RUNX1 DNA-Binding Mutants, Associated with Minimally Differentiated Acute Myelogenous Leukemia, Disrupt Myeloid Differentiation

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Abstract

Mutations in the *RUNX1* gene are found at high frequencies in minimally differentiated acute myelogenous leukemia. In addition to null mutations, many of the mutations generate Runx1 DNA-binding (RDB) mutants. To determine if these mutants antagonize wild-type protein activity, cDNAs were transduced into murine bone marrow or human cord blood cells using retroviral vectors. Significantly, the RDB mutants did not act in a transdominant fashion *in vivo* to disrupt Runx1 activity in either T-cell or platelet development, which are highly sensitive to Runx1 dosage. However, RDB mutant expression impaired expansion and differentiation of the erythroid compartment in which Runx1 expression is normally down-regulated, showing that a RDB-independent function is incompatible with erythroid differentiation. Significantly, both bone marrow progenitors expressing RDB mutants or deficient for *Runx1* showed increased replating efficiencies *in vitro*, accompanied by the accumulation of myeloblasts and dysplastic progenitors, but the effect was more pronounced in RDB cultures. Disruption of the interface that binds CBFβ, an important cofactor of Runx1, did not impair RDB mutant replating activity, arguing against inactivation of Runx1 function by CBFβ sequestration. We propose that RDB mutants antagonize Runx1 function in early progenitors by disrupting a critical balance between DNA-binding–independent and DNA-binding–dependent signaling. [Cancer Res 2007;67(2):537–45]

Introduction

The *RUNX1/AML1* gene encodes an important regulator of hematopoiesis and is the target of several genetic alterations during leukemogenesis. Its product is a DNA-binding protein that, together with a non-DNA-binding β subunit, forms a heterodimeric transcription factor termed the core-binding factor (CBF). CBF regulates transcription of several genes relevant to both myeloid and lymphoid development by associating with transcriptional cofactors, repressors, and other DNA-binding transcription factors in a promoter context-dependent fashion (1, 2). The conserved Runt homology domain at the NH_2 terminus of Runx1 is required for DNA binding and CBFβ heterodimerization, whereas the COOH terminus contains transcriptional activation and repressor domains. Binding to CBFβ confers both increased DNA-binding affinity and stability to Runx1 and is essential for most of its known functions (3).

Disruption of the *RUNX1* gene is one of the most common aberrations found in acute leukemia (1, 4). Most frequently, the *RUNX1* gene is disrupted by chromosomal translocations, which are associated with distinct acute leukemias. These include the translocation t(8;21), generating the *RUNX1/CBFA2T1* fusion gene (also known as *AML1/ETO*), associated with ~30% of acute myelogenous leukemia (AML) with a French-American-British (FAB) M2 phenotype (immature with differentiation), and the t(12;21), generating the *ETV6/RUNX1* (*TEL/AML1*) fusion gene, associated with 20% of pediatric pro-B-cell acute lymphoid leukemias. Significantly, the gene encoding CBFβ is also the target of chromosome aberrations [e.g., inv(16) and t(16;16)] in acute myelomonocytic leukemia with an eosinophil component (FAB M4Eo). The fusion proteins mediate their oncogenic activity in part by dominantly repressing Runx1 target genes (2, 5).

In addition to the translocations/inversions affecting gene members of the CBF family, insertions, deletions, and point mutations in the *RUNX1* gene have also been identified in myeloid disorders. Strikingly, these mutations are found at the highest incidence (~25%) in minimally differentiated AML (AML-M0) and are less commonly associated with other *de novo* AML subtypes (6, 7). However, similarly high incidences are found in radiation-associated and therapy-related myelodysplasia syndrome (MDS) and AML (tMDS and tAML; refs. 8, 9) as well as in the two *de novo* MDS subtypes: refractory anemia with extra blasts (RAEB)-1 and RAEB-2 (10, 11). In addition, *RUNX1* mutations have been identified in 13 of 14 pedigrees of a rare familial platelet disorder with predisposition for AML (FPD/AML; refs. 6, 12).

RUNX1 mutations found in AML1-M0 and FDP/AML fall into two basic categories: (*a*) null mutations, which result in no Runx1 protein due to either large DNA deletions or to the introduction of premature stop codons that are predicted to activate nonsense-mediated mRNA decay (13), and (*b*) Runt DNA-binding (RDB) mutations, which generate Runx1 proteins with impaired DNA binding but which can still bind CBFβ (10, 14–16). The relative incidence of these two mutation types is approximately 2:1, and although recent studies have also revealed COOH-terminal truncation mutations in MDS patients, these have not been reported in AML1-M0 or FDP/AML (9, 11).

Although most *RUNX1* mutations in the AML-M0 subtype are biallelic (14, 17, 18), arguing for a classic tumor suppressor gene, some are monoallelic and thus support the concept of a tumor suppressor gene that is haploinsufficient (19). Indeed, in FDP/AML, tMDS/tAML, and RAEB-1/RAEB-2, *RUNX1* mutations are almost exclusively monoallelic (14). Furthermore, mice hemizygous for *Runx1* show perturbed hematopoiesis as evidenced by increased

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numbers of multilineage progenitors and decreased levels of $CD4^+$ T cells and circulating platelets (20–22). Clearly, interacting genetic lesions or unknown cellular variables determine whether a single amorphic (null) mutation of the *RUNX1* gene is sufficient to inhibit tumor suppressor activity.

Another level of complexity is added by the proposal that the RDB mutations are antimorphic [i.e., antagonize wild-type (wt) function in a dominant or semidominant fashion]. This hypothesis stems from the fact that (*a*) mutations specifically disrupt the DNA interface but not the CBFβ interface, leading to a protein with increased stability and increased affinity for CBFβ, and thus may efficiently sequester CBFβ from wt Runx1 and (*b*) RDB mutants inhibit wt Runx1 transcriptional activation *in vitro* (10, 14–16). Further support comes from the observation that the RDB allele is duplicated by acquired trisomy 21 (the chromosome on which the *RUNX1* gene is located), arguing for a semidominant effect that would be facilitated by increased expression levels (17). Recently, it has also been reported that MDS and AML patients that are heterozygous for RDB mutations have a significantly shorter survival time than patients heterozygous for null mutations (9). Thus, this study was designed to determine if RDB mutants inhibit wt Runx1 function in murine and human primary hematopoietic cells and to determine their role in AML induction.

Materials and Methods

Retroviral constructs and mutant Runx1. The retroviral expression vector FMEV (SF91) as well as the CRE recombinase vector have been described previously (23). The different Runx1 mutants were generated using site-directed mutagenesis and validated by sequence analysis. Retroviral supernatants were produced by transient transfection of the Phoenix GP producer cell line as described previously (23). *Env* genes from either ecotropic murine leukemia virus or the feline endogenous virus RD114 were used for virus production.

Retroviral transduction of hematopoietic cells and transplantation. Bone marrow cells from C57BL/6J (B6) mice or $Runx1^{fl/fl}$ mice in a mixed B6/129J background were obtained by flushing cells from the femur and tibiae. Lin^{neg} bone marrow cells were isolated using a lineage depletion kit (Miltenyi, Bergisch Gladbach, Germany) following the manufacturer's protocol and expanded for 3 days in serum-free expansion medium (StemSpan, StemCell Technologies, Meylan, France) with the addition of 50 ng/mL murine stem cell factor (SCF), 20 ng/mL murine interleukin (IL)-3, 100 ng/mL human IL-11, and 100 ng/mL human Flt3 (hFlt3) ligand. Human $CD34^+$ cells were isolated from umbilical cord blood (obtained by an approved protocol of the local ethics committee after informed consent of the mothers) using EasySep (StemCell Technologies). Cells were cultured for 48 h in serum-free expansion medium supplemented with 20% BIT9500 (StemCell Technologies) and 100 ng/mL hFlt3 ligand, 100 ng/mL human SCF, 20 ng/mL human thrombopoietin, and 20 ng/mL human IL-6. For infections, nontissue culture plates coated with RetroNectin (TaKaRa, Shiga, Japan) were preloaded four times with retroviral supernatant by centrifugation at 2,000 rpm for 20 to 30 min at 4°C. Cells were added to the RetroNectin-coated plates and incubated overnight in serum-free expansion medium with cytokines. Infection was repeated on the next day. For bone marrow transplantation, 5×10^5 cells were injected into the tail vein of lethally irradiated mice (9 Gy).

