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RUNX1 mutations in clonal myeloid disorders: from conventional cytogenetics to next generation sequencing, a story 40 years in the making

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Abstract

Translocations and mutations in the core binding factor genes, *RUNX1* or *CBFB*, are found in acute myeloid and lymphocytic leukemia, therapy-related myeloid leukemia, myelodysplastic syndrome, chronic myelomonocytic leukemia, and in familial platelet disorder with predisposition to acute myeloid leukemia. Here we review the biochemical and biological properties of the normal Runx1 protein, discuss the nature of *RUNX1* mutations in myeloid leukemia, their prognostic significance, and the mutations that cooperate or co-exist with them in these various diseases.

Keywords

Runx1; AML; MDS; Hematopoietic Stem Cells

I. INTRODUCTION

In 1973 Janet Rowley, using new chromosome banding techniques, identified a reciprocal translocation between chromosomes 8 and 21 in two female patients with acute myeloid leukemia (AML).[1] That same month, Rowley published another paper demonstrating that the end of the long arm of chromosome 22 thought to be missing in the Philadelphia chromosome, a cytogenetic abnormality frequently associated with chronic myeloid leukemia, had in fact not been lost but was instead relocated to the end of chromosome 9.[2] These contemporaneous papers established that consistent chromosomal translocations could be correlated with specific leukemia subtypes. Eighteen years later the Acute Myeloid Leukemia 1 (AML1) residing at the breakpoint in t(8;21)(q22;q22) was cloned,[3] and later renamed *RUNX1*.[4] Hence the discovery of *RUNX1* mutations was an important milestone in the history of cancer genetics. Janet Rowley's keen powers of observation and intuition that translocations were causative in leukemia, and not simply correlative, begat an era of intensive research in cancer genetics, which may have reached its zenith with the application of next generation sequencing technology.

Fast forward nearly 40 years, and we now know much about the *RUNX1* gene and its encoded protein. Runx1 is sequence-specific DNA binding protein, and has an obligate non-

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DNA binding partner called core binding factor β (CBFβ), the gene for which is also targeted by translocations important in AML, the inv(16)(p13;q22) and t(16;16)(p13;q22).[5] AML with any of these three translocations is often referred to as "core binding factor leukemia". Runx1 has essential functions in normal hematopoiesis in the embryo and the adult. In addition to the t(8;21), translocations and mutations in *RUNX1* have been found in *de novo* and therapy-related AML, myelodysplastic syndrome (MDS), chronic myelomonocytic leukemia (CMML), acute lymphocytic leukemia (ALL), and in the autosomal dominant pre-leukemia syndrome familial platelet disorder with predisposition to acute myeloid leukemia (FPD/AML).

II. THE RUNX1 PROTEIN - DOMAIN STRUCTURE AND FUNCTION, AND INTERACTING PROTEINS

Runx1 is a sequence-specific DNA binding protein, and like most proteins of its ilk contains effecter domains linked by less structured sequences. By far the most well characterized domain of Runx1 is its DNA binding "Runt" domain, named after the first member of the family to be cloned, the *Drosophila* runt protein.[6; 7] (Figure 1) Multiple structures of the Runt domain have been solved.[8–11] The DNA and CBFβ interacting interfaces are on opposite sides of the Runt domain and do not overlap, and CBFβ does not touch the DNA. The primary role of CBFβ is to increase binding of Runx1 to DNA by stabilizing a particular conformation of the Runt domain.[11; 12] As will be described later, many missense mutations in the Runt domain have been identified in AML, MDS, CMML, and FPD/AML, the vast majority of which involve residues at the DNA binding interface.

