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SHORT COMMUNICATION

Runx1 deficiency permits granulocyte lineage commitment but impairs subsequent maturation

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First-hits in the multi-hit process of leukemogenesis originate in germline or hematopoietic stem cells (HSCs), yet leukemia-initiating cells (LICs) usually have a lineage-committed phenotype. The molecular mechanisms underlying this compartment shift during leukemia evolution have not been a major focus of investigation and remain poorly understood. Here a mechanism underlying this shift was examined in the context of Runx1 deficiency, a frequent leukemia-initiating event. Lineage-negative cells isolated from the bone marrow of *Runx1*-haploinsufficient and wild-type control mice were cultured in granulocyte-colony-stimulating factor to force lineage commitment. *Runx1*-haploinsufficient cells demonstrated significantly greater and persistent exponential cell growth than wild-type controls. Not surprisingly, the *Runx1*-haploinsufficient cells were differentiation-impaired, by morphology and by flow-cytometric evaluation for granulocyte differentiation markers. Interestingly, however, this impaired differentiation was not because of decreased granulocyte lineage commitment, as RNA and protein upregulation of the master granulocyte lineage-commitment transcription factor Cebpa, and Hoxb4 repression, was similar in wild-type and *Runx1*-haploinsufficient cells. Instead, RNA and protein expression of Cebpe, a key driver of progressive maturation after lineage commitment, were significantly decreased in *Runx1*-haploinsufficient cells. Primary acute myeloid leukemia cells with normal cytogenetics and *RUNX1* mutation also demonstrated this phenotype of very high CEBPA mRNA expression but paradoxically low expression of CEBPE, a CEBPA target gene. Chromatin-immunoprecipitation analyses suggested a molecular mechanism for this phenotype: in wild-type cells, Runx1 binding was substantially greater at the *Cebpe* than at the *Cebpa* enhancer. Furthermore, Runx1 deficiency substantially diminished high-level Runx1 binding at the *Cebpe* enhancer, but lower-level binding at the *Cebpa* enhancer was relatively preserved. Thus, Runx1-deficiency permits Cebpa upregulation and the exponential cell growth that accompanies lineage commitment, but by impairing activation of *Cebpe*, a key proliferation-terminating maturation gene, extends this exponential growth. These mechanisms facilitate germline cell or HSC of origin, yet evolution into LIC with lineage-committed phenotype.

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INTRODUCTION

Known first-hits in the multi-hit process of leukemogenesis are inactivating or dominant-negative mutations in *RUNX1*.[1] These first-hits originate in the germline in familial acute myeloid leukemia (AML, reviewed in the study by Owen *et al.*[2]) and in hematopoietic stem cells (HSCs) in acquired AML.[3–5] Germline or stem cell origin of the first hit is necessary as these cells live long enough that there is a feasible possibility of secondary genetic abnormalities (additional hits) that can cooperate for evolution into AML. However, some of these secondary genetic abnormalities occur in daughter cells with restricted lineage potential. This is evident in familial AML, in which neoplastic evolution does not occur in the germline cells of origin, but is also true of acquired AML: self-renewing AML cells, also known as leukemia-initiating cells (LICs) or leukemia stem cells, have a lineage-committed phenotype by numerous surface markers (CD34 + 38 +, CLL-1 +, CD71 +, CD90 −, c-Kit-),[6–16] by strikingly high expression of lineage-specifying transcription factors such as CEBPA, PU.1/SPI1 or GATA1,[17–19] by inactivation of polycomb repressor complex 2 components that usually suppress lineage programs,[20] and by the observation that highly recurrent secondary genetic abnormalities such as *FLT3* mutation are not detected in the HSC compartment.[5]

Thus, a first-hit is present or originates in HSC, but creates conditions that favor further neoplastic evolution not necessarily in the HSC compartment itself but in lineage-committed progenitors. The molecular mechanisms underlying this compartment shift during leukemia evolution have not been a major focus of investigation and are poorly understood. A better understanding of these mechanisms could provide guidance for novel

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treatments that exploit the lineage-committed cellular context of LIC to thereby spare uncommitted normal HSC.

