ZNF143 protein is an important regulator of the myeloid transcription factor C/EBPα

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The transcription factor C/EBPα is essential for myeloid differentiation and is frequently dysregulated in acute myeloid leukemia. Although studied extensively, the precise regulation of its gene by upstream factors has remained largely elusive. Here, we investigated its transcriptional activation during myeloid differentiation. We identified an evolutionarily conserved octameric sequence, CCCAGCAG, ~100 bases upstream of the CEBPA transcription start site, and demonstrated through mutational analysis that this sequence is crucial for C/EBPα expression. This sequence is present in the genes encoding C/EBPα in humans, rodents, chickens, and frogs and is also present in the promoters of other C/EBP family members. We identified that ZNF143, the human homolog of the Xenopus transcriptional activator STAF, specifically binds to this 8-bp sequence to activate C/EBPα expression in myeloid cells through a mechanism that is distinct from that observed in liver cells and adipocytes.

Altogether, our data suggest that ZNF143 plays an important role in the expression of C/EBPα in myeloid cells.

Pluripotent hematopoietic stem cells are capable of differentiating along myeloid, erythroid, or lymphoid pathways (1), via intermediate, more lineage-restricted progenitor cells. Once a cell has committed to the myeloid pathway, it can further develop into either monocytic or granulocytic lineages. This stepwise differentiation process requires the concerted action of several TFs, and aberrant regulation by these TFs has been shown to play a role in leukemia (2–4). One of the critical TFs during myelopoiesis is C/EBPα, which is a member of the bZIP family of TFs. This family of proteins contains a leucine zipper domain that is important in protein dimerization and DNA binding (5). Six C/EBP family members have been identified thus far. These proteins share homology in the DNA-binding C terminus while diverging from each other within their N-terminal domain (6). C/EBPα is indispensable for inducing the granulocytic differentiation of myeloid progenitors (7–9) and for the acquisition and maintenance of adult hematopoietic stem cell quiescence (10). It does so by binding to and repressing genes involved in stem cell renewal and by activating the promoters of a myriad of genes involved in myeloid differentiation (11, 12). In line with these observations, defects in C/EBPα have been reported in ~10% of patients with acute myeloid leukemia (13–15).

Although much is known about the general transcriptional machinery in eukaryotic cells, the events that are required for the transcription of specific genes that encode master controllers of differentiation, such as CEBPA, are not sufficiently understood. In human liver cell lines, C/EBPα was reported to

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The abbreviations used are: TF, transcription factor; TSS, transcription start site; contig, group of overlapping clones; NSC, non-silencing control.
autoregulate its promoter indirectly through an upstream stimulatory factor–binding site located at -268 bp relative to the transcription start site (TSS) (16). In addition to autoactivation, the murine Cebpa promoter also contains two repressive AP2 sites, one at -218 bp and the other in the 5′-untranslated region, and both sites must be occupied to fully suppress the transcription of C/EBPα (17).

Whereas most of the known regulatory regions within the CEBPA promoter have been described in hepatocytes or adipocytes, less is known about its regulation in hematopoietic cells. However, Runx1 was recently reported to activate the expression of C/EBPα by binding to a conserved site in the murine promoter and to a distal enhancer in hematopoietic cells (18). To further extend our knowledge with regard to myelopoiesis, we examined the proximal promoter of the human CEBPA gene in search of previously undescribed cis-regulatory elements. This analysis revealed a highly evolutionarily conserved 8-bp sequence in the CEBPA promoter that is crucial for its transcription. Using affinity chromatography, we were able to demonstrate that zinc finger protein 143 (ZNF143), a human homolog of Xenopus STAF (19, 20), bound this element. ZNF143 was previously shown to bind to >3000 non-coding RNAs and protein-coding genes genome-wide (21). Binding of ZNF143 to this conserved 8-bp regulatory sequence is crucial for the transcriptional activation of the CEBPA gene promoter in myeloid cells.

