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The C-terminal multimerization domain is essential for leukemia development by CBFβ-SMMHC in a mouse knockin model

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Chromosome 16 inversion, inv(16), is the signature chromosome abnormality in M4Eo subtype of acute myeloid leukemia (AML), which produces a fusion gene *CBFB-MYH11*[1]. We previously generated a knockin mouse model for *CBFB-MYH11* ($Cbfb^{+/MYH11}$)[2]. Heterozygous *Cbfb-MYH11* knockin mice have definitive hematopoiesis blockage and die at mid-gestation, which is similar to the phenotypes of $Runx1^{-/-}$ and $Cbfb^{-/-}$ mice[3, 4], indicating that CBFβ-SMMHC, the fusion protein encoded by *Cbfb-MYH11*, dominantly suppresses RUNX1 and CBFβ. Chimeric and conditional *Cbfb-MYH11* knockin mice develop AML when they acquire additional mutations[5].

An important domain of CBFβ-SMMHC is the C-terminal region of SMMHC, which catalyzes homo-dimerization and multimerization of the fusion protein that may be functionally important[6]. To test the function of this region, we previously generated knockin mice that expressed a truncated CBFβ-SMMHC missing C-terminal 95 amino acids. These mice would not develop leukemia, indicating the importance of C-terminal region for leukemogenesis[7].

The CBFβ-SMMHC C-terminal region contains an assembly competence domain (ACD), which is important for SMMHC multimerization[8, 9], as well as a transcriptional repression domain[8–10] . Specific amino acid residues in the helices D and E of ACD are important for multimerization of CBFβ-SMMHC but not for transcription repression[8]. To distinguish which domain is critical for the role of CBFβ-SMMHC in leukemia development, we generated the current mouse model with mutations in helices D & E of CBFβ-SMMHC to impair multimerization but leave the transcriptional repression domain intact.

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Conflict of interest
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Specifically, we mutated six charged residues of helices D and E to threonine, serine or alanine residues: D (NANRRKL to NSNRASL), E (QRELDEA to QAELTSA), as published previously[8]. We incorporated these mutations into the full length *Cbfb-MYH11* knockin construct[2] (Supplementary Figure S1a). Correct knockin was confirmed by southern blot hybridization (Supplementary Figure S1b) and the expression of the fusion protein CBFβ-SMMHC^{mDE} was detectable in BM cells, at a level similar to CBFβ-SMMHC when compared to CBFβ (Supplementary Figure S1c).

Mice carrying one copy of DE mutated *Cbfb-MYH11* ($Cbfb^{+/mDE}$) developed normally through adulthood, while $Cbfb^{mDE/mDE}$ embryos died in mid-gestation with central nervous system hemorrhage (Supplementary Table S1) and defective definitive hematopoiesis (Supplementary Figure S2a and b), similar to $Cbfb^{+/MYH11}$ embryos[2]. However, $Cbfb^{+/mDE}$ and $Cbfb^{mDE/mDE}$ embryos did not develop primitive hematopoiesis defect (Supplementary Figure S2c–e) as seen in $Cbfb^{+/MYH11}$ and $Cbfb^{\ C95/\ C95}$ mice[7].

We observed $Cbfb^{+/mDE}$ mice for up to two years and did not see any notable abnormality. Their peripheral blood (PB) cells were largely normal except for decreased B cells and an increased Mac1^{+}Gr-1^{+} myeloid cells in older mice (Supplementary Figure S3). Adult BM cells from $Cbfb^{+/mDE}$ mice showed no significant difference when compared to their wild type littermates in lineage differentiation, colony forming ability, cell viability, total cell count and apoptosis, except a slight increase of apoptosis in lin^{+} cells (Supplementary Figures S4–6). Importantly, none of the $Cbfb^{+/mDE}$ mice developed leukemia, even after treatment with ENU to induce additional mutations (Figure 1a).

It was shown previously that the DE mutations decreased the ability of CBFβ-SMMHC to form multimers and enter the nuclei[8]. We therefore examined the subcellular localization of endogenous CBFβ-SMMHC^{mDE} in the knockin mice. Immunofluorescence staining of PB cells from E11.5 $Cbfb^{mDE/mDE}$ embryos showed that RUNX1 was mostly localized in the nuclei while CBFβ-SMMHC^{mDE} was localized in both nuclei and cytoplasm (Figure 1b and c). On the other hand, RUNX1 and CBFβ were mostly co-localized in the nuclei of PB cells in WT embryos (Figure 1b and c). These observations were confirmed by western blot, since more CBFβ-SMMHC^{mDE} than CBFβ-SMMHC was detected in the cytoplasm of bone marrow cells from adult mice (Supplementary Figure S7a). In addition, transfected 293 cells showed similar reduction of co-localization between RUNX1 and CBFβ-SMMHC^{mDE} as well as more cytoplasmic localization of CBFβ-SMMHC^{mDE} (Figure 1d and e) and the western blot of transfected 293 cells (Supplementary Figure 7b) confirmed the expression of transfected proteins. The reduced co-localization between RUNX1 and CBFβ-SMMHC^{mDE} as well as more cytoplasmic localization of CBFβ-SMMHC^{mDE} suggested that CBFβ-SMMHC^{mDE} likely has reduced capacity to interact with RUNX1, which may be important for leukemogenesis by CBFβ-SMMHC.