RESULTS AND DISCUSSION

To investigate a basis for compartment shifts during evolution of myeloid cancers, lineage-negative HSC from wild-type and *Runx1* haploinsufficient mice[21] were cultured in granulocyte-colony-stimulating factor (G-CSF) to force lineage commitment. The *Runx1*-haploinsufficient cells demonstrated more rapid and persistent proliferation than wild-type controls (Figure 1a). This growth advantage was also apparent in semi-solid media supplemented with G-CSF: *Runx1*-haploinsufficient cells produced a higher number and larger-sized colonies than wild-type control (Figure 1b). The *Runx1*-haploinsufficient cells were differentiation-impaired: after 15 days of culture, *Runx1*-haploinsufficient cells included mitotic figures and immature forms with high nuclear-cytoplasmic ratios and decreased neutrophilic granulation, whereas wild-type cells demonstrated mostly mature granulocytes (Figure 1c). Furthermore, the granulocyte-lineage markers Ly6G and CD11b were upregulated to a much lesser extent in *Runx1*-haploinsufficient cells than in wild-type controls (Figure 1d).

To better understand the molecular mechanisms underlying the persistent proliferation and impaired differentiation, expression patterns of key hematopoietic transcription factors that determine lineage commitment and progressive maturation were examined. These were Hoxb4 that promotes self-renewal by HSC, Cebpa that drives granulocyte lineage commitment, and Cebpe that drives