The second most well-characterized domain in Runx1 is the transactivation domain, which is located midway between the Runt domain and the C-terminus, and is essential for Runx1's *in vivo* functions.[13–16] No structures of this domain have been solved, although computational analysis[17] predicts that parts of the transactivation domain and an adjacent inhibitory domain are likely to be structured. Multiple proteins have been identified that interact with sequences C-terminal to the Runt domain that presumably mediate its activities.[18–28] Mutations C-terminal to the Runt domain are also found in leukemia, and are primarily nonsense or frameshift mutations that result in the production of proteins lacking all or part of the transactivation domain. A few missense mutations have also been found, but their functional significance has not been established.[29; 30]

Less well-characterized sequences in the C-terminus of Runx1 affect Runx1's DNA binding potential. Specifically, deletion of C-terminal sequences causes Runx1 to bind DNA with an affinity approximately 40 fold greater than that of the full-length protein.[14; 31] Therefore Runx1 proteins lacking the inhibitory sequences can presumably out-compete binding of the functional full-length protein to DNA, and dominantly inhibit its activity. For simplicity's sake we will use the term "Runx1 mutations" to refer to all mutations other than translocations, including loss of function (amorphic) mutations, hypomorphic mutations, and antimorphic mutations that create dominant negative *RUNX1* alleles.

III. RUNX1 FUNCTION IN NORMAL HEMATOPOIESIS

Runx1's earliest role in development is for the differentiation of hematopoietic progenitors and stem cells (HSCs) from a small population of endothelial cells in the conceptus.[32–34] Because mutations in the germline caused mid-gestation lethality, conditional deletion strategies were necessary to ascertain its role in adult hematopoiesis. Deletion of Runx1 in adult HSCs caused multi-lineage blocks in B and T lymphoid development and megakaryocyte maturation, and thus the mice are lymphopenic and thrombocytopenic.[35–37] Notably, Runx1 loss in HSCs does not cause AML on its own, but establishes a pre-

leukemic state that predisposes to AML following the acquisition of secondary mutations.[38; 39] The effects of Runx1 loss on HSCs and progenitors are not entirely understood. One outcome is an increase in a population of cells in the mouse bone marrow that lacks lineage markers and expresses the HSC markers Sca-1 and c-Kit (LSK cells).[32; 35; 36; 38; 39] Runx1 loss also increases the number of granulocyte and megakaryocyte progenitors in the bone marrow and causes what has been alternatively described as a myeloproliferative disease or myelodysplasia,[35; 37] and in one study a lower penetrance lymphoma was noted.[37] Runx1 loss does not, however, cause a notable decline in functional long-term repopulating HSCs,[39; 40] which is probably a critical property contributing to the pre-leukemic state, as a mutant HSC that is rapidly lost from the bone marrow cannot provide a target population for secondary mutations. Runx1 loss enhances the ability of mouse hematopoietic progenitors to undergo serial replating in culture,[36] which is regarded as a measure of self-renewal activity and may also contribute to the maintenance of a dysfunctional progenitor population.

IV. RUNX1 MUTATIONS IN AML

A. t(8;21)

AML can be subdivided into several subtypes including AML with recurrent cytogenetic abnormalities, AML with multi-lineage dysplasia (this includes patients with an antecedent MDS or myeloproliferative disease), AML and MDS therapy related (following chemotherapy or radiation exposure), and AML not otherwise categorized.[41]. Translocations or loss of function *RUNX1* mutations have been found in all of these subtypes.

Characteristic genetic abnormalities include the t(8;21) and inv(16) in *RUNX1* and *CBFB*, respectively, each of which defines subgroups within the category of recurrent cytogenetic abnormalities, and confer a favorable prognosis. The t(8;21) breaks the *RUNX1* gene in intron 5, and results in fusion of the N-terminal portion of Runx1 (including the Runt domain, but minus the transactivation domain) to a protein most commonly known as ETO (encoded by *RUNX1T1*) (Figure 1)..[3; 42–45] ETO contains four domains conserved with its *Drosophila* homologue nervy, the structures for all of which, along with their interacting proteins, have been solved.[46–51] Mutations that specifically disrupt the interaction between individual domains in ETO and their associated proteins revealed that one domain in particular, nervy homology region 2 (NHR2, also known as hydrophobic heptad repeat or HHR) is critical for AML1-ETO's leukemogenic activity in retroviral transduction based assays.[52; 53] NHR2 forms a four-helix bundle (a dimer of dimers), and mutations that reduced the tetramer to dimer abrogated AML1-ETO's leukemogenic activity.[53] That oligomerization of AML1-ETO *per se* was important was demonstrated by the ability of an oligomerization domain from the forkhead binding protein to substitute for NHR2 and enable AML1-ETO to confer serial replating activity to primary bone marrow cells.[54]

