Letter to the Editor: CBFβ-SMMHC regulates ribosomal gene transcription and alters ribosome biogenesis

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Letter to the Editor: CBFβ-SMMHC regulates ribosomal gene transcription and alters ribosome biogenesis

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The Core Binding Factor (CBF) complex is a heterodimeric transcription factor comprising a CBFβ subunit and a variable DNA-binding RUNX subunit, usually RUNX1 in hematopoietic cells. Aside from its critical hematopoietic functions, CBF regulates the expression of ribosomal protein genes and ribosomal RNA (rRNA) in a cell context-dependent manner (1-3). Intriguingly, this function may have implications for the pathogenesis of acute myeloid leukemia (AML), as reduced ribosome biogenesis in RUNX1-deficient hematopoietic stem cells has recently been proposed to confer a survival advantage that favors outgrowth of pre-leukemic RUNX1-deficient clones (3). Furthermore, AML-associated fusion proteins that arise from translocations of CBF subunit genes have been shown to occupy nucleolar organizing regions at mitotic chromosomes (4, 5), suggesting that ribosomal homeostasis might be altered in CBF-AML.

The AML-associated *CBFβ-MYH11* translocation arises from either an inv(16)(p13;q22) or t(16;16)(p13;q22), and generates the CBFβ-SMMHC fusion protein. The oncoprotein has been thought to subvert wild-type CBF function primarily through cytoplasmic sequestration of RUNX1, and by the recruitment of transcriptional corepressors which inhibit the expression of CBF-regulated genes (6, 7). It is however likely that CBFβ-SMMHC has additional activities that contribute to AML oncogenesis. For example, we have shown that a significant proportion of cellular CBFβ-SMMHC is present in the nucleus, binds to chromatin and preferentially co-localizes with RUNX1 at the promoters of transcribed loci, suggesting that CBFβ-SMMHC may also participate in the activation of RUNX1 target genes (8).

We tested whether CBFβ-SMMHC affects the transcriptional regulation of ribosomal protein genes and ribosomal DNA (henceforth 'ribosomal genes'). We initially

performed chromatin immunoprecipitation followed by massive parallel sequencing (ChIP-seq) in an ME-1 cell line containing a tetracycline-inducible shRNA against *CBFβ-MYH11* (8) (Supplementary Figure S1). ChIP was carried out using anti-SMMHC and anti-RUNX1 antibodies, in order to define the DNA binding patterns of these proteins in the presence or absence of shRNA knockdown. We used the Genomic Regions Enrichment of Annotations Tool (GREAT, see Supplementary Methods) to characterize the functions of bound genes. Strikingly, unbiased gene ontology analysis identified 'structural constituent of ribosome' as the most highly-enriched molecular function annotation for both CBFβ-SMMHC- and RUNX1-associated loci, while 'ribosome' and 'ribosomal subunit' featured in the top five cellular component terms in each case (Figure 1A and Supplementary Figure S2). Analysis of a set of ribosomal genes that have previously been defined as high-confidence Runx1 binding sites in murine hematopoietic cells (3) revealed high rates of promoter occupancy by both CBFβ-SMMHC and RUNX1 (Figure 1B). Examination of individual sequencing tracks confirmed the presence of both proteins at the promoters of multiple ribosomal genes, which was further tested by ChIP-qPCR at selected loci, including at rDNA (Figure 1C and Supplementary Figures S3 and S4). In order to verify that these findings were representative of binding patterns in human blasts, we performed xenografting of primary human CBFβ-SMMHC+ AML cells in immunodeficient mice (see Supplementary Methods), and extracted chromatin from the resultant leukemias. ChIP-qPCR analyses revealed binding of both CBFβ-SMMHC and RUNX1 to ribosomal protein gene promoters and rDNA (Supplementary Figure S4), providing *in vivo* corroboration of the cell line results.

