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# The assembly competence domain is essential for inv(16)-associated acute myeloid leukemia

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Chromosomal rearrangements involving the two subunits of the heterodimeric transcription factor, core-binding factor (CBF), are the most commonly observed cytogenetic abnormalities in adult acute myeloid leukemia (AML). CBF is comprised of one of three potential DNA-binding α subunits (RUNX1, RUNX2, or RUNX3)[1] that interact with a non-DNA-binding subunit, CBFβ. The major rearrangement affecting CBFβ generates the inv(16)(p13.1q22), which fuses the first 165 amino acids of CBFβ with the coiled-coil rod domain of the smooth muscle myosin heavy chain (SMMHC) gene, *MYH11*.[2,3] The resulting CBFβ-SMMHC fusion protein retains the ability to interact with RUNX1, and functions by dominantly inhibiting normal CBF activity. This view is supported by studies where *CBFβ-MYH11* was knocked into the endogenous *CBFβ* locus, which resulted in an



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**CONFLICT OF INTEREST**

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**AUTHORSHIP**

Contribution: H-G.K., C.S.S., H.J.N., J.L., S.P., C.V.C., R.K., and L.G. designed and performed the experiments; D.C. provided biostatistical analysis; V.R. analyzed histology results; S.W.H., A.D.F., and C.A.K. designed experiments; H-G.K. and C.A.K. wrote the manuscript.

early embryonic lethality that phenocopied many of the developmental abnormalities observed in mice with homozygous deletions of either *RUNX1* or *CBFβ*.[4,5] CBFβ-SMMHC may dominantly inhibit CBF function by recruiting nuclear co-repressor molecules, including mSin3A and HDAC8, to a C-terminal region of SMMHC that includes the 28-amino-acid assembly competence domain (ACD), which mediates skeletal or smooth muscle myosin oligomerization and filament formation.[6–9]

To examine the role of the ACD in promotion of AML *in vivo*, we generated retroviral constructs that co-expressed a GFP reporter and either CBFβ-SMMHC or a CBFβ-SMMHC mutant lacking the 28-amino-acid ACD (Figure 1a). Bone marrow (BM) isolated from C57BL/6-CD45.2 animals treated with 5-fluorouracil to enrich for hematopoietic stem/progenitor cells (HSPC) was transduced with each retroviral vector and then transplanted into lethally-irradiated, congenic C57BL/6-CD45.1 mice. Western blot analysis of $GFP^+$ splenocytes isolated from 4–5-month post-transplant (PT) animals indicated that each fusion protein was expressed at equivalent levels (Figure 1b).

Analysis of transplant recipients showed that mice reconstituted with CBFβ-SMMHC-expressing cells (CBFβ-SMMHC mice) died of AML between 4–7 months PT (Figure 1c). Disease penetrance was 100% when $GFP^+$ cells exceeded 5% in peripheral blood (PB) at 3–4 weeks PT ($GFP^+$ chimerism ranged between 5.7–11.6%, mean=9.1%, n=16). $GFP^+$ chimerism in MIG control and ΔACD mice was stable over time (Figure 1d), with no MIG or ΔACD animal showing outward signs of sickness at least 12 months PT. The comparable expression levels of the ΔACD mutant and CBFβ-SMMHC indicated that this does not account for their differing potencies in promoting AML.

Moribund CBFβ-SMMHC animals displayed splenomegaly (Figures 1e) and hepatomegaly (data not shown) and showed effacement of normal splenic architecture with expanded splenic red pulp and infiltrating blasts (Figure 1f). Leukemic infiltrate was also noted in the sinusoidal space of the liver and alveolar septae of the lung (Figure 1f). Transfer of one million BM cells from two independent moribund CBFβ-SMMHC animals with AML into each of 5 lethally-irradiated secondary recipient mice showed that leukemia was transplantable, with secondary recipients becoming moribund more rapidly than primary recipients at 8–12 weeks PT (Figure 1g). All CBFβ-SMMHC animals exhibited an initial pre-leukemic period between 1–4 months PT where stable, low-level expression of CBFβ-SMMHC (based on the GFP surrogate marker) in PB was followed by rapid emergence of a $GFP^+$ blast population that uniformly expressed high CBFβ-SMMHC (Figure 1d). Invariably, animals that exhibited a spike in $GFP^+$ blasts succumbed to AML within 2–4 weeks.