On the other hand, the most C-terminal domain, NHR4, also known as the myeloid-Nervy-DEAF-1 (MYND), appears to restrain AML1-ETO's leukemogenic activity, as mutations that severely disrupt the NHR4 fold promote AML1-ETO's activity.[55] In fact, the full-length unaltered form of AML1-ETO is not by itself leukemogenic, and can only cause AML in mice when combined with another oncogene such as Fms-like tyrosine kinase 3 internal tandem duplication *(FLT3-ITD)*, *TEL-PDGFBR*, or activated *KIT*.[56–58] But either the deletion or mutation of NHR4 will allow AML1-ETO to induce leukemia in the absence of a co-transduced oncogene.[55] NHR4 binds the silencing mediator of retinoid and thyroid hormone receptor (SMRT) and nuclear receptor co-repressor (N-CoR) complexes, as well as a protein called SON, and presumably one or more of these complexes is responsible for dampening AML1-ETO's activity.[55; 59–61] Mutations involving NHR4 have not been found

in t(8;21) leukemia, thus this does not appear to represent a common mechanism for augmenting AML1-ETO's activity.[62]

Deletions of the NHR1 domain have yielded conflicting results, with one group reporting an effect on AML1-ETO's activity[63] and others not.[52; 54] The NHR1 deletion in the former study[63] was larger than in the latter two studies,[52; 54] and included sequences C-terminal to the conserved NHR1 domain that were previously shown to bind N-CoR and to contribute to leukemogenesis.[52; 64] The larger deletion prevented association with p300 and acetylation of two lysine residues within the Runx1 portion of the AML1-ETO protein.[63] Substituting one of those lysines with an arginine impaired AML1-ETO's leukemogenic activity, indicating that the ability to recruit p300 and become acetylated is important for AML1-ETO function.[63]

The two other interactions mediated by AML1-ETO that are essential for its activity are DNA and CBFβ binding by the Runt domain, although there is some disagreement about the importance of the latter.[52; 54; 65–67] Thus AML1-ETO has several surfaces amenable to targeting with small molecule or peptide inhibitors: the Runt domain:DNA interface, potentially the Runt domain:CBFβ interface, and NHR2 oligomerization. Inhibition of p300 or other histone acetylases may also provide a therapeutic option.[63]

Multiple lines of evidence indicate that the acquisition of AML1-ETO confers a different phenotype than Runx1 mutations. Genetic experiments in *Drosophila* showed that AML1-ETO behaves as a constitutive repressor, blunting the transcription of genes the Runx1 homologue lozenge activates, but additionally those that lozenge would normally repress.[68] The phenotype of conditional knock-in mice in which AML1-ETO expression is activated in the adult bone marrow resembles a somewhat milder version of Runx1 loss, as the mice had no evidence of lymphopenia or thrombocytopenia.[69] AML1-ETO conditional knock-in mice did, however, display some of the aberrations in progenitors seen upon Runx1 loss, including increased numbers of granulocyte-monocyte (GM) progenitors and enhanced serial replating activity. AML1-ETO in the conditional knock-in mice cooperated with the HIP1-PDGFBR oncogene to induce a very rapid myeloproliferative disease (MPD) that was not observed with either mutation alone.[70] As hematopoietic differentiation was not impeded in this AML1-ETO knock-in mouse model, this suggests that defects in the stem/progenitor pool caused by AML1-ETO are fundamental to the leukemic process. The phenotypes in mice correlate with the leukemic phenotypes, as AML1-ETO is found in the French-American-British (FAB) M2 subtype, also known as acute myeloblastic leukemia with maturation, whereas biallelic *RUNX1* mutations have been found in minimally differentiated acute myeloblastic leukemia, AML M0. Finally, as discussed in more detail below, *RUNX1* mutations confer a considerably worse prognosis than the t(8;21).