Methylcellulose replating assay and CRE-mediated excision of Runx1. Directly after infection, bone marrow cells were plated into methylcellulose (MethoCult GF-M3434, StemCell Technologies) at a cell density of 2×10^4 per plate. For serial replating, cells were harvested from the methylcellulose, and 2×10^4 cells per plate were replated at 7-day intervals. To validate *Runx1* excision after infection with a CRE-expressing retrovirus, DNA from harvested cells was subjected to PCR with the following primers: Timer-FLP1, GCCGGGTGCAATATTAAGTC; Timer-FLP2, TAGGGAGTGCTGCTTGCTCT; Runx1-CRE1, CTCTGGGAAACCAGGGAGTG; and Runx1-CRE2, AGTGGCCTTCCACTTTCAGC.

Fluorescence-activated cell sorting and histologic analysis of hematopoietic cells and organs. Peripheral blood smears and cytospins from bone marrow or colony assays (8×10^4 cells) were stained by the Pappenheim method (Sigma, Deisenhofen, Germany). Histologic inspection of hematopoietic tissue was done as described previously (23). Single-cell suspensions were prepared from hematopoietic tissue or colony assays after lysis of erythroid cells with PharmLyse (BD PharMingen, Hamburg, Germany) and incubation at 4°C for 30 min in PBS containing 2% FCS with phycoerythrin-, allophycocyanin-, or CyChrom-conjugated monoclonal antibodies (mAb; BD PharMingen). Nonspecific binding of mAbs was prevented by preincubation with Fc Block (BD PharMingen). Cells were washed with PBS and applied for analysis on a FACSCalibur (BD Biosciences, Heidelberg, Germany). GFP^{pos} cells from bone marrow or cord blood were sorted using an Aria cell sorter (BD Biosciences) and plated into methylcellulose (MethoCult GF-M3434 or MethoCult GF-H4434, StemCell Technologies) to evaluate progenitor numbers after 7 or 14 days, respectively.

Western blot analysis. Total bone marrow harvested from mice or cells obtained from colony assays were lysed in NP40 lysis buffer containing protease inhibitors. Cell extracts were analyzed by SDS-PAGE and Western blot analysis as described previously (24). To detect protein expression, a 1:1,000 dilution of the polyclonal anti-AML1 (N-20) and anti-green fluorescent protein (GFP; FL; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or a 1:10,000 dilution of the monoclonal anti-β-actin (clone AC-74, Sigma) antibody was used. The bound antibody was detected with the appropriate secondary antibody conjugated with horseradish peroxidase and visualized using the enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom).

Results

Generation of retroviral vectors expressing RDB mutants and transduction into murine bone marrow cells. Two different Runx1 mutations (K83N and R135G), which have been identified in patients with AML and/or FPD/AML, were introduced into a *RUNX1* cDNA. Consistent with the localization of these residues in one of two loops that contact DNA (βA′-B and βE′-F, respectively), both these mutations have previously been shown to impair DNA binding without perturbing the Runt fold or ability to bind CBFβ (10, 16, 25). The two RDB mutants were cloned into the bicistronic FMEV retroviral vector coexpressing GFP, which serves as a marker for transduced cells (depicted in Fig. 1*A*).

To evaluate the effects of the RDB mutants on hematopoiesis *in vivo*, lineage-depleted bone marrow cells were infected with retroviral vectors and transplanted into lethally irradiated mice. Between 18 and 30 weeks after transplantation, cells from peripheral blood, bone marrow, and spleen were examined for GFP and surface marker expression. The percentage of GFP-positive bone marrow cells ranged from 2% to 81% for all mice, with median values of 11%, 14%, and 10% for mice receiving the empty GFP vector ($n = 5$), the K83N ($n = 8$), or the R135G ($n = 6$) RDB mutant, respectively. As no obvious difference was observed between the two cohorts of RDB mutants, the results of these mice have been grouped together and collectively referred to as RDB.

To determine the levels of RDB mutant expression, protein was isolated from bone marrow cells from transplanted mice. The levels of Runx1 protein were significantly increased (>3-fold) in bone marrow of mice receiving RDB-transduced bone marrow (Fig. 1*B*). As the bone marrow cells were not sorted before analysis but GFP levels were from 50% to 80% in the mice analyzed (Fig. 1*C*; data not shown), we estimate at least a 4-fold increase in RDB levels over wt. Although exact quantifications between different experiments are

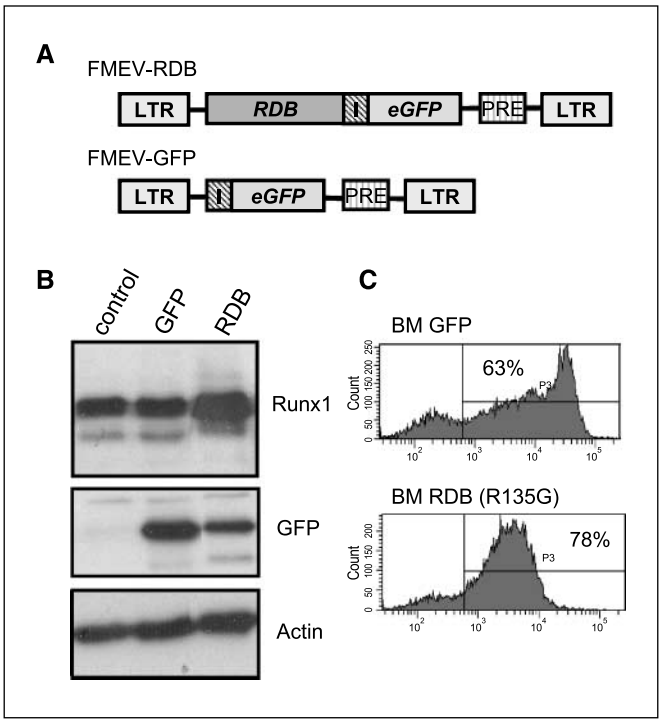

Figure 1. Retroviral transduction of RDB mutants into murine bone marrow cells. *A,* schematic representation of FMEV retroviral vectors. Transcription is driven by the long terminal repeat (*LTR*) from the Friend spleen focus-forming virus. An internal ribosomal entry site facilitates translation of the *eGFP* gene. The posttranscriptional regulatory element (*PRE*) increases transcription and translation efficiencies. *B,* Western blot analysis of protein isolated from bone marrow cells from a normal B6 mouse (control) or a mouse transplanted with bone marrow infected with either FMEV-GFP (*GFP*) or FMEV-RDB (*RDB*). The same blot was used to determine Runx1, GFP, or actin protein levels using appropriate antibodies. *C,* FACS analysis to determine the percentage of GFP^{pos} cells and mean GFP fluorescent levels of transduced and transplanted bone marrow (*BM*) cells. The cells used for this analysis correspond to the same bone marrow cells used for the Western blot analysis.

not possible, we observed only minor fluctuations in the mean GFP fluorescence of RDB-transduced bone marrow cells (a range of ~4-fold); thus, we are confident that the majority of animals contained cells where RDB expression levels were above wt levels.