FACS analysis of BM myeloid development showed similar Mac-1/Gr-1 staining profiles for MIG, ΔACD, or pre-leukemic (prior to the spike in $GFP^+$ PB blasts) CBFβ-SMMHC mice at 2–4 months PT (Supplementary Figure S1a). Differential counts and histochemical staining of cytospun, FACS-sorted $GFP^+$ BM cells from MIG and ΔACD mice were also normal, while both pre-leukemic and moribund CBFβ-SMMHC mice showed 5–10-fold increased frequencies of primitive myeloid cells (Supplementary Figure S1b, Supplementary Table S1). Since $GFP^+$ chimerism levels in pre-leukemic CBFβ-SMMHC mice were stable and

typically <10% (Figure 1d), total blast frequencies within the collective $GFP^+$ and $GFP^-$ BM fraction of pre-leukemic CBFβ-SMMHC mice were not appreciably increased.

Consistent with differential counts, plating of 10,000 $GFP^+$ BM cells FACS-sorted from each of 6 independent MIG, ΔACD, and pre-leukemic CBFβ-SMMHC animals into methylcellulose showed a 14.5-fold increase in myeloid CFU from pre-leukemic CBFβ-SMMHC mice ($p$=0.001) and a modest, although non-significant, 1.9-fold expansion from ΔACD animals ($p$=0.73)(Supplementary Figure S1c). Serial replating of an equivalent number of $GFP^+$ BM cells FACS-sorted from 3 additional MIG, ΔACD and pre-leukemic CBFβ-SMMHC mice showed an initial 3–4-fold increase in myeloid CFU in pre-leukemic CBFβ-SMMHC cultures but this difference diminished over two additional passages as previously reported using CBFβ-SMMHC knock-in BM cells.[10] Myeloid CFU numbers were similar between MIG control and ΔACD cultures in all passages (Supplementary Figure S1d). Together, these results showed that loss of the ACD completely impaired the ability of CBFβ-SMMHC to arrest early myeloid development.

Characterization of B lymphopoiesis in BM of pre-leukemic CBFβ-SMMHC mice at 2–4 months PT showed that CBFβ-SMMHC blocked B cell development prior to the first B-committed progenitor stage ($B220^+CD19^+$) (Supplementary Figure S1e, middle panel)[11], which was not observed in MIG control or ΔACD animals, indicating this block was ACD-dependent. Thymocyte development was suppressed by either CBFβ-SMMHC[12] or the ΔACD mutant ($p$<0.0001), with very low percentages of $GFP^+$ thymocytes in CBFβ-SMMHC- or ΔACD-reconstituted animals that had high percentages of $GFP^+$ BM cells at 2–4 months PT (Supplementary Figure S1f). These results indicate that sequences outside of the ACD contribute to CBFβ-SMMHC inhibition of thymocyte development.

One model to explain leukemic progression in pre-leukemic CBFβ-SMMHC mice could involve significant expansion of the HSPC compartment to increase the probability of secondary leukemogenic events in actively dividing HSPC. To address this, we compared the absolute number of KLS cells between MIG control and pre-leukemic CBFβ-SMMHC mice at 3–4 months PT and observed no significant difference (n=5, Supplementary Table S2). In leukemic CBFβ-SMMHC animals, KLS cell numbers modestly increased ~2-fold relative to MIG ($p$=0.044, two-tailed $t$-test) or pre-leukemic CBFβ-SMMHC ($p$=0.013) mice. This indicates that secondary events leading to leukemic progression in this spontaneous AML progression model do not require substantial prior KLS cell expansion.