The t(8;21) is the most common translocation in pediatric AML patients (10%–20%).[71–74] One study documented a prenatal origin of the t(8;21) from the Guthrie cards of half of the pediatric patients analyzed.[75] Two of the positive patients were between 10–12 years of age at the time of diagnosis, therefore harbored a pre-leukemic clone for more than a decade before developing AML. Patients in long-term remission can also harbor residual t(8;21)-containing cells in their bone marrow for many years.[45; 76] Eighteen percent of healthy individuals have t(8;21) containing cells detectable by polymerase chain reaction, and AML1-ETO transcripts were detected in 40% of cord blood samples.[77; 78] Thus the t(8;21) results in the acquisition of a long-lived pre-leukemic HSC that has no overt clinical manifestations.

The mutations that cause AML are often divided into two classes: class I mutations which activate signaling pathways, hence proliferation and survival, and class II mutations that

generally involve transcription factors and cause impaired differentiation, decreased apoptosis, and growth arrest. Another class of frequently mutated genes encodes epigenetic regulators. The class I mutations most frequently found in t(8;21) AML include *KIT, NRAS*, and *KRAS*. About 20% of t(8;21) patients have activating mutations in *KIT*, and exon 17 mutations have been found to confer an unfavorable prognosis in multiple studies.[79–81] *FLT3* is the most commonly mutated gene in AML, but *FLT3* mutations occur at a relatively low rate in t(8;21) leukemia.

B. AMORPHIC AND ANTIMORPHIC RUNX1 MUTATIONS IN AML

RUNX1 mutations were first described in AML M0 and FPD/AML,[82; 83] followed shortly thereafter in MDS,[84] and more recently in CMML.[85; 86] More recent larger-scale sequencing efforts are providing a more comprehensive picture of the frequency and scope of *RUNX1* mutations, their prognostic significance, and the co-existing mutations.

Two groups recently analyzed large numbers of AML patients for the presence of *RUNX1* mutations.[29; 30] A report from the German-Austrian AML study group, which evaluated 18 to 60 year old AML patients (primarily *de novo* AML, but including a smaller number of secondary and therapy-related AML patients) found *RUNX1* mutations in 53 of 945 (5.6%) cases.[29] An earlier study of an older 15- to 90-year old Taiwanese patient population with *de novo* AML reported a higher incidence of *RUNX1* mutations (13.2%, 62 of 470 patients).[30] It was suggested by authors of the former study that the higher frequency of *RUNX1* mutations in the Taiwanese study could be caused by their inclusion of older patients, as the mutation frequency increases with age. In both studies most *RUNX1* mutations were frameshift mutations, the remainder included missense, nonsense, in-frame, or silent mutations, and the vast majority were mono-allelic. The mutations were primarily located in the Runt domain and C-terminal to the Runt domain. Both groups reported *RUNX1* mutations were mainly found in the cytogenetic intermediate-risk group, and closely associated with trisomy 8. No *RUNX1* mutations were found in the favorable risk group with characteristic genetic abnormalities that include t(8;21) and inv(16). In univariate analyses *RUNX1* mutations were found to be associated with refractory disease and inferior event free, relapse free, and overall survival. Allogeneic hematopoietic stem cell transplant improved the outcome of patients with *RUNX1* mutations,[29; 30] while patients who instead received repetitive cycles of high-dose cytarabine or autologous hematopoietic stem cell transplant relapsed or died.[29] *RUNX1* mutations were associated with the presence of *MLL-PTD* mutations in both studies and *IDH1/IDH2* in one study,[29] but inversely correlated with *CEBPA* and *NPM1* mutations.[29; 30] No significant correlation was found with *FLT3, NRAS, KRAS, KIT, PTPN11, or WT1* mutations, despite the fact that several of these, and in particular *KIT* mutations are frequent in t(8;21) AML. Both groups found *RUNX1* mutations highly associated with AML M0; one group also reported association with M1[30] and the other with M2 morphologies.[29]