We then determined whether the presence of CBFβ-SMMHC affected the levels of ribosomal gene transcription. RT-qPCR analysis showed that expression of a set of

CBFβ-SMMHC and RUNX1-bound genes was consistently lower after *CBFβ-MYH11* knockdown, while the levels of pre-rRNA also fell, indicating concomitant reduced transcription of rDNA (Figure 1D). In contrast, transcription of ribosomal genes that were not bound by CBFβ-SMMHC and RUNX1 did not change after shRNA knockdown (Figure 1D), which, along with the fact that the levels of RUNX1 binding did not alter significantly after *CBFβ-MYH11* inhibition (Figure 1B), suggests that the observed gene expression changes were directly linked to the presence of CBFβ-SMMHC and/or CBFβ-SMMHC-associated transcriptional regulatory molecules at these promoters. Tetracycline treatment of a different ME-1 line that does not contain an inducible shRNA did not result in any significant changes in ribosomal transcription or protein levels (Supplementary Figure S5), indicating that tetracyclines do not alter ribosome biogenesis in this cellular context. We also found that ectopic expression of CBFβ-SMMHC caused variable increases in ribosomal gene expression in three additional cell lines (Supplementary Figure S6), indicating that the oncoprotein can activate ribosomal gene transcription, even in cellular milieus that are not necessarily permissive for its function.

We next used a microarray data set (see Supplementary Methods) to examine the expression of ribosomal genes in a large cohort of primary AML samples. Gene set enrichment analysis (GSEA) using the same set of high confidence RUNX1 targets that were enriched for CBFβ-SMMHC binding in ME-1 cells revealed significantly increased levels of ribosomal transcripts in *CBFβ-MYH11+* cases, compared with the rest of the AML patient cohort (Figure 1E). These results therefore provide further support for the hypothesis that CBFβ-SMMHC activates the expression of ribosomal genes in leukemic blasts *in vivo*. We additionally found that *RUNX1-ETO+* samples had similar levels of

ribosomal transcripts as *CBFβ-MYH11+* cases (Supplementary Figure S7), suggesting that subversion of ribosomal gene expression might be a shared feature of CBF-AML.

We then addressed the impact of *CBFβ-MYH11* inhibition on cellular ribosome content. We initially analyzed the levels of a panel of ribosomal proteins by western blotting. Surprisingly, despite causing a decrease in the corresponding mRNAs, CBFβ-SMMHC depletion resulted in increased amounts of proteins of both the small and large ribosomal subunits (Figure 2A). Consistent with this finding, *CBFβ-MYH11* inhibition led to increases in both ribosome content as measured by polysome profiling (Figure 2B), and protein synthesis rates as assessed by measurement of puromycilation in a SunSET assay (Figure 2C and Supplementary Methods). Interestingly, this increase in translational capacity was associated with the presence of higher levels of proteins implicated in the initiation of protein synthesis such as the components of the Eukaryotic Initiation Factor 4F (eIF4F) complex eIF4E and eIF4G, the helicase DDX3X and the poly A binding protein PABP (Supplementary Figure S8).

The observed increases in ribosome content that follow *CBFβ-MYH11* inhibition suggest the involvement of mechanisms that maintain ribosome levels, despite the decreases in rRNA and ribosomal protein mRNAs. We initially considered whether ribosomal protein levels might be preserved through increased translation of the corresponding transcripts. As the translation of ribosomal protein mRNAs is co-regulated (9), analysis of the polysomal distribution of a single transcript can be used as a reliable measure of overall ribosomal protein mRNA translation. We found no changes in the proportions of polysome-associated *RPS6* mRNA (Supplementary Figure S9), suggesting that the efficiency of translation of ribosomal transcripts is not increased by CBFβ-SMMHC depletion. We therefore proceeded to use *in vivo* labeling with ^{3}H-Uridine to measure the

rate of maturation of the pre-rRNA precursor into mature 18S and 28S rRNA, as an indication of 40S and 60S ribosome synthesis. Figure 2D shows that in cells depleted of *CBFβ-MYH11*, 18S and 28S rRNA accumulate at significantly faster rates as compared with control cells. In keeping with this, western blotting revealed that major nucleolar proteins involved in ribosome assembly were increased following *CBFβ-MYH11* inhibition (Figure 2E). These findings therefore provide an explanation for the observed increases in ribosome content in CBFβ-SMMHC-depleted cells, despite the gene expression effects.