Results

Identification of a regulatory region essential for expression of C/EBPα in myeloid cells

To identify regulatory elements required for myeloid expression of human C/EBPα, we mapped the minimal promoter region necessary for gene expression in the immature myeloid cell line, U937. We performed transient transfections of luciferase reporter constructs to test the activity of a series of five deletion constructs extending from -2.2 kb to -78 bp relative to the TSS (Fig. 1A). We observed that most of the transactivation was directed by a region from -110 to -95 is crucial for transcriptional activation. PXP1 is the empty vector. Error bars, S.D.

Figure 1. Identification of the promoter region essential for C/EBPα expression in myeloid cells. A, C, and E, schematic representation of the human CEBPA promoter and luciferase reporter constructs used to study CEBPA transcriptional activation. The numbers indicate the position of the 5′-end of each construct relative to the TSS at position +1. The rectangle labeled LUC represents the luciferase gene. A, luciferase map of CEBPA promoter fragments within -2200 bp relative to the TSS. C, deletion constructs within the 438-bp proximal promoter. Δ followed by a number designates the location of internal deletions shown in the schematics. E, deletion constructs within the 438-bp proximal promoter. B, D, and F, graphs depicting luciferase activity in U937 cells in relative luciferase units (RLU). Note that stepwise deletions demonstrate that the region between -110 and -95 is crucial for transcriptional activation. PXP1 is the empty vector. Error bars, S.D.
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Figure 2. Protein binding to a conserved motif centered around −100 bp. A, schematic representation of the probes designed to pinpoint the region of the CEBPA promoter in which binding occurs. B, EMSAs of the region between positions −124 and −71 using the three overlapping probes (I, II, and III). Note that only probe II (lanes 5–8) generated two complexes that could be outcompeted by a 100-fold excess of unlabeled double-stranded self-oligonucleotide (lane 7). S, self-competitor (non-labeled probe); N, non-self-competitor (unrelated DNA probe). C, EMSA demonstrates binding in nine different cell lines when using probe II. Probe II alone and with self-competitor (S) are loaded as controls in lanes 1 and 3, respectively. D, presence of the conserved 8-base sequence “CCCAGCAG” (gray) shared with several C/EBP family members across different species (h, human; m, mouse; r, rat; c, chicken). The locations of the sequences relative to the TSS are indicated.

A specific DNA–protein interaction occurs between −112 and −88 of the CEBPA promoter

Based on our deletion studies, we hypothesized that a TF bound to the proximal promoter between −121 and −78. To further narrow down the binding region, we generated a series of overlapping double-stranded oligonucleotide probes (Fig. 2A) and performed EMSAs using nuclear extracts from U937 cells, which express C/EBPα. Probe II (−112 to −88; Fig. 2B, lanes 5–8) generated two complexes (lane 6) that could be outcompeted by a 100-fold excess of non-labeled (“cold”) specific self-probe (lane 7). These complexes could not be outcompeted by a cold nonspecific probe containing an SP1 site from the PU.1 promoter (lane 8). Probe I (−124 to −101) did not generate a specific shifted band on the gel (Fig. 2B, lane 2). Probe III (−96 to −71; lanes 9–12) generated a complex that could be partially outcompeted with excess unlabeled self-probe (lane 11) but also by the non-self-probe used as a negative control (lane 12), indicative of a nonspecific interaction. Probe II overlaps with portions of both probes I and III but has a unique core region. Given that this probe generated a complex of greater intensity and specificity, we concluded that a specific DNA–protein interaction does indeed occur within this region of the CEBPA promoter.

EMSA on eight other cell lines demonstrated a similar binding pattern (Fig. 2C), indicating that this factor is widely expressed. Two bands were observed in some cell lines. Given that the assay is performed under non-denaturating conditions, it is difficult to assess whether the probe is binding two different factors, the same factor with different protein modifications, or a single factor that is partially degraded.