An analysis of 111 pediatric AML cases identified five with *RUNX1* mutations, in addition to twenty t(8;21) and sixteen inv(16) cases, bringing the total of core binding factor mutations in this pediatric AML cohort to 36.9%.[87] If one combines the frequencies of *RUNX1* mutations in adult AML (13% in the unselected Taiwanese study), with the t(8;21) (7% in a Cancer and Leukemia Group B (CALGB) study with a median age of 52; and inv(16) (8% in the same CALGB study),[88] this results in an overall frequency of core binding factor mutations in adult AML of approximately 28%. *RUNX1* mutations, t(8;21), and inversion 16 are mutually exclusive.

V. RUNX1 MUTATIONS IN MDS

MDS is a clonal stem cell disorder characterized by ineffective production of myeloid lineage cells with associated dysplasia that can involve one or more myeloid lineages. There are multiple subcategories of MDS, including refractory cytopenia with unilineage dysplasia, refractory anemia with ringed sideroblasts with associated thrombocytosis, refractory cytopenia with multilineage dysplasia, refractory anemia with excess blasts I and II, 5q- syndrome, myelodysplasia unclassifiable, and refractory cytopenia of childhood. Approximately one third of MDS patients will progress to AML over time. Fewer than half of MDS patients have chromosomal abnormalities, and balanced translocations are rare.

Nevertheless the first report of a *RUNX1* mutation in MDS was a balanced translocation, the t(3;21)(q26.2;q22).[89] However loss of function *RUNX1* mutations are far more common in MDS, and numerous reports have documented them.[84; 90; 91] At the time of writing the most recent report was a mutational screen in 439 MDS patients for a broad array of cancer-associated genes, in which mutations in *RUNX1* along with 17 other genes were identified.[92] *RUNX1* mutations were the third most frequent (8.7%), surpassed only by mutations in the epigenetic regulators *TET2* (20.5%) and *ASXL1* (14.4%). A multivariate analysis that included risk stratification using the International Prognostic Scoring System[93] showed that mutations in *RUNX1, ASXL1, TP53, EZH2*, and *ETV6* were independent predictors of poor overall survival in all but the highest risk category. Mutations in *RUNX1, TP53*, and *NRAS* correlated with severe thrombocytopenia and elevated blast counts, but not with neutropenia or anemia. Loss of function Runx1 mutations in mice affect megakaryocyte but not granulocyte or erythroid differentiation, consistent with the MDS phenotype seen in human patients with *RUNX1* mutations. A 13.8% frequency of *RUNX1* mutations was reported in an earlier study of 188 MDS + CMML patients.[94] Samples from MDS patients who progressed to secondary AML (s-AML) were analyzed for mutations at both stages.[94; 95] In most cases *RUNX1* mutations were present in both the MDS and s-AML samples, and in a smaller number of cases *RUNX1* mutations were found in the s-AML but not in the antecedent MDS. Thus *RUNX1* mutations are likely to be early events in many cases, but can also be later events in disease progression. Conversion from mono- to biallelic *RUNX1* mutations was also observed in several s-AML samples, either through acquisition of an independent mutation or uniparental disomy.[95]

In the fourteen samples in the Bejar et al.[92] study that had mutations in addition to *RUNX1*, they were most often in *TET2* (12), *ASXL1* (12), *EZH2* (8), and *NRAS* (6), and there was no overlap with mutations in *TP53, JAK2, ETV6, IDH1/2, NPM1, GNAS, BRAF, PTEN*, or *CDKN2A*. Thus although the types of mutations in *RUNX1* found in AML and MDS were similar, the cooperating mutations were distinct. In general, very few activated tyrosine kinases were identified in MDS, confirming previous hypotheses that MDS is generally associated with class II mutations and mutations in epigenetic regulators, and MPD with class I mutations.