Even though $Cbfb^{mDE/mDE}$ and $Cbfb^{+/MYH11}$ embryos had a similar phenotype at midgestation, they had very different gene expression patterns, with 188 and 1725 differentially expressed genes, respectively (FDR<0.01, Fold change>2). Moreover, most differentially expressed genes in $Cbfb^{+/MYH11}$ embryos were up-regulated while almost all differentially expressed genes in $Cbfb^{mDE/mDE}$ embryos were down regulated (Figure 2a).

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Interestingly, canonical pathway and disease/biological functions were also affected in opposite directions in *Cbfb*$^{mDE/mDE}$ and *Cbfb*$^{+/MYH11}$embryos (Supplementary Figure S8).

We performed RNA-Seq experiments to compare the expression profile of C-KIT^{+} cells in the adult *Cbfb*$^{+/mDE}$ mice with C-KIT^{+} cells in *Cbfb*$^{+/MYH11}$ and WT mice. As shown in Figure 2b, gene expression profile of *Cbfb*$^{+/mDE}$ cells overlapped with that of WT cells, but differed from *Cbfb*$^{+/MYH11}$ cells. There were 5641 differentially expressed genes (FDR<0.05, Fold change 1.5) in *Cbfb*$^{+/MYH11}$ cells, compared to the WT cells, accounting for 36.46% of all expressed genes. Between *Cbfb*$^{+/mDE}$ and WT cells, only 82 genes (82 of 14932, 0.55%) were differentially expressed (Figure 2c).

Among the 82 differentially expressed genes in the *Cbfb*$^{+/mDE}$ cells, 50 (60%) (Supplementary Table S2) were also differentially expressed in the *Cbfb*$^{+/MYH11}$ cells (Figure 2d). Interestingly, some genes were differentially expressed in opposite directions, e.g., Socs2 was 2X down in *Cbfb*$^{+/mDE}$ cells, but 17.6X up in *Cbfb*$^{+/MYH11}$cells (Supplementary Table S2).

Furthermore, *Cbfb*$^{+/MYH11}$ cells expressed significantly higher number of genes, while *Cbfb*$^{+/mDE}$ cells expressed similar number of genes as the WT cells (Figure 2e and Supplementary Figure S9). In addition, *MYH11* and *MYH11*^{mDE} expression was detected in *Cbfb*$^{+/MYH11}$ and *Cbfb*$^{+/mDE}$ cells, respectively, from the RNA-Seq data.

Overall our data demonstrated that the C-terminal multimerization domain, especially the 6 charged aa residues, is essential for leukemogenesis by CBFβ-SMMHC. The mechanism is still unclear, but likely through reduced RUNX1 sequestration, leading to fewer gene expression changes. Our findings point to an important target for developing specific therapeutic approaches for this type of leukemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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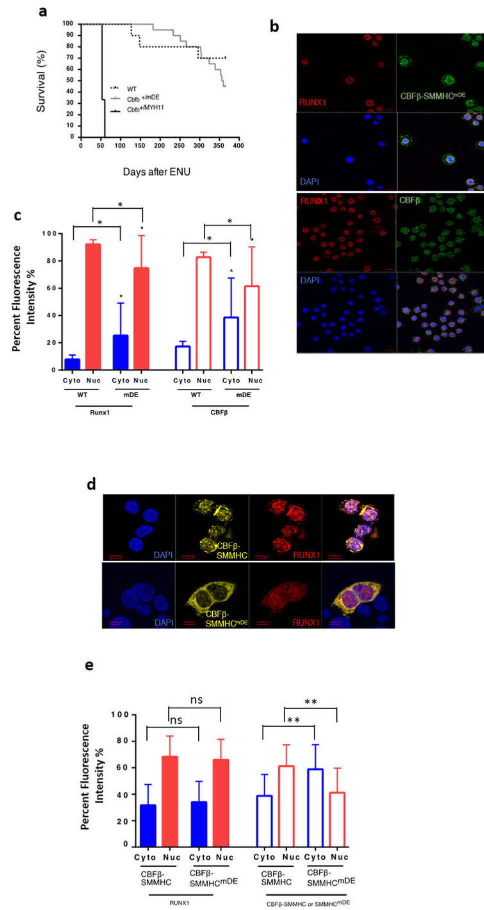

Figure 1.