No evidence for RDB-mediated dominant-negative suppression of Runx1 function on hematopoiesis *in vivo*. Previous studies have shown that inactivation or down-regulation of Runx1 (and CBFβ) activity has the most severe effects on the lymphoid lineages, particularly on T-cell development (3, 20, 26–28). Due to the fact that ~70% of the leukocytes in murine blood are mature lymphoid cells, we first sought to determine if the RDB-transduced cells would be underrepresented in the blood compared with bone marrow. As shown in Fig. 2*A*, the ratio between the percentages of GFP^{pos} cells in blood versus bone marrow in mice receiving RDB bone marrow cells was similar to that as in control mice. This was in striking contrast to our previous studies of mice receiving bone marrow cells transduced with the *RUNX1* fusion gene AML1/ETO or TEL/AML1, both of which severely disrupt T-cell development and, to a lesser extent, B-cell development (23, 29).

Marker analysis confirmed that the RDB^+ blood cells were composed of mature T cells ($CD4^+/CD3^+$ or $CD8^+/CD3^+$) and mature B cells ($B220^{hi}/IgM^+$) in proportions equivalent to nontransduced cells in the same mouse (Fig. 2*B*; data not shown). Furthermore, no skewing in the proportion of CD4 versus CD8 T cells was observed, which has been reported in $Runx1^{+/-}$ mice (20, 21). Myeloid differentiation also seemed not to be affected by RDB expression, as RDB^+/GFP^+ blood cells with high levels of CD11b and Gr1 expression, typical of mature granulocytes in the blood, were found at expected levels (data not shown). In conclusion, RDB mutant expression in bone marrow progenitors, at levels equivalent or up to an estimated 3-fold higher than wt Runx1, did not have an obvious antagonistic effect on either myeloid or lymphoid differentiation or maturation.

The lineage distribution of transduced cells in the bone marrow was also analyzed to determine the effect of RDB expression. Lineage markers were used to determine the proportion of cells in the myeloid (CD11b/Gr1), erythroid (TER119), and B-cell (B220/IgM) compartments. For this analysis, both the GFP^{pos} (RDB^+) and GFP^{neg} (RDB^-) compartments in each mouse were determined as well as the GFP^{pos} cells in control mice. No significant difference in the proportion of B-lymphoid cells nor myeloid cells in the different fractions was observed (data not shown). Normal levels of myeloid progenitors were also confirmed by myeloid colony assays, where the average number of colony-forming cells (CFC) in the GFP^{pos} population sorted from RDB and GFP bone marrow from transplanted mice was 20 ± 9.5 and 17 ± 4.9, respectively (mean of two independent experiments done in triplicate.) This is in contrast to bone marrow progenitors from mice hemizygous for *Runx1* or carrying two deleted alleles, which show an increase in CFC (20, 26, 28). Furthermore, no expansion of the megakaryocytic compartment ($CD41^{pos}/CD117^{pos}$) in the RDB population was observed, which has been observed in *Runx1*-deficient mice (20). This was also confirmed by histochemical and immunohistochemical analysis of bone marrow sections in RDB mice ($n = 2$) with >40% GFP-positive cells (data not shown).

In summary, the myeloid (including megakaryocytic) and lymphoid compartments of RDB-expressing bone marrow population were indistinguishable from either nontransduced cells or GFP^+-transduced cells in the bone marrow. These results argue against RDB mutations being strongly antimorph for known wt Runx1 functions in these lineages.

Ectopic expression of mutant Runx1 leads to impaired erythropoiesis in murine and human cells. In contrast to the myeloid and lymphoid compartments in the bone marrow, we consistently observed a reduction in the proportion of $TER119^+$ erythroid cells within the RDB population (Fig. 3*A*) compared with either the nontransduced population in the same mouse (GFP^{neg}; $P < 0.003$, $n = 8$, paired t test) or the GFP^{pos} population in control-transduced mice ($P < 0.03$, $n = 6$, unpaired t test). Significantly, Runx1 expression is down-regulated during erythropoiesis (30, 31), and thus, this effect cannot be attributed to antagonizing Runx1 activity. To more closely evaluate the significance of this finding, we evaluated the effect of RDB expression on erythropoiesis in human cord blood cells, due to the high levels of erythroid progenitors in this source. Human $CD34^+$ cord blood cells were infected with pseudotyped vectors expressing either one of the two RDB mutants, wt Runx1, or an empty control vector. After sorting for GFP expression, cells were analyzed in colony assays in methylcellulose under conditions supporting myelopoiesis and erythropoiesis. Strikingly, the ratio of erythroid to myeloid colonies was significantly reduced in cultures expressing RDB (Fig. 3*B*). This drastic decrease in erythroid colonies supports the hypothesis that expression of RDB within the erythroid compartment impairs differentiation and expansion of these cells. Interestingly, this was observed with both the RDB mutants as well as wt Runx1.

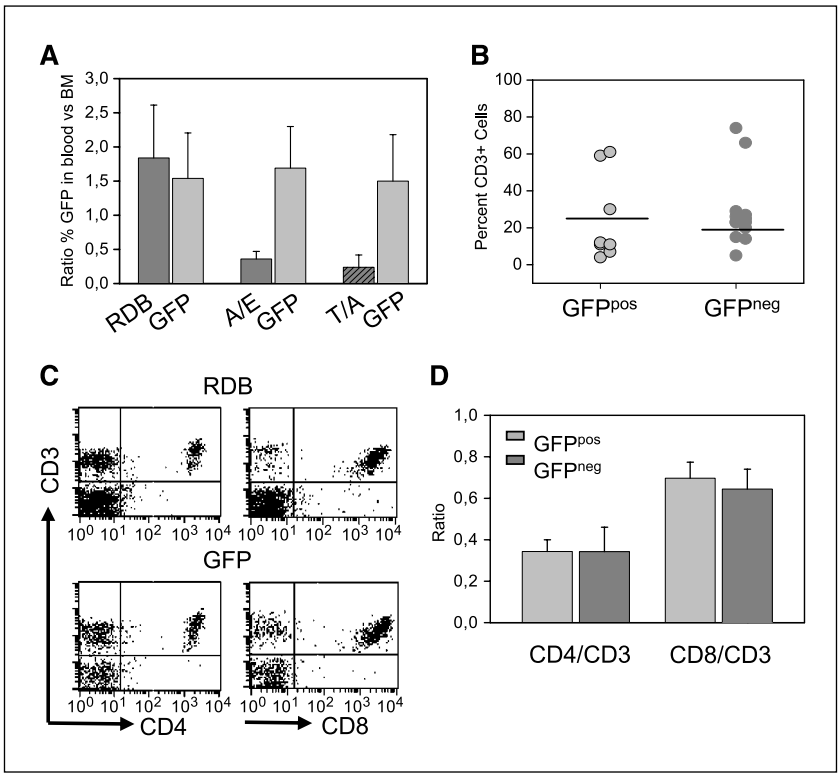

Figure 2. RDB expression does not impair differentiation of lymphoid cells into mature blood cells. *A, columns,* mean ratio of the percentage of GFP^{pos} cells in the blood versus bone marrow was determined in mice receiving RDB-transduced ($n = 5$) or GFP-transduced ($n = 7$) transplants; *bars,* SD. For comparison, results from previous experiments in which mice receiving bone marrow transplants transduced with either AML1/ETO (*A/E*) or TEL/AML (*T/A*) and their respective GFP controls are shown (23, 29). *B,* the proportion of T cells expressing RDB within the transduced blood population (GFP^{pos}) are similar to the T-cell proportion in the nontransduced blood population (GFP^{neg}). Blood cells were stained with the CD3 antibody, and the proportion of cells within the GFP-positive and GFP-negative gates was determined. *Dot,* value from a single mouse. *Horizontal line,* mean value of all mice. *C* and *D,* no deviation in the relative proportions of $CD4^+$ and $CD8^+$ T cells within the RDB-expressing cell population was observed. *C,* representative FACS analysis of blood cells stained for CD3 and either CD4 and CD8 and gated on the GFP^{pos} population from either RDB- or GFP-transplanted mice. *D, columns,* mean ratio of either CD4 or CD8 single positive cells to the total number of $CD3^+$ cells within either the GFP^{pos} or GFP^{neg} pools of RDB-transduced mice ($n = 7$); *bars,* SD.