An intriguing observation is that loss of function *RUNX1* mutations in MDS are highly correlated with previous exposure to radiation, both therapeutic and accidental, the latter in atomic bomb survivors and individuals who lived in close proximity to the Semipalatinsk nuclear test site in what today is Kazakhstan.[90; 96] The close association of *RUNX1* mutations with radiation suggest either that the *RUNX1* gene is particularly sensitive to DNA damage following radiation, or that preexisting *RUNX1* mutations may predispose patients to MDS following DNA damage.

RUNX1 mutations were also recently described in Fanconi anemia (FA) patients, who have a 30%–40% probability of developing MDS and AML by age 40.[97] A screen of 57 FA bone

marrows for chromosome copy number changes and mutations in commonly MDS/AML genes (*TET2*, *CBL*, *NRAS*, *TP53*, *RUNX1*, *CEBPA*, *NPM1*, *FLT3*, and *MLL*) found that the somatic acquisition of only three abnormalities correlated with MDS/AML in FA patients: 3q+, 7/7q−, and *RUNX1* translocations, deletions, and mutations.[98]

VI. RUNX1 MUTATIONS IN CMML

CMML has overlapping features of MDS and myeloproliferative neoplasms, including peripheral monocytosis > 1×10^9/L, <20% blood or bone marrow blasts, and bone marrow dysplasia in one or more myeloid lineage, and progresses to AML in 15–20% of patients. CMML is a relatively rare disease, thus sequencing studies of the scale described above for AML and MDS have not been performed. In a smaller scale analysis by the Munich Leukemia Group, mutations in *TET*, *CBL*, *NRAS*, *KRAS*, *JAK2*, *RUNX1*, and *MPL* were interrogated in 81 CMML samples.[99] The majority (72.8%) of CMML samples had a mutation in *TET*, *CBL*, *NRAS*, *KRAS*, *JAK2*, or *RUNX1*, with *RUNX1* mutations in 8.6% of patients. *RUNX1* mutations in this study were not found to be of prognostic relevance. Another analysis was performed in a Taiwanese population, and *RUNX1* mutations were found in 30/81 patients (37%). Both were unselected groups with a preponderance of elderly patients. In the Taiwanese cohort, there was a trend toward faster progression to AML in the *RUNX1* mutated group, which was especially pronounced when *RUNX1* mutations occurred in the C-terminus.[86]

VII. RUNX1 MUTATIONS IN FPD/AML

FPD/AML is an autosomal dominant disorder caused by mutations in *RUNX1*. Many but not all FPD/AML patients have low platelet counts or platelet activation defects.[100; 101] The penetrance of MDS/AML in FPD/AML patients is >40%, with a median age of incidence of 33 years.[102] Large intragenic deletions in *RUNX1* in FPD/AML established haploinsufficiency is one mechanism for the disease,[83] but mutations are also frequently found in the Runt domain. FPD/AML is clearly an intriguing syndrome and an improved understanding of the pathogenesis of MDS and AML in this disorder would seem to be key for unraveling the mechanisms underlying *RUNX1* mutant AML and MDS in general. However the ability to gain more insight into the pathogenesis of MDS/AML in FPD/AML has been hampered by small patient numbers and the heterogeneity of the disease presentation.

VIII. BIOCHEMICAL AND FUNCTIONAL ANALYSES OF RUNX1 MUTATIONS

The majority of *RUNX1* mutations can be categorized based on their potential impact on the protein (Table 1). These include : 1) large deletions; 2) mutations resulting in truncation within the Runt domain; 3) missense mutations in the Runt domain at the DNA interface that affect DNA but not CBFβ binding; 4) missense mutations in the Runt domain at the CBFβ interface that affect CBFβ but not DNA binding; 5) missense mutations in the Runt domain that affect both DNA and CBFβ binding through destabilizing the Runt domain fold; 6) mutations that truncate Runx1 C-terminal to the Runt domain and remove all or part of the transactivation domain; and 7) missense mutations C-terminal to the Runt domain (rare). It has been hypothesized that these various mutations would have different biological effects, with some behaving as loss of function (amorphic) mutations, others as hypomorphic mutations, and some as antimorphic mutations that could create dominant interfering Runx1 proteins.