A highly conserved 8-bp element within the proximal promoter of different C/EBPs is essential for promoter activation

The results obtained in the luciferase assays and EMSA experiments strongly suggested the presence of an important regulatory region between −112 and −88 upstream of the human CEBPA TSS. Therefore, we examined its sequence conservation between different species. We identified an octameric sequence motif (CCCAGCAG) that was fully conserved among human, mouse, rat, and chicken CEBPA proximal promoters (Fig. 2D). In humans, it is located from position −106 to −99. This octameric sequence is also present in human CEBPD and CEBPE promoters, but not in CEBPB or CEBPG promoters. Interestingly, we also observed a similar motif in the Xenopus CEBPA promoter, with all but the seventh base conserved.
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Figure 3. The identified protein binds to the conserved CCCAGCAG site. A, methylation interference analysis of DNA contact points of the DNA-binding factor to the C/EBPα promoter demonstrates that a protein makes specific contact with this region. Lanes G and G+A are sequence standards prepared from unmethylated probe by DNA sequencing. Lane F is free DNA, and lane X is DNA from the observed DNA–protein complex. Both samples were subjected to piperidine-mediated cleavage of methylated guanosine residues. The asterisks indicate a direct interaction between the DNA and protein at bases −101, −104, and possibly −105. B, mutation of the conserved CCCAGCAG core region abolishes binding. U937 nuclear extract was incubated with radiolabeled probe II or a mutant derivative (probe M). FP, free probe; **, specific band. C, schematic representation of the WT and mutant luciferase constructs used to test the effect of the full CCCAGCAG sequence on promoter activity. Mutations were introduced in three constructs containing various segments of the CEBPA promoter region. The associated graph reports luciferase activity of each construct in U937 cells after transient transfection. D, UV cross-linking determines the molecular weight of the DNA binding factor at ~80–95 kDa. A double-stranded oligonucleotide containing BrdU was synthesized and radiolabeled. Samples were electrophoresed on a 6% non-denaturing gel, and two complexes were excised and further separated on a 10% SDS-polyacrylamide gel. Lane 1, upper complex, estimated to be 95 kDa; lane 2, lower band, estimated to be 80 kDa. Error bars, S.D.

(CCCAGCGC), at −108 to −101 bp relative to the TSS, suggesting a spatial and sequential conservation of this motif.

To identify which residues are crucial for protein binding, methylation interference assays were performed using the antisense strand of probe II (Fig. 3A). Comparing the patterns produced by the free probe and the retarded band, which represents the DNA–protein complex, we observed three guanosine residues at positions −105, −104, and −101 (Fig. 3A, lane 3) with diminished intensity relative to the free probe (Fig. 3A, lane 4), indicating their close contact with a DNA-binding protein. No other protected regions were observed, showing that this specific site is critical for the formation of the observed complex. We then introduced mutations into these three guanosine residues (G → T at positions −101, −104, and −105) and used this mutated probe to perform EMSA. We assessed the effects on DNA binding but only observed partial loss (data not shown). Next, we performed EMSAs using probes that harbored different numbers of mutated nucleotides. These mutational analyses demonstrated that binding could be abolished only upon mutating at least six of the eight bases in the core sequence CCCAGCAG (Fig. 3B) (data not shown). Fig. 3B demonstrates that alteration of the ACCCAGCAG sequence in probe II to TTCACCAA abolishes the interaction (lanes 1–4). This unlabeled mutant probe was unable to compete with the observed complex when using the wild-type labeled oligonucleotide, probe II (Fig. 3B, lane 8).

These mutant sequences were then cloned into various luciferase constructs. As shown in Fig. 3C, the basic pattern among the wild-type constructs is consistent with that observed previously (Fig. 1C). The luciferase activity of the −443/+124, −181/+124, and −125/+124 constructs was consistently higher than that of the −97/+124 and −78/+124 constructs, leading us to further focus on the region surrounding position −100. Additionally, mutation of the CCCAGCAG site significantly decreased luciferase activity in each of the three mutant constructs to a level comparable with that of the −78 bp minimal sequence (Fig. 3C). These results support our hypothesis that the sequence between −106 and −99 is important for promoter activity of these constructs in luciferase assays.