Thus, this can be explained neither by a dominant-negative effect of RDB mutations nor by the transactivation of genes regulated by Runx1 in a DNA-binding–dependent fashion.

RDB mutants impart increased replating efficiency and impair terminal differentiation of murine bone marrow progenitors. An oncogenic effect of the Runx1 fusion protein AML1/ETO on primary murine bone marrow progenitors was first observed by evaluation of the replating capacity of AML1/ETO-expressing bone marrow cells, leading to immortalization of the cultures (32). Ichikawa et al. (28) have also shown that *Runx1*-null bone marrow cells also have an increased replating capacity compared with wt bone marrow cells, but no immortalization was observed. We thus sought to determine if the RDB mutants imparted increased replating potential to murine bone marrow cells. As controls, bone marrow cells were infected with vectors expressing wt Runx1, AML1/ETO, and GFP alone. Cultures with infection frequencies between 16% and 30% were used for the assay and serially replated in methylcellulose at 1-week intervals. Cells were not sorted, as monitoring for the percentage of GFP^+ cells provides an indicator for a selective advantage. Colony number and morphology, as well as cell composition, was determined by visual examination, Pappenheim-stained cytospins, and fluorescence-activated cell sorting (FACS) analysis after each replating, when possible.

As shown in Fig. 4*A*, expression of both RDB mutants led to increased replating capacity and immortalization of murine bone marrow cells. The effect was less pronounced than that observed for AML1/ETO, which consistently resulted in higher colony numbers. Colonies were tight and compact for both RDB and AML1/ETO cultures and were morphologically distinct to mast cell colonies observed in control cultures. Morphologic and FACS analysis of cells from the dispersed colonies showed maintenance of myeloid progenitors (i.e., $CD11b^{hi}/Gr1^{lo}$ progeny) in the RDB and AML1/ETO cultures throughout the replating experiments (Fig. 4*B*). Strikingly, as early as two replatings, the accumulation of blast cells was observed in both the AML1/ETO and RDB cultures, in addition to cells with dysplastic morphology, which were more abundant in RDB cultures (Fig. 4*B*). This was in contrast to control cultures in which, after the first replating, cells consistently expressed either the mast cell markers $Sca1^+/Kit^{hi}$ or, with decreasing frequency with subsequent replatings, the myeloid marker CD11b, consistent with their mast cell and macrophage morphology, respectively (Fig. 4*B*). In accord with a positive selection for RDB-transduced cells, a striking increase in the GFP^{pos} cells was observed in RDB cultures, with cultures being composed of >85% GFP cells after the first or second replating (Fig. 4*C*). This was in contrast to control GFP cultures, where the proportion of GFP^{pos} cells varied in a stochastic fashion. Interestingly, a negative selection for bone marrow cells receiving wt Runx1 was observed as evidenced by the consistent loss of GFP expression after two replatings (observed in four independent experiments; data not shown).

Significantly, although the percentage of RDB-transduced cells consistently increased during replating, no dramatic increase in the mean GFP fluorescence levels was observed, arguing against selection of a clone with exceptionally high expression levels (Fig. 4*C*). This was confirmed by Western blot analysis of Runx1 protein levels in cells isolated after multiple replatings. Although Runx1 protein was no longer detectable in uninfected controls, which were composed primarily of mast cells, Runx1 levels were ~2-fold higher in RDB-infected cultures compared with AML1/ETO controls, which are composed of similar cell types (myeloid progenitors; Fig. 4*D*).

In summary, similar to AML1/ETO, RDB expression imparts increased replating capacity to a few early progenitors. Differentiation is impaired in these cells as evidenced by the accumulation

of myeloblasts and cells with dysplastic morphology. These cells are also not transplantable *in vivo* (data not shown). These results suggest that RDB expression disrupts the differentiation program, whereby cells retain their capacity to proliferate and form colonies at a frequency of approximately 5×10^{-3}.

Disruption of the CBFβ interface in the RDB mutants does not disrupt their ability to promote replating. To investigate the possibility that the RDB effect on replating and immortalization was due to sequestering of CBFβ and thus resulting in the reduced activity of Runx1 (or one of its homologues), we mutated either one or both of two amino acid residues (T161 and N109), which specifically reduce CBFβ equilibrium binding constants by 40- and 60-fold, respectively (33). The activity of either K83N or wt Runx1 proteins containing one of these mutations was determined. Replating activity was evaluated by determining the proportion of GFP^+ cells in the replated cultures and confirming their myeloid progenitor phenotype by morphology and FACS. Cells expressing the single T161A or N109D mutants did not show impaired differentiation, resulting in cultures composed primarily of mast cells after three replatings (Fig. 5). Interestingly, we also do not observe negative selection against cells expressing these CBFβ interaction mutants, in contrast to wt RUNX1 cells, suggesting that the negative effect of RUNX1 on proliferation requires CBFβ interaction. In contrast, double mutants containing K83N and either T161A or N109D mutations behaved similarly to single K83N mutants (Fig. 5), in which >85% of the cells after three replatings were GFP^+ and maintained CD11b expression, consistent with a predominance of dysplastic and blastic myeloid cells. Triple mutants (T161A, N109D, and K83N) also lead to increased replating of myeloid progenitors and the accumulation of $CD11b^+$ cells. The results above indicate that the marked effect of RDB expression on differentiation as assayed in a replating assay is not due to a simple dominant-negative effect mediated by sequestering CBFβ and/or very high expression levels.

Runx1-deficient cells have a lower replating capacity than RDB-expressing cells. We next sought to determine if the replating phenotype observed after RDB expression was the same as that observed in bone marrow cells lacking *Runx1*. We thus transduced murine bone marrow from $Runx1^{fl/fl}$ mice, in which exon 5 is flanked by loxP sites, with retroviral vectors that express either the CRE recombinase, a RDB mutant, or GFP alone. Bone marrow cells were then subjected to a replating assay in methylcellulose as described above. The excision of the *Runx1* gene by CRE recombinase was confirmed by PCR using primers specific for the deleted allele (Fig. 6*A*).

Bone marrow cells receiving CRE showed prolonged replating capacity compared with GFP controls; however, in contrast to RDB infections, no colony formation was observed after the fourth round of replating (Fig. 6*B*). FACS and morphologic analysis of the second replating also confirmed that, in contrast to control cultures that were solely composed of long-living macrophages and mast cells, the CRE cultures still showed myeloid (predominantly monocytic) and erythroid cells at various stages of differentiation (Fig. 6*C*). However, this was distinct to the RDB cultures that contained predominantly immature myeloid progenitors and no obvious erythroid precursors (Fig. 6*C*). The striking difference in erythroid cells between the RDB and CRE bone marrow cultures derived from the $Runx1^{fl/fl}$ mouse strain is consistent with the earlier observation that RDB selectively impairs erythroid differentiation. Importantly, immature myeloid progenitors were also observed in the third and fourth replatings of the CRE cultures, but their prevalence was lower than that observed in RDB cultures (in three independent experiments). Thus, although Runx1 deficiency also results in an increased replating efficiency and disruption of the normal myeloid differentiation signaling, the effect is more pronounced in RDB cultures.