In summary, our results suggest that CBFβ-SMMHC has complex actions on human ribosome biogenesis at both the genomic and post-transcriptional level. These findings differ significantly from the reported effects of Runx1 haploinsufficiency in murine hematopoietic cells (3), strongly suggesting that CBFβ-SMMHC-related subversion of ribosomal homeostasis is not solely due to RUNX1 inhibition. As the regulation of ribosomal gene transcription by RUNX factors is cell context-dependent (1-3), it is probable that additional lineage-specific factors modulate the activation and repression of these loci in different tissue types, including in CBFβ-SMMHC-positive leukemic blasts. We also cannot exclude the possibility that RUNX1 may have activities that are independent of CBFβ-SMMHC in this cellular context, or that either factor might additionally affect upstream regulators of ribosomal biogenesis.

Our data also raise the question of whether alterations in cellular ribosome content might contribute to the oncogenesis of CBF-AML. Mutations in ribosomal structural genes characterize a range of bone marrow failure syndromes that are often accompanied by deregulated expression of p53 (reviewed in (10)), while ribosomal protein gene mutations are also found in acute leukemia (reviewed in (11)). There is

long-standing evidence that ribosomal homeostasis critically influences cellular differentiation in a range of cell types across different species (12-14), suggesting that subversion of ribosomal function is likely to impair hematopoietic development. Our findings therefore provide further theoretical support for the investigation of the contribution of altered ribosomal biogenesis to an oncogenic differentiation block.

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References:

1. Young DW, Hassan MQ, Pratap J, Galindo M, Zaidi SK, Lee SH, et al. Mitotic occupancy and lineage-specific transcriptional control of rRNA genes by Runx2. Nature. 2007 Jan 25;445(7126):442-446.

2. Ali SA, Zaidi SK, Dacwag CS, Salma N, Young DW, Shakoori AR, et al. Phenotypic transcription factors epigenetically mediate cell growth control. Proc Natl Acad Sci U S A. 2008 May 6;105(18):6632-6637.

3. Cai X, Gao L, Teng L, Ge J, Oo ZM, Kumar AR, et al. Runx1 Deficiency Decreases Ribosome Biogenesis and Confers Stress Resistance to Hematopoietic Stem and Progenitor Cells. Cell Stem Cell. 2015 Aug 6;17(2):165-177.

4. Bakshi R, Zaidi SK, Pande S, Hassan MQ, Young DW, Montecino M, et al. The leukemogenic t(8;21) fusion protein AML1-ETO controls rRNA genes and associates with nucleolar-organizing regions at mitotic chromosomes. J Cell Sci. 2008 Dec 1;121(Pt 23):3981-3990.

5. Lopez-Camacho C, van Wijnen AJ, Lian JB, Stein JL, Stein GS. CBFbeta and the leukemogenic fusion protein CBFbeta-SMMHC associate with mitotic chromosomes to epigenetically regulate ribosomal genes. J Cell Biochem. 2014 Dec;115(12):2155-2164.

6. Liu PP, Hajra A, Wijmenga C, Collins FS. Molecular pathogenesis of the chromosome 16 inversion in the M4Eo subtype of acute myeloid leukemia. Blood. 1995 May 1;85(9):2289-2302.

7. Lutterbach B, Hou Y, Durst KL, Hiebert SW. The inv(16) encodes an acute myeloid leukemia 1 transcriptional corepressor. Proc Natl Acad Sci U S A. 1999 Oct 26;96(22):12822-12827.