The zinc finger, ZNF143, binds to the CCCAGCAG site in the CEBPA promoter

Next, we determined the molecular mass of the DNA-binding protein that interacts with this region by UV cross-linking (22). Two BrdU-labeled bases were incorporated in the sequence of probe II at the putative DNA-binding site located between positions −106 and −99. After performing EMSA, excising the bands, and exposing them to UV light, we loaded the gel slices on an SDS-polyacrylamide gel. As shown in Fig. 3D, the lower band migrates with apparent molecular mass of 95 kDa, and the upper band migrates similar to a 105-kDa protein. Attempts to identify the protein using a variety of screen-
ing techniques, including FROGS (23) and yeast one-hybrid (24), were unsuccessful. In addition, EMSAs using control sequences demonstrated that the protein was not Sp1 or a member of the STAT family. Finally, we identified the protein binding to the CCCAGCAG site by partially purifying a HeLa nuclear extract using column chromatography in combination with SDS-PAGE and EMSA. HeLa cells were used, as they can be grown in large quantities in spinner flasks and therefore often used to isolate TFs. We first partially purified the DNA-binding activity of the crude nuclear extract by anion-exchange chromatography using a Mono Q column. Approximately 90% of the DNA-binding activity was eluted from the column, leading to a 100-fold purification of the factor compared with crude nuclear extract (Fig. 4A). This fraction was subsequently separated on an 8% SDS-polyacrylamide gel, 12 slices of different sizes in the 100-kDa range were excised (Fig. 4B), and the polypeptides within were renatured and tested for binding in an EMSA using probe II. Fraction 5 contained the highest binding activity (Fig. 4C), so we submitted the gel slice corresponding to this fraction for mass spectrometry analyses.

Simultaneously, motif analysis on 566 bp surrounding the TSS of the human CEBPA gene was performed with the MatInspector tool of Genomatix. This is a software tool that utilizes a large library of matrix description for TF binding sites to locate matches in DNA sequences. MatInspector suggested that the *Xenopus* protein STAF could bind to the CCCAGCAG sequence (Table 1). This finding was in close agreement with our mass spectrometric findings, as the human homolog of STAF, ZNF143 (19, 20), was identified in fraction 5 (Table 2). Subsequently, using EMSA, we demonstrated that a protein within fraction 5 bound to a synthetic ZNF/STAF consensus binding site (Fig. 4D) as well as to two other published ZNF143 probes (Fig. 4E). EMSA using HeLa extracts with ZNF143 antibodies demonstrates that ZNF143 binds to the CCCAGCAG motif in the *CEBPA* promoter in vitro (Fig. 4F). Note that the negative control, normal rabbit serum, did not supershift the complex (lanes 4 and 8).

Figure 4. The seven-zinc finger TF ZNF143 binds to the CCCAGCAG site of the CEBPA promoter. A, fractionation of the binding activity on a Q column. B, size fractionation of the binding activity by SDS-PAGE. Twelve fragments were selected for further analysis. C, EMSA of size-fractionated extract using the probe from the −112 to −86 region of the CEBPA promoter demonstrates binding to fractions 4, 5, and 6. D, EMSA demonstrating binding of the protein in fraction 5 to a probe containing the STAF consensus binding site, CCCAGCAG. E, EMSA demonstrating binding to three different ZNF143 probes in U937 cells and that all three probes undergo a similar supershift as probe II (Fig. 2, A and B) by adding the anti-ZNF143 antibody. F, EMSA demonstrates that ZNF143 binds to the CCCAGCAG motif in the *CEBPA* promoter in vitro. Note that the negative control, normal rabbit serum, did not supershift the complex (lanes 4 and 8).