Discussion

Runx1 is a critical hematopoietic regulator, where threshold levels are necessary for various functions, including the maintenance of long-term repopulating stem cells and development and terminal differentiation of T cells, B cells, and megakaryocytes (3, 20, 26–28). Due to the occurrence of monoallelic amorphic mutations in FPD/AML, RAEB-1/RAEB-2, and tMDS/tAML patients, *RUNX1* has also been postulated to be a haploinsufficient tumor suppressor gene. However, the high frequency of biallelic *RUNX1* mutations in AML1-M0 suggests that "less is better yet" for AML induction in which myeloid differentiation is almost completely blocked. In accord with this hypothesis, RDB mutations

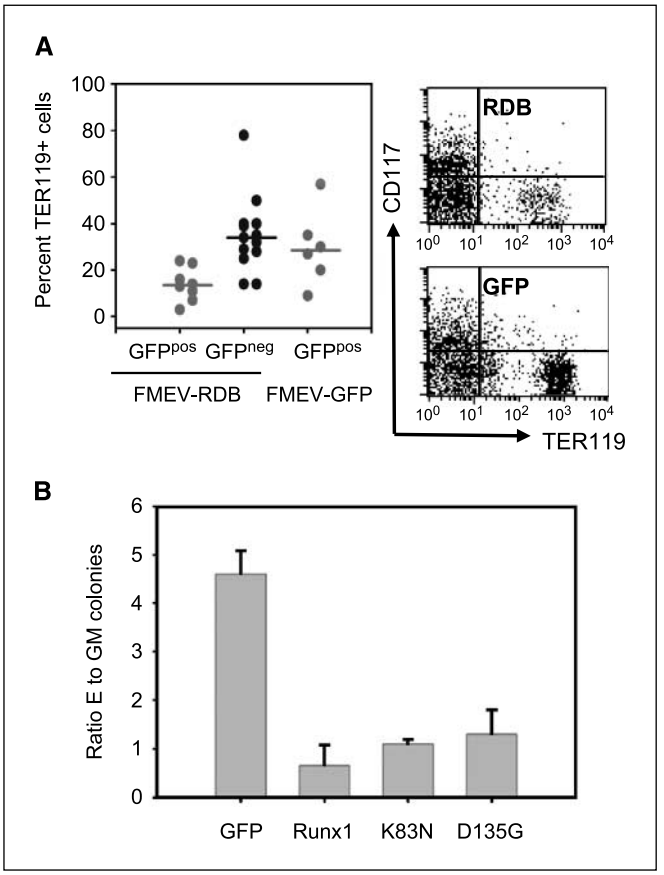

Figure 3. A decrease in the relative levels of erythropoiesis is found within the RDB-expressing population in murine bone marrow and in human cord blood cells. *A,* bone marrow cells of mice receiving FMEV-RDB–transduced or FMEV-GFP–transduced bone marrow were stained with the erythroid marker TER119 to determine the relative proportion of this lineage in either the transduced (GFP^{pos}) or nontransduced (GFP^{neg}) population. *Dot,* independent mouse. *Vertical line,* median value. *Right,* representative FACS data. *B,* human $CD34^+$ cord blood cells transduced with indicated vector and sorted for GFP expression were analyzed by colony assays to determine their potential to differentiate into the erythroid compartment. *Columns,* mean ratio of erythroid [e.g., blast-forming unit (erythroid) and colony-forming unit (CFU) in culture] to myeloid (granulocyte-macrophage CFU, granulocyte CFU, and macrophage CFU) colonies from two independent experiments; *bars,* SE.