Matheny *et al.*[103] compared different categories of missense mutations in the Runt domain using both biophysical and genetic approaches, and could confirm that mutations that perturbed CBFβ binding or the Runt domain fold resulted in hypomorphic *Runx1* alleles in

mice, while a mutation that severely impaired DNA but not CBFβ binding generated a weakly antimorphic allele. The mechanism for the antimorphic activity was not clear, but could involve sequestering a limiting protein with a Runx1:CBFβ heterodimer that cannot bind DNA. The majority of leukemia mutations in the Runt domain are at the DNA interface, indicating that severe disruption of Runx1 activity is more likely to be pathogenic.

Watanabe et al.[104] compared mutations in the Runt domain that affected DNA binding to a truncation C-terminal to the Runt domain that removed the transactivation domain by overexpressing the mutant proteins in a bone marrow transplant model. Both induced MDS, but with different properties, in that the DNA binding mutant caused leukocytosis while the C-terminal truncation mutant caused leukopenia. Thus, different mutations will indeed contribute different biological properties to the Runx1 protein, and presumably to disease phenotype.

Most *RUNX1* mutations are mono-allelic, and unfortunately a disease caused by mono-allelic mutations has been very difficult to model in the mouse. Although moderately (15%) decreased platelet counts were reported in mice haploinsufficient for *RUNX1*,[105] this was not reproduced in another lab,[103] potentially due to differences in genetic background of the mice. More pronounced thrombocytopenia was observed in mice homozygous for a hypomorphic *RUNX1* allele,[103] suggesting that reducing the effective dosage by more than 50% may provide a strategy for more faithfully modeling at least some aspects of *RUNX1* haploinsufficiency in human disease. Hence one of the more interesting unresolved questions is why mono-allelic *RUNX1* mutations would confer a more adverse phenotype than t(8;21)? One possible explanation is that mono-allelic *RUNX1* mutations tend to occur in older AML patients, who may have accumulated more cooperating mutations than younger patients with t(8;21).

IX. CONCLUSION

Patients with t(8;21) and inv(16) for the most part do well with standard induction and high dose cytarabine consolidation alone without the need for allogeneic stem cell transplantation. However even for these so-called favorable prognosis core binding factor leukemias, long-term leukemia-free survival is only 50%.[106] Prognosis for patients with *RUNX1* mutations, which typically fall into the intermediate risk cytogenetic categories (normal and non-complex), is even worse. Our increased knowledge of core binding factor mutations and function has not yet resulted in novel therapeutic approaches. This may be due, in part, to *RUNX1's* role as a class II mutation that may be involved in altering the expression of a multitude of target genes. This is illustrated by the recognition that t(8;21) AML is associated with its own unique gene methylation profile that is predictive of outcome.[107] Similarly, the presence or absence of a *RUNX1* mutation in AML M0 can be ascertained through the use of gene expression profiling, because *RUNX1* mutations result in altered expression of key target genes in a reproducible manner.[108] Given the multitude of potential Runx1 targets, one approach would be to focus on therapies that alter expression of many target genes simultaneously, as is the case for hypomethylating agents in MDS. A second approach would be to focus on combinatorial therapies, simultaneously targeting the class II effects of *RUNX1* and the class I mutations in tyrosine kinases that frequently accompany *RUNX1*, such as the *FLT3-ITD* or activated *KIT*. One such phase I trial is currently underway at MD Anderson Cancer Center, in which the hypomethylating 5-azacitadine is being tested in combination with the FLT3 inhibitor sorafenib in relapsed and refractory AML. A third approach would be to incorporate knowledge about specific targets of Runx1 in AML: a so-called "target the target" approach. For example, a recent study showed that *AKT3* and *RARA* are upregulated in AML M0,[108] providing a rationale for further investigation into the efficacy of small molecules targeting these pathways. Given

the difficulties in modeling leukemias with mono-allelic *Runx1* deficiency in the mouse (described earlier), more robust *Runx1* deficient leukemia models may need to be created by crossing *Runx1* haploinsufficient mice with mice carrying tyrosine kinase mutations that are known to cooperate with *Runx1*. Such improved models may ultimately allow us to better "target the target."

