1's DNA-binding-dependent and -independent activities, and that disruption of this balance leads to leukemogenesis.

This model could potentially apply to both CBFβ–SMMHC and AML1–ETO. In the case of AML1–ETO, it is clear that binding DNA is required for its leukemic activity [Kwok et al., 2009; Roudaia et al., 2009; Yan et al., 2009]. However, it is not known if the fusion protein affects RUNX1's DNA-binding-independent functions, thus upsetting the balance between the two activities. Interestingly, it was recently shown by chromatin immunoprecipitation that AML1–ETO is associated with promoters lacking a known RUNX1 binding site, but enriched for sites of other hematopoiesis-related transcription factors [Gardini et al., 2008]. This finding is consistent with the possibility that AML1–ETO can form complexes with other transcription factors that provide the DNA-binding activity and target gene specificity.

FINAL THOUGHTS

With the development of imatinib for the treatment of chronic myeloid leukemia (CML) in patients with the BCR-ABL translocation [Druker et al., 1996, 2001a,b], much attention has been focused on the development of drugs that specifically target the fusion proteins arising from other recurrent chromosomal abnormalities. However, the development of such drugs depends on a clear understanding of the molecular mechanisms of these oncogenes. In the case of the CBF leukemias, recent findings have indicated that the activity of these fusion proteins is more complex than originally thought. Both CBFβ–SMMHC and AML1–ETO appear to repress transcription of some CBFβ/RUNX1 target genes but also activate transcription of an alternate set of target genes. The identity of the genes in this alternate set as well as the co-factors involved in activating their transcription have yet to be determined. However, this line of inquiry promises to yield important insights into the oncogenic mechanism of both fusion proteins, and ultimately, the development of new therapies for inv(16) and t(8:21) leukemia.

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