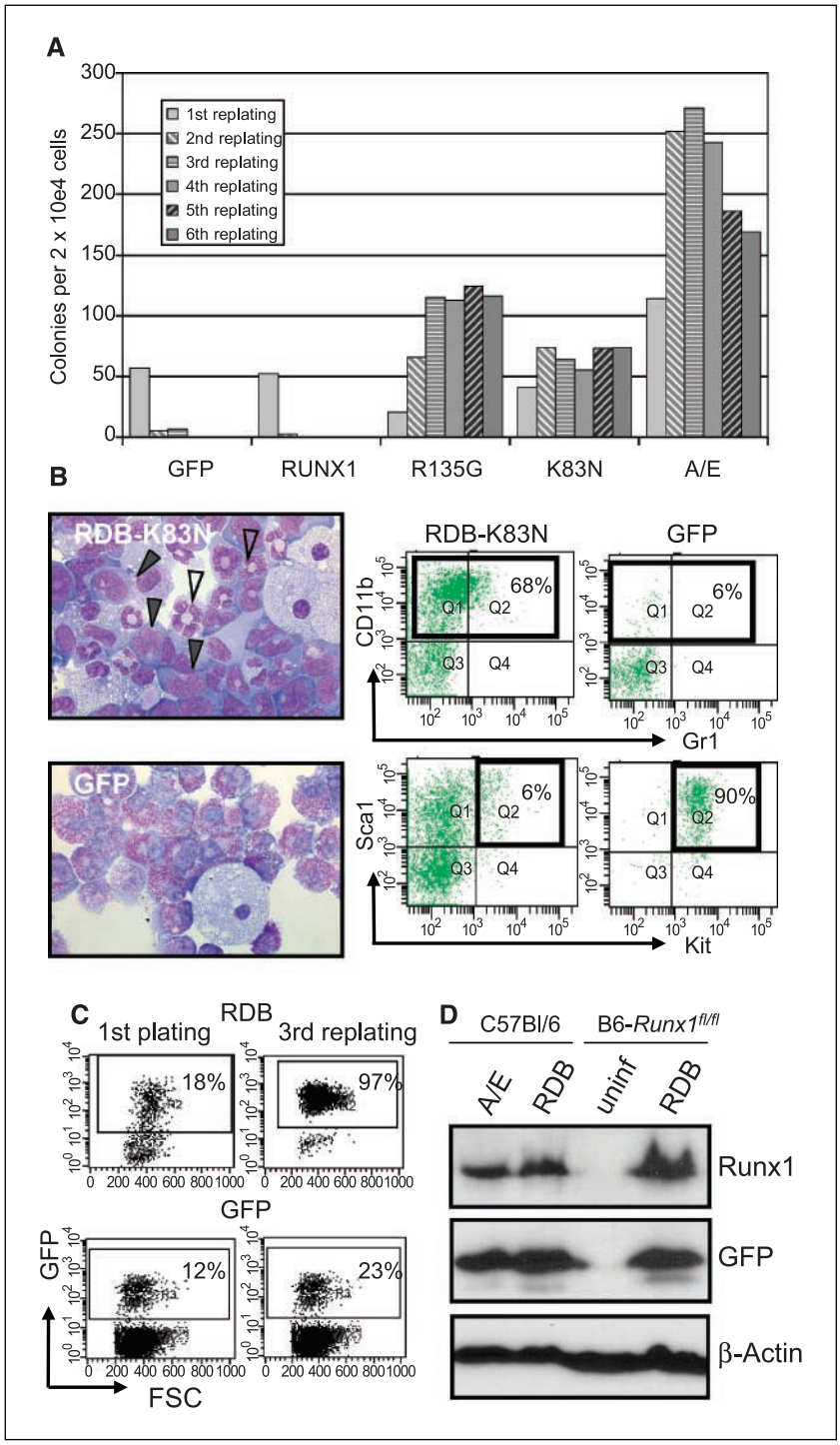

Figure 4. A replating assay reveals a striking effect of RDB expression on early myeloid progenitors. Bone marrow cells were infected with virus expressing the indicated protein and analyzed by replating in methylcellulose. *A,* unlike control cultures infected with either FMEV-GFP or FMEV-Runx1, cultures infected with FEMV-RDB or FMEV-AML1/ETO (*A/E*) showed replating capacity for over nine replatings (only six replatings are shown). Similar results were obtained in four independent experiments. *B,* morphology and FACS analysis of cells obtained from replating assays. Cytospins stained by the Pappenheim method show the accumulation of blast-like cells (*closed arrowheads*) but also cells with a dysplastic morphology (*open arrowheads*) in RDB cultures (second replating). In contrast, GFP control cultures showed predominantly mast cells and macrophages (second replating). FACS analysis confirmed the maintenance of clonogenic cells that cause myeloid progenitors and precursors (CD11b/Gr1) in RDB cultures (GFP^{pos}) but not in GFP control cultures (GFP^{pos}), which primarily cause mast cell colonies or show residual macrophages (results from first replating are shown). *C,* replating experiments resulted in the selection of RDB-transduced cells as shown by analysis of GFP expression after the first plating and third replating. Strikingly, there is no selection for exceptionally high levels of RDB expression as determined by the surrogate GFP marker. *D,* Western blot analysis confirms that RDB expression levels in replating assays are similar to endogenous Runx1 levels in early progenitors as observed in a parallel culture expressing AML1/ETO. In contrast, Runx1 was not detectable in uninfected cultures subjected to replating, which were composed entirely of mast cells at the time of harvesting. Cells were harvested from parallel cultures in the indicated mouse strain after the third replating.

have been proposed to be antimorphic, generating mutant proteins that antagonize wt activity in a dominant or semidominant fashion. Interestingly, we found no evidence for down-regulation of wt Runx1 activity when RDB was retrovirally expressed in bone marrow cells in a mouse model. In contrast, we saw a striking effect of RDB expression in a replating assay, which selected for cells with impaired differentiation and increased proliferation capacity. Although bone marrow progenitors from *Runx1*-deficient mice also had increased replating activity, this phenotype was less dramatic and did not lead to ready immortalization. Thus, and as discussed below, our data support the hypothesis that RDB mutant proteins have a gain of function most likely through their ability to carry out only a specific set of Runx1 functions.

Theoretically, RDB mutants could antagonize wt Runx1 activity in a dominant or semidominant fashion if they possess increased affinity for the common cofactor CBFβ or are present at increased levels, due to either changes in protein stability (normally mediated by CBFβ binding) or transcription/translation rates. Examples for each of these possibilities have been described for various RDB mutants (10, 14, 16), particularly K83N, which eliminates a binding

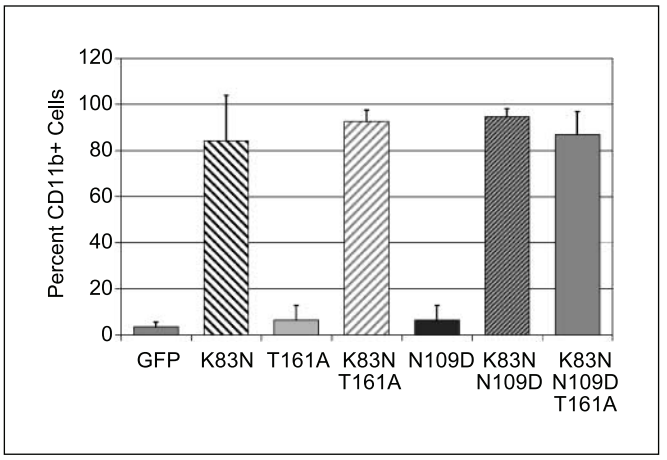

Figure 5. Disruption of the CBFβ interface does not impair RDB activity. Replating experiments were done using the indicated Runx1 mutants. Percentage of cells expressing the myeloid marker CD11b within the GFP^{pos} population at the second or third replating for each Runx1 construct or GFP control. *Columns,* mean of two independent experiments; *bars,* SD.

site for ubiquitination (34). However, our analysis of two cohorts of mice expressing different RDB mutants did not reveal any disruption of T-cell or platelet development nor were increased levels of myeloid progenitors observed, all characteristics of haploid levels of Runx1. An obvious explanation may be that the expression levels required to inhibit wt Runx1 function were not obtained by our retroviral system. However, protein analysis showed that RDB expression levels were at least 3-fold higher than wt levels (i.e., levels that should have been sufficient to inactivate Runx1 function to haploid levels if the RDB had weakly dominant-negative activity). Although we cannot rule out that RDB expression levels in specific cell types (e.g., megakaryocyte precursors and T cells) are insufficient due to variable transcription rates of the FMEV retroviral enhancer/promoter in different cell types, we find this unlikely as earlier work has shown robust expression in all hematopoietic lineages (35).

If RDB mutants do not efficiently down-regulate wt Runx1 function, why was a striking effect seen in the *in vitro* replating assay less pronounced to that observed in AML1/ETO cultures but more pronounced than *Runx1*-deficient bone marrow progenitors? Again, expression levels could play an important role. Importantly, however, although there was a strong selection for cells expressing RDB mutants, there was no significant selection for cells with high RDB expression levels during the replating assays. Indeed, RDB levels were at most 1-fold higher than endogenous Runx1 levels as estimated by comparing Runx1 levels in cells with the same progenitor phenotype. Thus, the apparent antagonistic effect was not due to aberrantly high levels of RDB expression.

Also arguing against a classic dominant-negative effect, our analysis showed that RDB activity was not impaired in the replating assay by disrupting CBFβ binding, a critical cofactor of wt Runx1. Thus, if RDB directly antagonizes wt Runx1 activity, it is through a mechanism not involving CBFβ sequestration. Conceivably, competition for another interacting factor could also lead to dominant-negative activity. It is important to note that neither of the two CBFβ-binding mutations tested disrupted RDB function. Due to its direct hydrogen bonding interactions with CBFβ, the T161A mutation would be expected to specifically disrupt binding to CBFβ but not disrupt the overall Runt domain structure (15, 33, 36). In contrast, however, the N109D leads to a more radical disruption by perturbing the overall folding architecture of the Runt domain (15, 33, 36, 37). This has been shown to lead to loss not only of CBFβ binding but also of low-affinity DNA binding (15, 25, 37) and thus may also disrupt other protein-protein interactions occurring within the Runt domain. It will be important to determine if the N109D mutation alleviates interaction with