To characterize BM subsets that function as leukemia-initiating cells (LIC) in pre-leukemic and leukemic CBFβ-SMMHC mice, we double-FACS sorted to >98% purity the KLS or myeloid progenitor cell (MPC, c-$Kit^+Lin^-Sca\text{-}1^-$) subsets from individual moribund CBFβ-SMMHC animals (designated β1-β5, Figure 2a) and transplanted graded cell doses into lethally-irradiated congenic mice. Approximately 5,000 $GFP^+$ MPC represented a limiting dilution dose of LIC from all moribund CBFβ-SMMHC mice (n=3), with 75–80% of secondary recipients developing AML by 3–4 months PT (Figure 2a). Since KLS cells only modestly expanded in CBFβ-SMMHC animals, we only obtained enough double-sorted cells from individual moribund mice to transfer 1,000 cells into multiple secondary recipients. At this dose, there was no evidence of AML or $GFP^+$ cells at least 5 months PT

using cells from 5 primary donor mice (Figure 2a). It remains possible that LIC exist within the KLS subset if more cells were available for transplant.

To address whether LIC existed in the MPC subset of 2–4-month pre-leukemic CBFβ-SMMHC mice, we transferred either 5,000 or 20,000 double-sorted MPC into multiple lethally-irradiated recipient mice and observed no evidence of AML or $GFP^+$ cells at least 5 months PT (Figure 2b). This suggests that MPC only function as LIC after they have acquired additional oncogenic changes that result in acute AML.

To understand how ACD loss blocks development of AML, we performed microarray analysis using RNA isolated from c-$Kit^+Lin^-Sca\text{-}1^+Flt3^-$ (KLSF) cells that were double-sorted and then transduced with MIG, ΔACD, or CBFβ-SMMHC retroviruses for 24 hours prior to re-sorting $GFP^+$ cells. Unsupervised cluster analysis showed a striking similarity between MIG and ΔACD samples, with only 20 genes differentially regulated >2-fold ($p<0.005$) between the groups (Supplementary Figure S2). In contrast, 771 genes were differentially regulated between MIG and CBFβ-SMMHC (GEO accession # GSE85659), with many changes that might contribute to CBFβ-SMMHC-associated AML (Figure 2C, Supplementary Table S3). These differences included significant downregulation of myeloid transcription factors including C/EBPα, Sox4, Hoxa9, Mef2C, and Irf8/ICSBP (decreased 4.5-, 4.3-, 4.2-, 3.0-, 3.0-fold, respectively), which might account for MPC accumulation in pre-leukemic CBFβ-SMMHC mice (Supplementary Table S1). Other factors implicated in myeloid development and leukemogenesis that were up-regulated by CBFβ-SMMHC included Fosb, Egr2, c-Jun, WT1, Egr1, and Prdm16, (3.6-, 2.5-, 2.3-, 2.3-, 2.1-, and 2.0-fold, respectively). Some factors promote monocytic differentiation, including fos/jun complexes and early growth factor (Egr1 and Egr2)[13,14], which may contribute to the myelomonocytic phenotype of $inv(16)^+$ human AML. The highly similar expression profiles between MIG and ΔACD in KLSF cells strongly suggests that ACD loss completely abrogates CBFβ-SMMHC activity in HSPC.