Lack of leukemia development in *Cbfb*$^{+/mDE}$ mice and decreased ability of CBFβ-SMMHC^{mDE} protein to sequester RUNX1. (**a**) Survival curves of wildtype (WT) (N=10), *Cbfb*$^{+/mDE}$ (N=20), and *Cbfb*$^{+/MYH11}$ (N=3) mice after ENU treatment. All *Cbfb*$^{+/MYH11}$ mice died from leukemia around 2 months after ENU treatment. No leukemia development was observed in *Cbfb*$^{+/mDE}$ and WT mice. *Cbfb*$^{+/mDE}$ vs WT: $p = 0.3447$; *Cbfb*$^{+/MYH11}$ vs WT or *Cbfb*$^{+/mDE}$: $p < 0.0001$. (**b**) Immunofluorescence staining of E11.5 PB cells by anti-CBFβ (green) and anti-RUNX1 (red) antibodies. DAPI (blue) was used for nuclear staining. PB cells from a C*bfb*$^{mDE/mDE}$ mouse (upper panel) showed both cytoplasmic and nuclear stainings of CBFβ-SMMHC^{mDE} and mainly nuclear staining of RUNX1. PB cells from a WT mouse (lower panel) showed that CBFβ is mainly co-localized in the nuclei with RUNX1. (**c**) and (e) Protein subcellular distributions in immunofluorescence stained E11.5 PB cells (c) and transfected 293 cells (e). Data presented as a percentage of the fluorescence intensity of each protein in each cellular fraction, cytoplasmic (Cyto) and nuclear (Nuc), compared to total fluorescence intensity of each protein in both cellular fractions. n=70 for WT cells and n=30 for C*bfb*$^{mDE/mDE}$ (mDE) cells. n=37 for cells transfected with mCherry-labeled RUNX1 and EYFP-labeled CBFβ-SMMHC; n=30 for cells transfected with mCherry-labeled RUNX1 and EYFP-labeled CBFβ-SMMHC^{mDE}. (**d**) 293 cells transfected with mCherry-labeled RUNX1 (red) and EYFP-labeled CBFβ-SMMHC or CBFβ-

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SMMHC^{mDE} (yellow). Upper panel: RUNX1 and CBFβ-SMMHC showed co-localization in both nucleus and cytoplasm. Lower panel: RUNX1 localized mainly to the nuclei while CBFβ-SMMHC^{mDE} mainly stayed in the cytoplasm. Statistical significance was calculated for the differences in fluorescence intensity of RUNX1 (left panel) and CBFβ (right panel) in PB cells between wildtype and *Cbfb*$^{mDE/mDE}$ embryos (**c**) and for the differences in fluorescence intensity of RUNX1 (left panel) and CBFβ-SMMHC/CBFβ-SMMHC^{mDE} (right panel) in 293 cells transfected with CBFβ-SMMHC or CBFβ-SMMHC^{mDE} (**e**). *: p=0.0004, **: p<0.0001, ns: not significant. Scale bars in panels **b** and **d** = 10 μM.

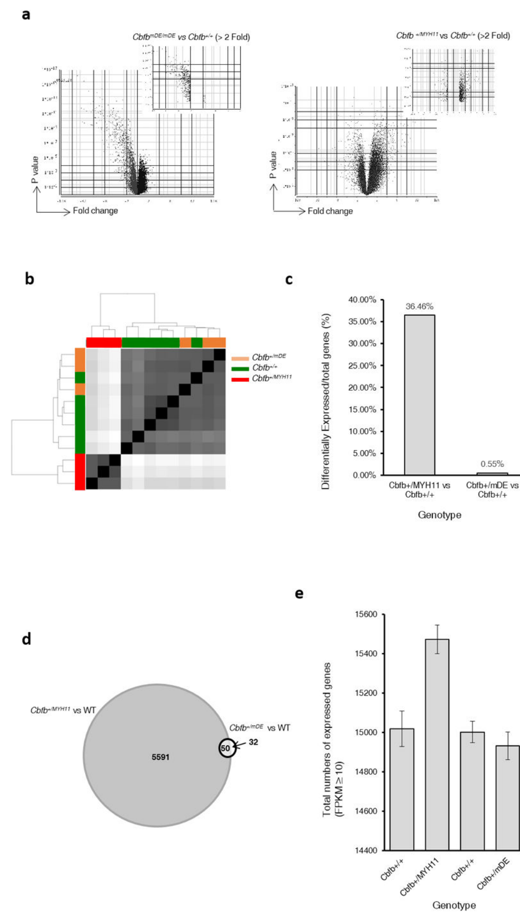

Figure 2.

Gene expression changes in *Cbfb*$^{+/mDE}$ and C*bfb*$^{mDE/mDE}$ mice. (**a**) Volcano plots showing gene expression profile differences in PB cells between *Cbfb*$^{mDE/mDE}$ and *Cbfb*$^{+/+}$ embryos (left panel) and between *Cbfb*$^{+/MYH11}$ and *Cbfb*$^{+/+}$ embryos (right panel). (**b**) Principal component analysis of RNA-Seq data shows that the gene expression profile of *Cbfb*$^{+/mDE}$ C-KIT^{+} cells is more similar to C-KIT^{+} cells in *Cbfb*$^{+/+}$mice. (**c**) Percentages of differentially expressed genes vs. all expressed genes (average of three samples). (**d**) Venn diagram of differentially expressed genes in *Cbfb*$^{+/MYH11}$ and *Cbfb*$^{+/mDE}$ C-KIT^{+} cells (p 0.05, fold change 1.5). (**e**) Total numbers of expressed genes (obtained with featureCounts[11]; average FPKM from three samples 10) in C-KIT^{+} cells from mice of the indicated genotype.

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