8. Mandoli A, Singh AA, Jansen PW, Wierenga AT, Riahi H, Franci G, et al.. CBFB-MYH11/RUNX1 together with a compendium of hematopoietic regulators, chromatin

modifiers and basal transcription factors occupies self-renewal genes in inv(16) acute myeloid leukemia. Leukemia. 2014 Apr;28(4):770-778.

9. Thoreen CC, Chantranupong L, Keys HR, Wang T, Gray NS, Sabatini DM. A unifying model for mTORC1-mediated regulation of mRNA translation. Nature. 2012 May 3;485(7396):109-113.

10. Fumagalli S, Thomas G. The role of p53 in ribosomopathies. Semin Hematol. 2011 Apr;48(2):97-105.

11. Armistead J, Triggs-Raine B. Diverse diseases from a ubiquitous process: the ribosomopathy paradox. FEBS Lett. 2014 May 2;588(9):1491-1500.

12. Ramagopal S, Ennis HL. Regulation of synthesis of cell-specific ribosomal proteins during differentiation of Dictyostelium discoideum. Proc Natl Acad Sci U S A. 1981 May;78(5):3083-3087.

13. Agrawal MG, Bowman LH. Transcriptional and translational regulation of ribosomal protein formation during mouse myoblast differentiation. J Biol Chem. 1987 Apr 5;262(10):4868-4875.

14. Gao R, Chen S, Kobayashi M, Yu H, Zhang Y, Wan Y, et al. Bmi1 promotes erythroid development through regulating ribosome biogenesis. Stem Cells. 2015 Mar;33(3):925-938.

15. Ban N, Beckmann R, Cate JH, Dinman JD, Dragon F, Ellis SR, et al. A new system for naming ribosomal proteins. Curr Opin Struct Biol. 2014 Feb;24:165-169.

Figure Legends:

Figure 1: CBFβ-SMMHC regulates ribosomal gene transcription.

(A) Ontology analysis of genes proximate to CBFβ-SMMHC binding sites in ME-1 cells. ChIP-seq peaks were analyzed using the Genomic Regions Enrichment of Annotations Tool (GREAT). The top results for annotations of 'molecular function' and 'cellular component' are shown. Ontologies relating to ribosomal composition and assembly are listed in red. Images are adapted from the GREAT website.

(B) Heat maps displaying RUNX1 and CBFβ-SMMHC tag densities at 139 ribosomal genes in ME-1 cells without and with shRNA knockdown (KD) of *CBFβ-MYH11*. Maps show tag density in a 1kb region centered on the transcriptional start site of ribosomal genes that have previously been defined as high-confidence RUNX1-binding sites (3).

(C) CBFβ-SMMHC and RUNX1 binding to ribosomal genes in ME-1 cells. ChIP-seq tracks for four genes are shown. Images were generated using the University of California Santa Cruz Genome Browser tool. Arrows indicate transcription start site and direction. Comparison with binding patterns in KD cells is shown in Supplementary Figure S3.

(D) RT-qPCR analysis of transcription of ribosomal protein genes and pre-RNA in ME-1 cells. Expression in *CBFβ-MYH11* KD cells was calculated relative to control. The mean and standard deviation for 3 experimental replicates are shown. Expression of a gene known to be upregulated following *CBFβ-MYH11* inhibition (*RUNX3*) is also depicted.

(E) Gene Set Enrichment Analysis (GSEA) of primary AML transcriptome data, using a set of ribosomal genes that have previously been defined as high-confidence RUNX1 binding sites (3). Expression of ribosomal genes was significantly higher in CBFβ-

SMMHC positive leukemia (n = 18) compared with the rest of the AML cohort (n = 198). FDR = False discovery rate.

Figure 2: CBFβ-SMMHC depletion increases ribosome formation and mRNA translation due to increased processing of pre-RNA to mature rRNA.

(A) Western blot analysis of ribosomal proteins in ME-1 cells without and with *CBFβ-MYH11* knockdown (KD). Figures indicate relative levels in KD cells, after normalization to β-actin expression. Ribosomal proteins are named using the nomenclature of Ban et al (15), and the corresponding gene names are shown in brackets.