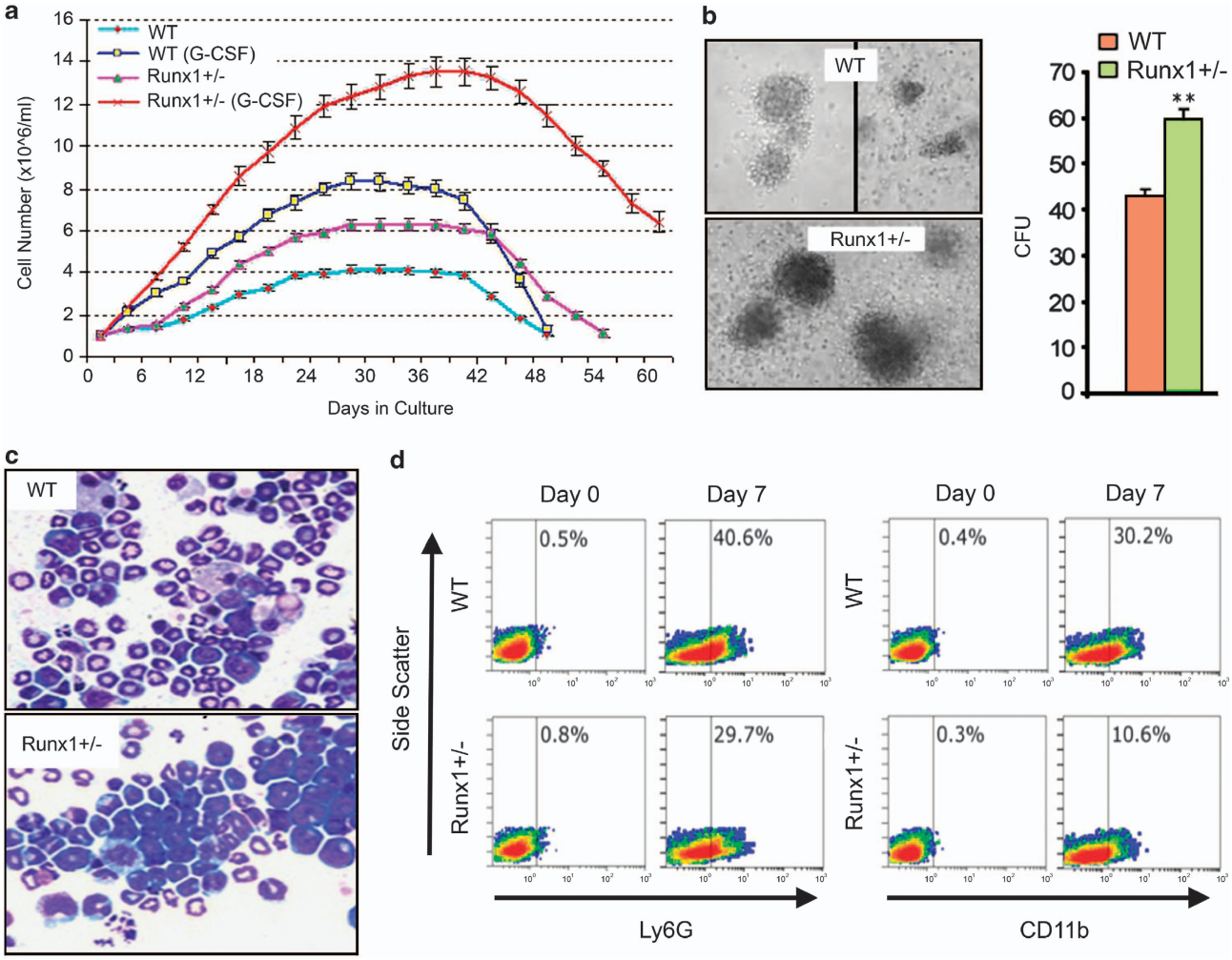

Figure 1. Runx1-haploinsufficient cells are able to lineage commit but have impaired subsequent maturation. Lineage-negative (Lin −) cells were isolated from bone marrow of *Runx1* +/− (haploinsufficient) and wild-type (WT) C57BL/6 mice using MACS Lineage Cell Depletion Kit (#130-090-858, Miltenyi Biotec Inc., Auburn, CA, USA). (**a**) Cell counts. Cytokine supplementation: 10 ng/ml of mSCF and 100 ng/ml of thrombopoietin (non-differentiation promoting conditions) or 10 ng/ml of murine stem cell factor, 10 ng/ml of mIL-6 and G-CSF 20 ng/ml (PeproTech Inc., Rocky Hill, NJ, USA). Cell numbers were determined by automated cell counter every 3 days during 60 days of culture. Mean ± standard deviation. Experiments performed in triplicate. (**b**) Colony formation by Runx1 +/− and WT Lin − cells in semi-solid media. 2×10^4cells/ml of methylcellulose (MethoCult GF M3434; Stem Cell Technologies, Vancouver, Canada). Colonies counted and imaged by inverted microscope on day 10 (LeicaDMI6000B inverted microscope, Leica Microsystems, Wetzlar, Germany). Experiments performed in triplicate. ** $p < 0.01$, Wilcoxon test. (**c**) Runx1 +/− and WT cell morphology after 15 days of culture with G-CSF. Cytospins were generated with a Shandon CytoSpinIII (Thermo Scientific, Pittsburgh, PA, USA; 500 r.p.m., 5 min). Air-dryed cells fixed with 100% methanol were Giemsa-stained for visualization using a Leica DMR light microscope equipped with CCD camera (Leica Microsystems), magnification ×630. (**d**) Ly6G and CD11b granulocyte-lineage marker expression in *Runx1* +/− and WT Lin − cells on day 0 (D0) and day 7 (D7) of culture with G-CSF. Expression measured by Coulter Epics XL-MCL flow cytometer and CXP software (Beckman-Coulter, Brea, CA, USA). Antibodies used were anti-mouse Ly6G-FITC (eBiosciences, San Diego, CA, USA; #551460), anti-mouse CD11b-PE (eBiosciences, #120112), and isotype-matched immunoglobulin control.

late granulocyte maturation and terminates proliferation.[22–27] Repression of *Hoxb4* and activation of *Cebpa* by G-CSF was similar in wild-type and *Runx1*-haploinsufficient cells (Figure 2a). Despite the upregulation of Cebpa, *Cebpe* activation was significantly decreased in the *Runx1*-haploinsufficient cells, even after 15 days of culture with G-CSF (Figure 2a). These observations were recapitulated at the protein level (Figure 2b). This pattern of gene expression is also a characteristic of primary AML cells with mutated *RUNX1* and normal cytogenetics: *CEBPA* was on average >30-fold more expressed than *CEBPE* and >6-fold more expressed than *HOXB4* (Figure 2c). Chromatin-immunoprecipitation analysis showed a basis for differential *Cebpa* and *Cebpe* activation in Runx1-deficient cells: in wild-type cells, Runx1 binding was substantially greater at the *Cebpe* than at the *Cebpa* enhancer[28,29] (Figure 2d). Furthermore, Runx1-deficiency substantially diminished high level Runx1 binding at the *Cebpe* enhancer,