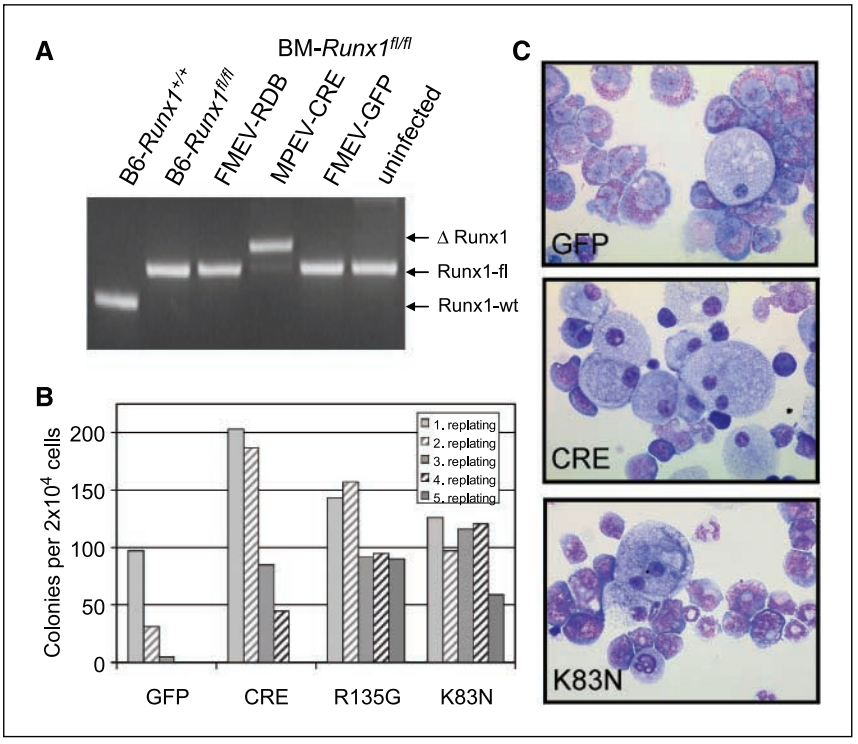

Figure 6. Excision of the *Runx1* gene by the CRE recombinase leads to increased replating capacity of bone marrow cells but with a distinct morphology. *A,* DNA was isolated from bone marrow cells of $Runx1^{fl/fl}$ mice that were infected with the indicated virus and then sorted for GFP expression. As controls, DNA from tail biopsies from either B6-$Runx1^{fl/fl}$ or B6-$Runx1^{+/+}$ mice was also analyzed. PCR fragments show the expected length in MPEV-CRE-infected cultures that show excision of exon 5. *B,* replating assays were done on infected bone marrow cells as described. Number of colonies per 2×10^4 cells after each round of replication. One experiment of three independent experiments. *C,* cytospins were made from colonies isolated from the second replating and subjected to Pappenheim staining. Microscopic inspection shows that the CRE-infected cultures are morphologically distinct from RDB (*K83N*) cultures, which are composed primarily of dysplastic myeloid progenitors.

other known hematopoietic transcription factors [e.g., PU.1, CAAT/enhancer binding protein α (C/EBPα), c-Jun, and GATA-1], whose activity is regulated by interaction with the Runt domain (38–41). However, with the exception of C/EBPα (42), this interaction is reported to be mediated by DNA binding and thus would not be expected to occur with the RDB mutants. Alternatively to protein-protein interactions occurring within the Runt domain, RDB antagonistic function may involve proteins that interact with the activation and repressor domains within the COOH-terminal half of Runx1 (43–45).

An interesting twist is the possibility that the observed effect may not be due to the direct inhibition of wt Runx1 but to the disruption of an intricate balance of multiple Runx1 functions. Runx1 is thought to act primarily as a transcription organizer by recognizing and binding of cognate *cis*-regulatory sequences within important target genes. However, growing evidence suggests that Runx1 and its orthologues can also be recruited to gene promoters/enhancers in a DNA-binding–independent fashion (42, 46). Thus, some functional activities of Runx1 may not be disrupted by RDB mutations. Importantly, we observed both *in vivo* and *in vitro* that ectopic expression of both RDB and wt RUNX1 impaired erythropoiesis, which is normally accompanied by the down-regulation of *Runx1*. This result suggests that down-regulation of a DNA-binding–independent Runx1 function is necessary for normal erythroid differentiation. Although the mechanism is not clear, it is tempting to speculate that this occurs through Runx1 binding to the erythroid transcription factor GATA-1, an interaction also conserved in the *Drosophila* homologues serpentine and lozenge (40, 47).

DNA-binding–independent activity of RDB may also be responsible for the disruption of myeloid differentiation observed in the replating assays. Conceivably, normal levels of DNA-binding activity may override DNA-binding–independent activity; however, if this balance is disrupted, subtle but significant differentiation controls may be deregulated. The importance of DNA-binding–independent functions has been shown for several transcription factors important in developmental control (48–50). Significantly, a DNA-binding–independent function of the Runx1 *Drosophila* homologue Runt has also been shown to be necessary in regulating the segment polarity gene *engrailed* (46). An important endeavor in the future will thus be to separate DNA-binding–dependent and DNA-binding–independent functions of Runx1 in hematopoiesis.

The effect of RDB mutant expression on myeloid differentiation was only observed in a replating assay that allows for the selection and expansion of a relatively rare event; thus, it is perhaps not surprising that an effect was not observed *in vivo*. However, we propose that the observed disruption of normal differentiation may lead to a small pool of preleukemic cells, which are the target of secondary mutations leading to MDS and/or AML. Thus, in line with the dual functions of Runx1 in tumorigenesis (4), we propose that RDB mutations inactivate tumor suppressor activity but simultaneously retain oncogenic activity by specifically inactivating DNA-binding–dependent function but retaining DNA-binding–independent function.

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References

1. Mikhail F, Sinha K, Saunthararajah Y, Nucifora G. Normal and transforming functions of RUNX1: a perspective. J Cell Physiol 2006;207:582–93.
2. Durst K, Hiebert S. Role of RUNX family members in transcriptional repression and gene silencing. Oncogene 2004;23:4220–4.
3. Miller J, Horner A, Stacy T, et al. The core-binding factor β subunit is required for bone formation and hematopoietic maturation. Nat Genet 2002;32:645–9.
4. Blyth K, Cameron E, Neil J. The RUNX genes: gain or loss of function in cancer. Nat Rev Cancer 2005;5:376–87.
5. Zelent A, Greaves M, Enver T. Role of the TEL-AML1 fusion gene in the molecular pathogenesis of childhood acute lymphoblastic leukaemia. Oncogene 2004;23:4275–83.
6. Osato M. Point mutations in the RUNX1/AML1 gene: another actor in RUNX1 leukemia. Oncogene 2004;23:4284–96.
7. Roumier C, Fenaux P, Lafage M, et al. New mechanisms of AML1 gene alteration in hematological malignancies. Leukemia 2003;17:9–16.
8. Harada H, Harada Y, Tanaka H, Kimura A, Inaba T. Implications of somatic mutations in the AML1 gene in radiation-associated and therapy-related myelodysplastic syndrome/acute myeloid leukemia. Blood 2003;101:673–80.
9. Christiansen D, Andersen M, Pedersen-Bjergaard J. Mutations of AML1 are common in therapy-related myelodysplasia following therapy with alkylating agents and are significantly associated with deletion or loss of chromosome arm 7q and with subsequent leukemic transformation. Blood 2004;104:1474–81.
10. Imai Y, Kurokawa M, Izutsu K, et al. Mutations of the AML1 gene in myelodysplastic syndrome and their functional implications in leukemogenesis. Blood 2000;96:3154–60.
11. Harada H, Harada Y, Niimi H, et al. High incidence of somatic mutations in the AML1/RUNX1 gene in myelodysplastic syndrome and low blast percentage myeloid leukemia with myelodysplasia. Blood 2004;103:2316–24.
12. Minelli A, Maserati E, Rossi G, et al. Familial platelet disorder with propensity to acute myelogenous leukemia: genetic heterogeneity and progression to leukemia via acquisition of clonal chromosome anomalies. Genes Chromosomes Cancer 2004;40:165–71.
13. Conti E, Izaurralde E. Nonsense-mediated mRNA decay: molecular insights and mechanistic variations across species. Curr Opin Cell Biol 2005;17:316–25.
14. Osato M, Asou N, Abdalla E, et al. Biallelic and heterozygous point mutations in the runt domain of the AML1/PEBP2αB gene associated with myeloblastic leukemia. Blood 1999;93:1817–24.
15. Tahirov T, Inoue-Bungo T, Morii H, et al. Structural analyses of DNA recognition by the AML1/Runx-1 Runt domain and its allosteric control by CBFβ. Cell 2001;104:755–67.
16. Michaud J, Wu F, Osato M, et al. *In vitro* analyses of known and novel RUNX1/AML1 mutations in dominant familial platelet disorder with predisposition to acute myelogenous leukemia: implications for mechanisms of pathogenesis. Blood 2002;99:1364–72.
17. Preudhomme C, Warot-Loze D, Roumier C, et al. High incidence of biallelic point mutations in the Runt domain of the AML1/PEBP2αB gene with M0 acute myeloid leukemia and in myeloid malignancies with acquired trisomy 21. Blood 2000;96:2862–9.
18. Silva G, Morolli B, Storlazzi C, Anelli L, Wessels H. Identification of RUNX1/AML1 as a classical tumor suppressor gene. Oncogene 2003;22:538–47.
19. Cook W, McCaw B. Accommodating haploinsufficient tumor suppressor genes in Knudson's model. Oncogene 2000;19:3434–8.
20. Sun W, Downing J. Haploinsufficiency of AML1 results in a decrease in the number of LTR-HSCs while simultaneously inducing an increase in more mature progenitors. Blood 2004;104:3565–72.
21. Hayashi K, Natsume W, Watanabe T, et al. Diminution of the AML1 transcription factor function causes differential effects on the fates of CD4 and CD8 single-positive T cells. J Immunol 2000;165:6816–24.
22. Woolf E, Xiao C, Fainaru O, et al. Runx3 and Runx1 are required for CD8 T cell development during thymopoiesis. Proc Natl Acad Sci U S A 2003;100:7731–6.
23. Schwieger M, Löhler J, Friel J, et al. AML1-ETO inhibits maturation of multiple lymphohematopoietic lineages and induces myeloblast transformation in synergy with ICSBP deficiency. J Exp Med 2002;196:1227–40.
24. Meyer J, Jücker M, Ostertag W, Stocking C. Carboxyl-truncated STAT5b is generated by a nucleus-associated serine protease in early hematopoietic progenitors. Blood 1998;91:1901–8.
25. Li Z, Yan J, Matheny C, et al. Energetic contribution of residues in the Runx1 runt domain to DNA binding. J Biol Chem 2003;278:33088–96.