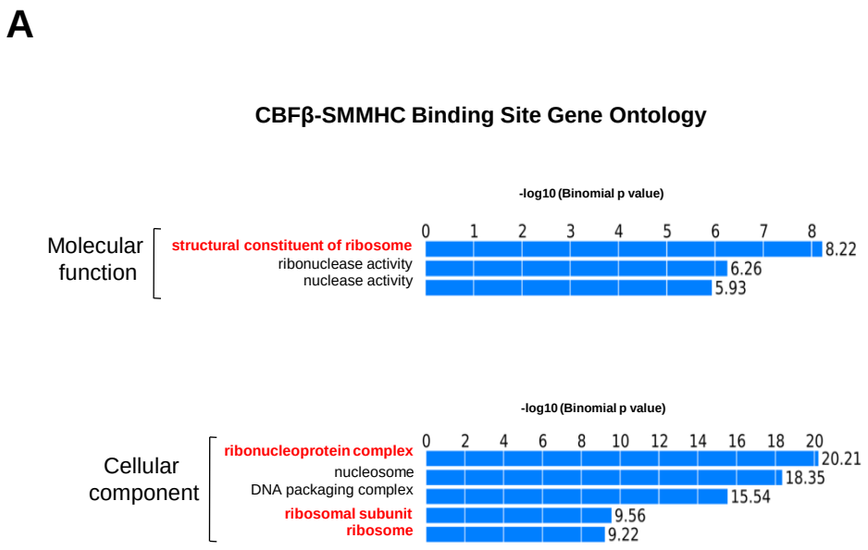
(B) Polysome profiling. Sucrose gradient fractionation of ME-1 cell lysates showed increased polysomes after *CBFβ-MYH11* KD (red line). Polysome fractions are annotated. LMW = Low Molecular Weight. HMW = High Molecular Weight. OD = Optical Density.

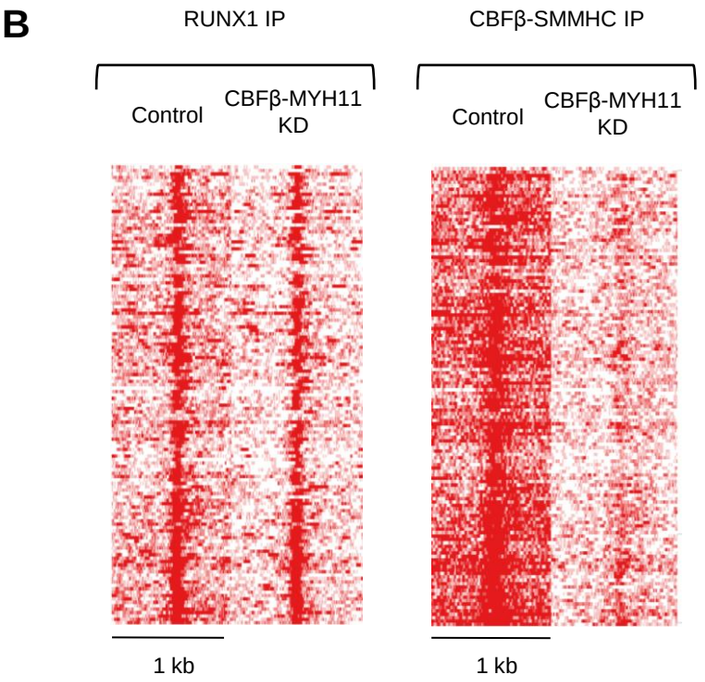
(C) Assessment of protein translation by puromycilation (SUnSET assay). Western blot analysis reveals increased puromycin incorporation, indicating increased translation, in ME-1 cells after *CBFβ-MYH11* KD, as compared with control cells.