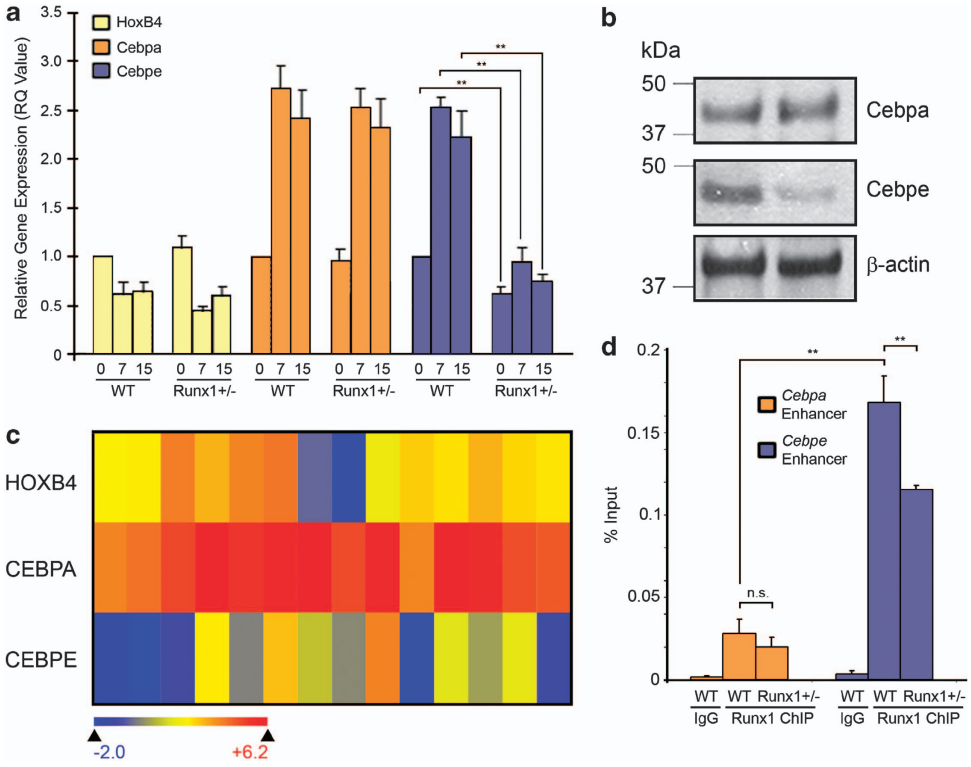

Figure 2. Unequal impact of Runx1 deficiency on Runx1 binding at the *Cebpa* and *Cebpe* enhancers and on *Cebpa* and *Cebpe* activation in response to G-CSF. (**a**) Time-course expression of Hoxb4 (key stem cell transcription factor), Cebpa (key lineage-commitment transcription factor) and Cebpe (key late-differentiation transcription factor) in WT and Runx1 +/− lineage-negative (Lin −) cells cultured with G-CSF for 15 days. SYBR Green bound to double-stranded DNA was detected in real-time with the 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Relative expression values were calculated by raising 2 to the power of the negative value of delta-delta CT for each sample. Primer sequences: HoxB4: forward (F): 5′-CCAGAATCGGCGCATGA-3, reverse (R): 5′-CCCGAGCGGATCTTGGT-3′; Cebpa: F: 5′-CA AAGCCAAGAAGTCGGTGGACAA-3′; R: 5′-TCATTGTCACTGGTCAACTCCAGC-3′; Cebpe: F: 5′-GCTACAATCCCCTGCAGTACC-3′; R: 5′-TGCCTTCTT GCCCTTGTG-3′; GAPDH: F: 5′-ACCACACTCCATGCCATCAC-3′, R:5′-TCCACCACCCTGTTGCTGTA-3′. Mean ± standard deviation. $^{**}P<0.01$, Student's *t*-test. Experiments performed in triplicate. (**b**) Cebpa and Cebpe protein expression in WT and Runx1 +/− Lin − cells cultured for 15 days with G-CSF. Western blot analyses with the following primary antibodies: anti-Cebpa, anti-Cebpe (Santa Cruz Biotechnology, Dallas, TX, USA; #SC-61, #SC-25770), anti-β-actin (Sigma-Aldrich, St Louis, MO, USA, A3854); secondary antibodies: anti-Rabbit (GE Healthcare, Waukesha, WI, USA; NA934) and anti-Mouse (GE Healthcare, NXA931) at 1:5000 and 1:10 000 dilutions, respectively. (**c**) Gene expression of CEBPA, CEBPE and HOXB4 in primary AML cells with normal cytogenetics and RUNX1 mutation (TCGA database, $n=14$). Heatmap by Arraystar software. (**d**) Chromatin immunoprecipitation (ChIP) analysis of Runx1 +/− and WT cells to evaluate Runx1 binding at the *Cepba* and *Cebpe* enhancers. Enhancers of *Cebpa* and *Cebpe* were identified by others using ChIP coupled with deep sequencing.[28] ChIP performed using ChIP Assay Kit (cat#17-295, EMD Millipore, Billerica, MA, USA). 5×10^6 cells bone marrow (BM) cells from WT and Runx1 +/1 fresh BM cells were resuspended in 10 ml of media and formaldehyde was added to a final concentration of 1% followed by 10 min incubation at 37 °C. The media containing formaldehyde was removed and the cells were washed twice with ice-cold PBS containing protease inhibitor (cat#P8340, Sigma-Aldrich) and phosphatase inhibitor cocktails (cat#P0044, Sigma-Aldrich). Crosslinked cells were resuspended in SDS lysis buffer (Millipore, #20–163) and incubated for 10 min on ice. These cell pellets were sonicated at 15 s pulses with 45 s hold on ice for a total of 10 min of pulsing at full power (Fisher-Scientific Model #550 Sonic Dismembrator equipped with a microtip probe). Genomic DNA fragments of 200-bp and 1-kb in size were obtained (confirmed by agarose gel electrophoresis). After centrifugation at 4 °C, the supernatant was pre-cleared with salmon sperm DNA/Protein A agarose-50% slurry (Millipore, #16–157) for 30 min at 4 °C with agitation. A 10% aliquot (vol) of the precleared protein/DNA mixture was removed and used for subsequent reverse transcription–PCR input quantification. 