26. Growney J, Shigematsu H, Li Z, et al. Loss of Runx1 perturbs adult hematopoiesis and is associated with a myeloproliferative phenotype. Blood 2005;106:494–504.
27. Putz G, Rosner A, Nuesslein I, Schmitz N, Buchholz F. AML1 deletion in adult mice causes splenomegaly and lymphomas. Oncogene 2006;25:929–39.
28. Ichikawa M, Asai T, Saito T, et al. AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cell in adult hematopoiesis. Nat Med 2004;10:299–304.
29. Fischer M, Schwieger M, Horn S, et al. Defining the oncogenic function of the TEL/AML1 (ETV6/RUNX1) fusion protein in a mouse model. Oncogene 2005;24: 7579–91.
30. Lorsbach R, Moore J, Ang S, et al. Role of RUNX1 in adult hematopoiesis: analysis of RUNX1-IRES-GFP knock-in mice reveals differential lineage expression. Blood 2004;103:2522–9.
31. North T, Staca T, Matheny J, Speck N, de Bruijn M. Runx1 is expressed in adult mouse hematopoietic stem cells and differentiating myeloid and lymphoid cells, but not in maturing erythroid cells. Stem Cells 2004;22: 158–68.
32. Okuda T, Cai Z, Yang S, et al. Expression of a knocked-in AML1-ETO leukemia gene inhibits the establishment of normal definitive hematopoiesis and directly generates dysplastic hematopoietic progenitors. Blood 1998;91:3134–43.
33. Zhang L, Li Z, Yan J, et al. Mutagenesis of the Runt domain defines two energetic hot spots for heterodimerization with the core binding factor β subunit. J Biol Chem 2003;278:33097–104.
34. Huang G, Shigesada K, Ito K, et al. Dimerization with PEBP2β protects RUNX1/AML1 from ubiquitin-proteasome-mediated degradation. EMBO J 2001;15:723–33.
35. Wahlers A, Zipfel P, Schwieger M, Ostertag W, Baum C. *In vivo* analysis of retroviral enhancer mutations in hematopoietic cells: SP1/EGR1 and ETS/GATA motifs contribute to long terminal repeat specificity. J Virol 2002;76:303–12.
36. Yan J, Liu Y, Lukasik S, Speck N, Bushweller J. CBFβ allosterically regulates the Runx1 runt domain via a dynamic conformational equilibrium. Nat Struct Biol 2004;11:901–6.
37. Akamatsu Y, Tsukumo S, Kagoshima H, Tsurushita N, Shigesada K. A simple screening for mutant DNA binding proteins: application to murine transcription factor PEBP2α subunit, a founding member of the Runt domain protein family. Gene 1997;31:111–7.
38. Westendorf J, Yamamoto C, Lenny N, et al. The t(8;21) fusion product, AML-1-ETO, associates with C/EBP-α, inhibits C/EBP-α-dependent transcription, and blocks granulocytic differentiation. Mol Cell Biol 1998; 18:323–33.
39. Petrovick M, Hiebert S, Friedman A, et al. Multiple functional domains of AML1: PU.1 and C/EBPα synergize with different regions of AML1. Mol Cell Biol 1998;18:3915–25.
40. Elagib K, Racke F, Mogass M, et al. RUNX1 and GATA-1 coexpression and cooperation in megakaryocytic differentiation. Blood 2003;101:4333–41.
41. Hess J, Porte D, Munz C, Angel P. AP-1 and Cbfa/Runt physically interact and regulate parathyroid hormone-dependent MMP13 expression in osteoblasts through a new osteoblast-specific element 2/AP-1 composite element. J Biol Chem 2001;276:20029–38.
42. Pabst T, Mueller B, Harakawa N, et al. AML1-ETO downregulates the granulocytic differentiation factor C/EBP-α in t(8;21) myeloid leukemia. Nat Med 2001;7:1–8.
43. Levanon D, Goldstein R, Bernstein Y, et al. Transcriptional repression by AML1 and LEF-1 is mediated by the TLE/Groucho corepressors. Proc Natl Acad Sci U S A 1998;95:11590–5.
44. Bruhn L, Munnerlyn A, Grosschedl R. ALY, a context-dependent coactivator of LEF-1 and AML-1, is required for TCRα enhancer function. Genes Dev 1997;11:640–53.
45. Yagi R, Chen L, Shigesada K, Murakami Y, Ito Y. A WW domain-containing yes-associated protein (YAP) is a novel transcriptional co-activator. EMBO J 1999;18: 2551–62.
46. Wheeler J, VanderZwan C, Xu X, et al. Distinct *in vivo* requirements for establishment versus maintenance of transcriptional repression. Nat Genet 2002;32:206–10.
47. Waltzer L, Ferjoux G, Bataille L, Haenlin M. Cooperation between the GATA and RUNX factors Serpent and Lozenge during *Drosophila* hematopoiesis. EMBO J 2003;22:6516–25.
48. Porcher C, Liao E, Fujiwara L, Zon L, Orkin S. Specification of hematopoietic and vascular development by the bHLH transcription factor SCL without direct DNA binding. Development 1999;126:4603–15.
49. Scully K, Rosenfeld M. Pituitary development: regulatory codes in mammalian organogenesis. Science 2002;295:2231–5.
50. Zappavigna V, Sartori D, Mavilio F. Specificity of HOX protein function depends on DNA-protein and protein-protein interactions, both mediated by the homeo domain. Genes Dev 1994;8:732–44.