We have demonstrated that the 28-amino-acid ACD is essential for promotion of AML by CBFβ-SMMHC. Since the ACD mediates both oligomerization and binding of nuclear co-repressor molecules like mSin3A and HDAC8,[6,7] it is presently not possible to distinguish whether the loss of either (or both) activities is responsible for the AML-promoting activity of CBFβ-SMMHC. Genetic ablation or pharmacologic inhibition of HDAC8 significantly suppresses the incidence and kinetics of AML development using the CBFβ-SMMHC knock-in mouse model, as well as LIC activity of human $inv(16)^+$ AML cells,[15] which suggests that targeting HDAC8 binding to the ACD may be a promising approach for treatment of $inv(16)^+$ AML in the clinic.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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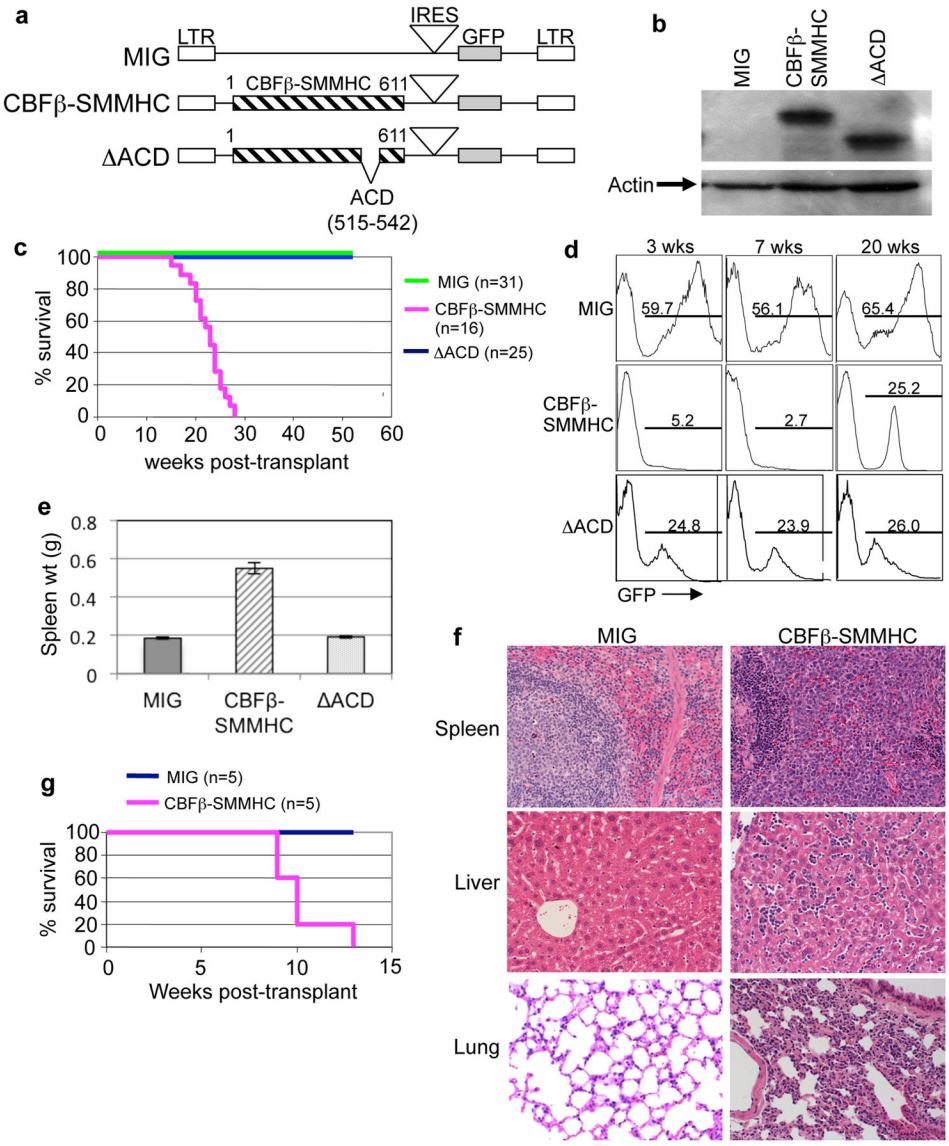
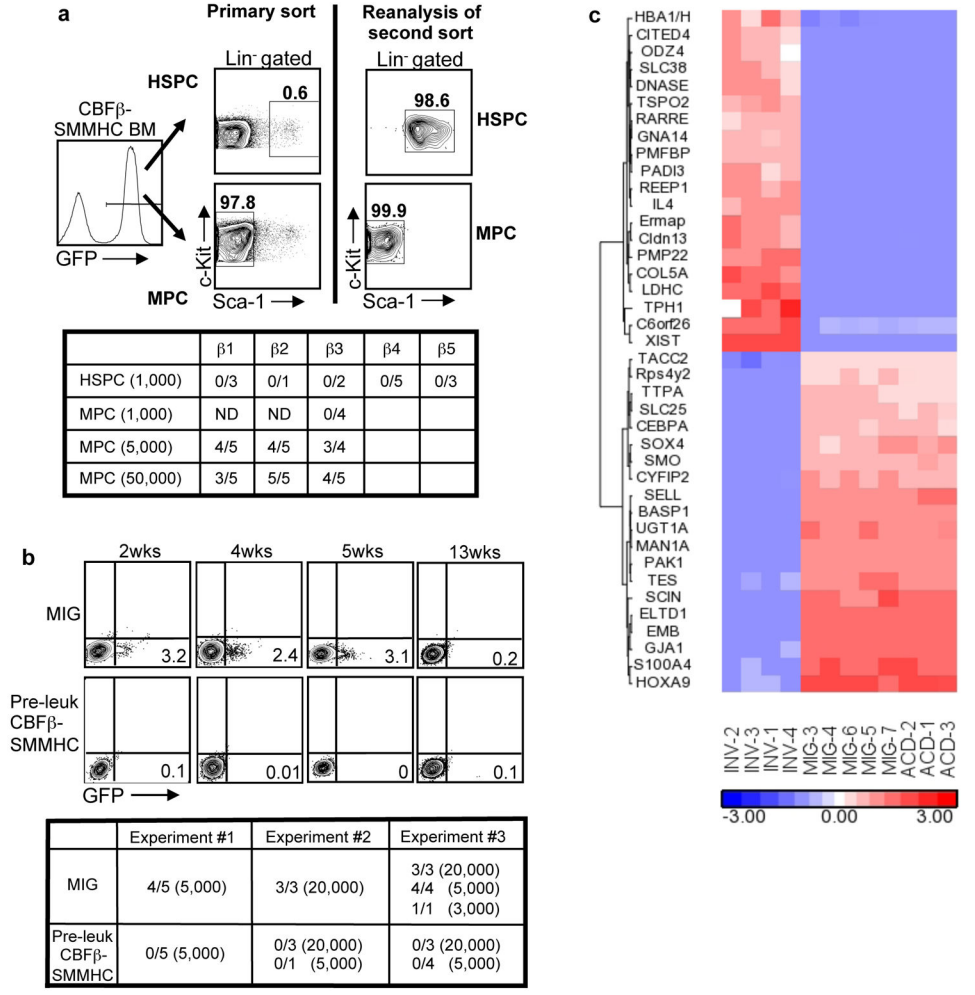