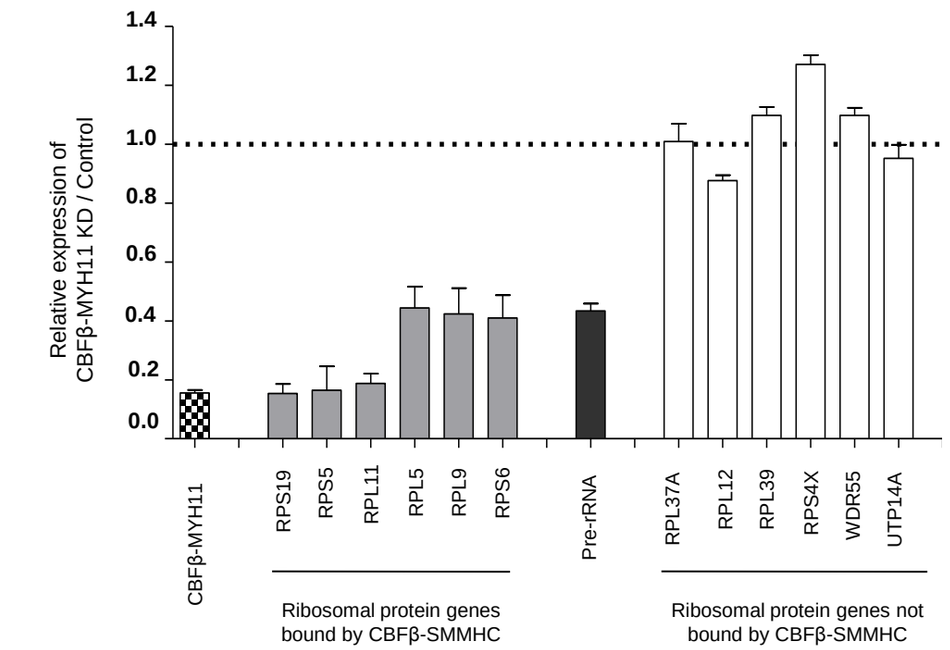
(D) Top panel: Northern blot autoradiogram showing ^{3}H-labelled RNA from ME-1 cells without and with *CBFβ-MYH11* KD, incubated for the indicated times with [5,6-^{3}H]Uridine. Increased rates of maturation of the pre-rRNA precursor into mature 18S and 28S rRNA are seen with *CBFβ-MYH11* KD. Bottom panel: Ethidium bromide staining prior to gel transfer to membrane, showing equal RNA loading for each sample.

(E) Western blot analysis of nucleolar proteins involved in ribosomal assembly in ME-1 cells. Figures indicate relative levels in KD cells, after normalization to β-actin expression.

Figure 1







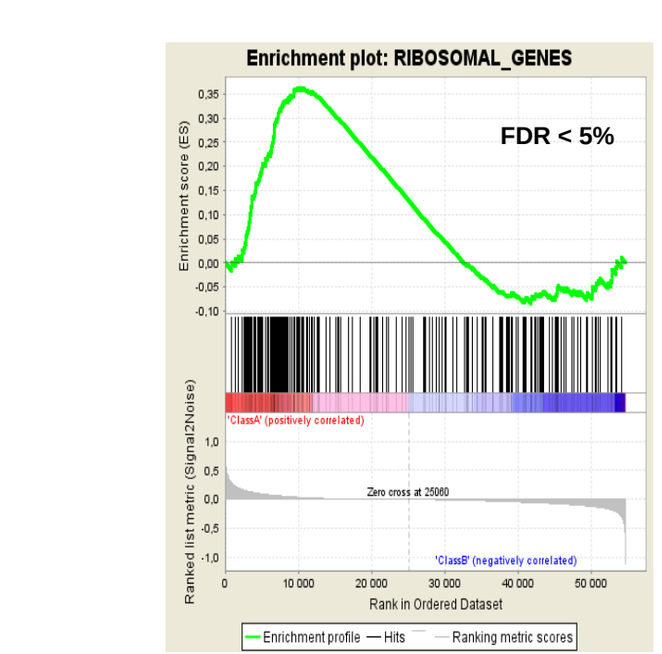


Figure 2