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| Probe | z1 | z2 | z3 | Probe II |
|-------|----|----|----|----------|
| U937 NE | +  | +  | +  | +        |
| ZNF143 Ab | -  | -  | -  | -        |
| Rabbit serum | -  | -  | -  | -        |

| Probe | z1 | Probe II |
|-------|----|----------|
| HeLa NE | +  | -        |
| ZNF143 Ab | -  | +        |
| Rabbit serum | -  | -        |
**Table 1**

Potential TF-binding sites within the human CEBPα promoter

| TF family | Opt. | Position | Strand | Core similarity | Matrix similarity | Sequence |
|-----------|------|----------|--------|-----------------|------------------|----------|
| AP4       | 0.93 | 2–18     | (+)    | 1.00            | 0.948            | ttcacgcCCAGacccag |
| LEF/TCF   | 0.94 | 7–23     | (+)    | 1.00            | 0.964            | ttcctcCCAAAgaacg |
| CHRF      | 0.92 | 10–22    | (+)    | 1.00            | 0.928            | gcgTTGgaagag |
| PURA      | 0.97 | 242–254  | (+)    | 1.00            | 0.986            | gcgcgggcgTCCagcc |
| XBBF      | 0.89 | 252–270  | (+)    | 1.00            | 0.907            | gcgccgCggctcg |
| CDEF      | 0.87 | 258–270  | (+)    | 1.00            | 0.881            | gcgcggCCgccg |
| INSM      | 0.90 | 269–281  | (+)    | 1.00            | 0.943            | gcgCTcGGGacgcc |
| C2P2F     | 0.90 | 270–288  | (+)    | 1.00            | 0.902            | gcgCTGgagcgcgcctc |
| ZFSE      | 0.95 | 275–285  | (+)    | 1.00            | 0.957            | gcgACGGccg |
| PAX5      | 0.73 | 282–310  | (+)    | 0.789           | 0.735            | ggccgccgaacccgGgc |
| SP1       | 0.88 | 300–314  | (+)    | 1.00            | 0.890            | ctygGcGgccaac |
| CTCF      | 0.80 | 302–326  | (+)    | 0.789           | 0.858            | gcgcggggaagggag |
| STAF      | 0.77 | 328–350  | (+)    | 1.00            | 0.794            | aggcGCGgacgagcgcgc |
| ZBF1      | 0.87 | 336–358  | (+)    | 1.00            | 0.931            | gcgcggcCgCgccgcgagc |
| EGRF      | 0.79 | 342–358  | (+)    | 0.864           | 0.769            | gctgctgcGGacggc |
| CTCF      | 0.80 | 346–370  | (+)    | 1.00            | 0.838            | cncggcccgccagGGacag |
| NR2F      | 0.82 | 355–379  | (+)    | 0.779           | 0.827            | gcgcggcggagccGGc |
| BNCF      | 0.85 | 358–375  | (+)    | 1.00            | 0.942            | aggggctctCGTGCgcc |
| OAZF      | 0.73 | 377–393  | (+)    | 0.750           | 0.745            | tcGCTCctcaggtcgc |
| EGRF      | 0.86 | 380–396  | (+)    | 1.00            | 0.860            | actctaggGGGcgc |
| STAF      | 0.76 | 389–411  | (+)    | 0.904           | 0.801            | tataCGCggacgccgcgc |
| CDEF      | 0.87 | 390–402  | (+)    | 1.00            | 0.873            | gcgACGGcgcg |
| WHNF      | 0.95 | 390–400  | (+)    | 1.00            | 0.951            | gcgACGGccg |