**Figure 1.**

The ACD domain is necessary for CBFβ-SMMHC-associated AML. (a) Structure of retroviral constructs. LTR, long terminal repeat; IRES, internal ribosome entry site; GFP, green fluorescent protein; ACD, assembly-competence domain. (b) Western blot analysis of $2 \times 10^6$ GFP$^+$ splenocytes isolated from a 4–5-month PT ΔACD and moribund CBFβ-SMMHC animal. (c, g) Kaplan-Meier survival curve for primary (c) and secondary transplant recipients (g). The representative experiment shown in (g) was done using one million bone marrow cells from a moribund CBFβ-SMMHC animal. (d) Peripheral blood analysis of GFP chimerism in representative MIG control (n=31), CBFβ-SMMHC (n=16) and ΔACD (n=25) animals. GFP$^+$ chimerism in MIG control and ΔACD mice was stable over time and ranged from 29.7–87.5%, mean=56.8%, for MIG control mice (n=31); and 10.4–51.0%, mean=25.0%, for ΔACD mice (n=25). GFP chimerism levels in CBFβ-SMMHC mice remained stable and low preceeding an acute phase marked by rapid GFP$^+$

cell expansion in the periphery. (e) Splenomegaly in all moribund CBFβ-SMMHC animals (n=10) versus transplant-age matched MIG controls (n=10) and ΔACD mice (n=8)($p<0.05$). Spleen weight is shown in grams. (f) Histopathology of representative MIG control (n=10) and moribund CBFβ-SMMHC animals (n=10). Leukemic infiltrate is notable in splenic red pulp, the liver sinusoids, and the alveolar septae of the lung in all sick CBFβ-SMMHC mice (all images are at 100X magnification).




| | β1 | β2 | β3 | β4 | β5 |
|---|---|---|---|---|---|
| HSPC (1,000) | 0/3 | 0/1 | 0/2 | 0/5 | 0/3 |
| MPC (1,000) | ND | ND | 0/4 | | |
| MPC (5,000) | 4/5 | 4/5 | 3/4 | | |
| MPC (50,000) | 3/5 | 5/5 | 4/5 | | |

| | Experiment #1 | Experiment #2 | Experiment #3 |
|---|---|---|---|
| MIG | 4/5 (5,000) | 3/3 (20,000) | 3/3 (20,000)<br>4/4 (5,000)<br>1/1 (3,000) |
| Pre-leuk CBFβ-SMMHC | 0/5 (5,000) | 0/3 (20,000)<br>0/1 (5,000) | 0/3 (20,000)<br>0/4 (5,000) |

**Figure 2.**

Myeloid progenitor cells from leukemic CBFβ-SMMHC mice contain leukemia-initiating cell (LIC) activity. (a) Hematopoietic stem/progenitor cells (HSPC) of the phenotype c-Kit$^+$Lin$^-$Sca-1$^+$ and myeloid progenitor cells (MPC) of the c-Kit$^+$Lin$^-$Sca-1$^-$ phenotype were FACS-sorted once and then re-sorted from the GFP$^+$ fraction of BM from moribund CBFβ-SMMHC animals (n=5, β1-β5) prior to transplantation at varying doses into lethally-irradiated secondary recipient mice. The numbers of transplanted cells per recipient mouse and the total number of multi-lineage donor reconstituted mice at 5 months PT is shown. ND=not done. (b) MPC from pre-leukemic CBFβ-SMMHC animals do not have LIC activity. Differing numbers of MPC double-FACS sorted from 3 MIG or 3 pre-leukemic CBFβ-SMMHC animals (indicated in parentheses) were transplanted into multiple lethally-irradiated secondary recipient mice in 3 independent experiments. The total number of mice transiently reconstituted with donor-derived (GFP$^+$) cells in peripheral blood (numerator) among the total number of transplanted mice (denominator) was assessed at the indicated time points post-transplantation, with representative FACS analysis shown for one reconstituted animal tranplanted with 20,000 cells from either an MIG control or pre-leukemic CBFβ-SMMHC animal. (c) Hierarchical cluster analysis indicating the top 20

differentially up- and down-regulated genes in KLSF cells by CBFβ-SMMHC (INV) compared with MIG control (absolute fold-difference is shown in Supplementary Table S3). Hierarchical clustering was done using the gene expression profiles of KLSF cells that were transduced with either the MIG control (n=5), ΔACD (n=3), or CBFβ-SMMHC (INV) (n=4) retroviruses for 24 hours prior to re-sorting $GFP^+$ (transduced) cells for RNA isolation and expression analysis using Affymetrix 430 2.0 GeneChip arrays. False discovery rate (FDR) *p*-value correction was applied. The color scale indicates log2-transformed normalized intensity ranging from low in blue and high expression in red.