10 μg of anti-Runx1 ChIP grade antibody (cat#ab23980, Abcam, Cambridge, MA, USA) was added to the remaining protein/DNA mixture and incubated at 4 °C overnight with rotation. Salmon sperm DNA/Protein A agarose-50% slurry was added and incubated for 1 h at 4 °C followed by washes with the ChIP assay kit provided buffers. The immunoprecipitated (IP) complexes were reverse crosslinked at 65 °C for 4 h and DNA was recovered by phenol/chloroform extraction protocol (Sigma). The IP products were amplified and quantified using real-time PCR. ChIP primer sequences are:[21] *cepba* enhancer left primer, 5′- TTCCCGTTTCTGAAATCTGC-3′, *cebpa* enhancer right primer, 5′-GGTTGTGGCAAGAAGGTCAC-3′, *cebpe* enhancer left primer, 5′-GTGTCATGGTCACCCTAGCC-3′ and *cebpe* enhancer right primer, 5′-CTGGAGCTAGCAGGGGTTTT-3′. Mean ± standard deviation from three independent ChIP experiments. $^{**}P<0.001$, NS = not significant, Tukey–Kramer HSD test.

but lower level binding at the *Cebpa* enhancer was relatively preserved (Figure 2d). In other words, RUNX1 is more abundant at, and presumably more important, for regulating the *Cebpe* enhancer than the *Cebpa* enhancer.

Consistent with the present results, *Runx1*-haploinsufficient mice have decreased HSC but increased myeloid progenitors compared with wild-type controls.[21] Similarly, a murine knock-in model of point-mutated *Runx1* demonstrated unaltered HSC emergence but defects in multiple committed hematopoietic lineages, and hematopoietic cells containing the leukemia fusion protein RUNX1-ETO demonstrated delayed granulocytic differentiation.[30,31] Previously, we demonstrated that even CD34 + 38 − subsets of primary AML cells demonstrate this pattern of very high CEBPA but relatively low HOXB4 and CEBPE expression compared with normal hematopoietic precursors, promyelocytes and neutrophils.[18,32,33] Ideally, leukemia treatments would suppress malignant clones but preserve normal HSC needed to restore blood counts. High expression of key transcription factors that drive differentiation (for example, CEBPA, PU.1) could be a difference between LIC and normal HSC that facilitates this goal, especially since the proliferation-terminating differentiation genes usually activated by these transcription factors, although aberrantly suppressed in leukemia cells (for example, *CEBPE*), are genetically intact and thus in theory available for activation. For example, high baseline CEBPA expression explains rapid restoration of CEBPE expression and cycle exit by maturation of primary AML cells treated with corepressor antagonizing drugs (for example, decitabine) or with FLT3 inhibitors.[34] The same treatments preserve self-renewal of uncommitted normal HSC that do not express high levels of lineage-specifying transcription factors at baseline.[35–37]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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