The incentive to engage in research with the goal of unlocking the secrets of the pathogenesis of AML with *RUNX1* mutations would also be increased if we had an improved understanding of the prognostic relevance of *RUNX1* mutations in specific therapeutic situations. How does *RUNX1* mutation status affect response to hypomethylating agents? FLT3 inhibitors? HDAC inhibitors? Therapy with high dose anthracyclines or high dose cytarabine? In MDS? In AML? These questions remain unanswered, as the prognostic relevance of *RUNX1* mutations have really only been examined on a global level. Our knowledge about the role of *RUNX1* in AML has clearly made great strides since Dr. Rowley identified the reciprocal translocation between chromosomes 8 and 21 nearly 40 years ago, but much remains undiscovered.

Abbreviations

ALL	acute lymphocytic leukemia
AML	acute myeloid leukemia
AML1	acute myeloid leukemia 1
CALGB	Cancer and Leukemia Group B
CBFB	Core Binding Factor Beta
CMML	chronic myelomonocytic leukemia
FAB	French-American-British
FLT3-ITD	Fms-like tyrosine kinase 3 internal tandem duplication
FPD-AML	familial platelet disorder with predisposition to acute myeloid leukemia
GM	granulocyte-monocyte
HHR	hydrophobic heptad repeat
HSCs	hematopoietic stem cells
MDS	myelodysplastic syndrome
MYND	myeloid-nervy-DEAF-1
N-CoR	nuclear receptor co-repressor
NHR1	nervy homology region 1
NHR2	nervy homology region 2
NHR4	nervy homology region 4
s-AML	secondary AML
SMRT	silencing mediator of retinoid and thyroid hormone receptor

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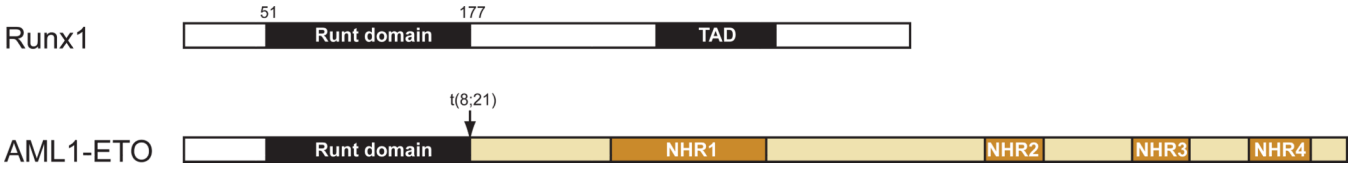

Figure 1.
Schematic diagram of Runx1 and AML1-ETO. White/black represent sequences from Runx1, and gold from ETO. TAD, transactivation domain ; NHR1-4, nervy homology domains 1–4.

Table 1

RUNX1 mutations in AML, MDS, and CMML, and FPD/AML

Mutation	Affects				Type
	DNA binding	CBFβ binding	Runt domain fold	Transactivation	
Large deletion	yes	yes	yes	yes	Amorphic
Truncation before or within Runt domain	yes	yes	yes	yes	Amorphic
Missense mutation in Runt domain at DNA interface[1]	yes	yes or no	yes or no	yes	Antimorphic or Amorphic
Missense mutation in Runt domain at CBFβ interface[2]	no	yes	no	yes	Hypomorphic
Missense mutation in Runt domain, not at DNA or CBFβ interface[2]	yes	yes	yes	yes	Hypomorphic
Truncation C-terminal to Runt domain	no	no	no	yes	Antimorphic
Missense mutation C-terminal to Runt domain[2]	no	no	no	yes[3]	Antimorphic[3]

[1] Common in leukemia. Mutations that affect DNA but not CBFβ binding result in antimorphic alleles, and are more common than those that affect both DNA and CBFβ binding.

[2] Rare in leukemia

[3] Presumed, not tested