**Table 2**

Mass spectrometric data of identified proteins present within fraction 5

| No. of peptide matches | Average cross-correlation | Gene          |
|------------------------|--------------------------|---------------|
| 38                     | 2.9993                   | UPSP:ACTN1_HUMAN |
| 33                     | 3.526                    | UPSP:ACTN4_HUMAN |
| 30                     | 3.515                    | UPSP:SND1_HUMAN |
| 29                     | 3.469                    | UPSP:SYA_HUMAN |
| 23                     | 3.508                    | UPSP:UBE1_HUMAN |
| 21                     | 2.889                    | UPTR:O14980_HUMAN |
| 16                     | 3.347                    | UPSP:HPS4L_HUMAN |
| 16                     | 3.322                    | UPSP:ABCF1_HUMAN |
| 14                     | 3.312                    | UPSP:HXX2_HUMAN |
| 10                     | 2.139                    | UPTR:Q7E6N6_HUMAN |
| 10                     | 3.070                    | UPSP:SPQ_HUMAN |
| 10                     | 3.031                    | UPSP:CSE1_HUMAN |
| 8                      | 3.476                    | UPSP:AP1B1_HUMAN |
| 7                      | 2.288                    | UPSP:JDE_HUMAN |
| 6                      | 3.438                    | UPSP:PM2_HUMAN |
| 6                      | 3.238                    | UPSP:SEC8_HUMAN |
| 5                      | 3.165                    | UPSP:MSH2_HUMAN |
| 3                      | 3.052                    | UPSP:HXX1_HUMAN |
| 2                      | 3.590                    | UPSP:CTN1_HUMAN |
| 2                      | 3.532                    | UPSP:UPB5_HUMAN |
| 1                      | 6.492                    | UPTR:Q6P665 |
| 1                      | 4.637                    | UPSP:ZNF143_HUMAN |
| 1                      | 4.322                    | UPTR:Q6NSX51_HUMAN |
| 1                      | 4.263                    | GP:AK044451_1 |
| 1                      | 4.231                    | UPSP:CORO7_HUMAN |
| 1                      | 3.941                    | UPTR:Q515SH7_HUMAN |
| 1                      | 3.730                    | UPTR:Q6MZH1_HUMAN |
| 1                      | 3.721                    | UPSP:SEC3HUMAN |
| 1                      | 3.687                    | UPSP:HSI05_HUMAN |
| 1                      | 3.496                    | UPTR:Q8NWG2_HUMAN |
| 1                      | 3.077                    | UPTR:Q6Q6NO64_HUMAN |
| 1                      | 3.001                    | UPTR:Q9UGQ5_HUMAN |
| 1                      | 2.895                    | UPTR:Q5T5N3_HUMAN |
| 1                      | 2.800                    | UPTR:Q9BWN4_HUMAN |
| 1                      | 2.709                    | UPSP:YS64_HUMAN |
| 1                      | 2.659                    | UPSP:XP07_HUMAN |
| 1                      | 2.639                    | UPSP:PSA_HUMAN |
| 1                      | 2.500                    | UPSP:TOP3A_HUMAN |
| 1                      | 2.434                    | UPSP:MCMI_HUMAN |
| 1                      | 2.316                    | UPSP:GSP1_HUMAN |
| 1                      | 2.301                    | UPSP:IKF2_HUMAN |
| 1                      | 2.213                    | UPTR:Q5T7F5_HUMAN |
| 1                      | 2.030                    | UPSP:GCC1_HUMAN |

**ZNF143 translocates the human CEBPα promoter in Schneider cells**

To test whether ZNF143 is able to transactivate the human CEBPα promoter, we performed transient transfection studies in *Drosophila* S2 Schneider cells. This cell line was utilized because the presence of binding activity for ZNF143 or the related family member ZFN76 (19) could not be detected by EMSA (Fig. 6A, lane 1). Overexpression of ZNF143 in Schneider cells led to the ~10-fold transactivation of the −179 bp promoter, whereas overexpression of ZFN76, as well as the empty vector, generated only low luciferase activity (Fig. 6B). In addition, mutating the ZNF143 site reduced transactivation 5-fold. Altogether, we show that ZNF143 can transactivate the CEBPα promoter by binding directly to a site within it.

**ZNF143 is required for CEBPα expression in myeloid cells**

Finally, we investigated whether ZNF143 could regulate C/EBPα expression. We identified two shRNA sequences that reduced ZNF143 transcription. Transduction of these shRNA constructs into U937 myeloid cells reduced ZNF143 expression at both the mRNA and protein level by 80% in comparison with a non-silencing control (NSC) (Fig. 7, A–C). In addition, ZNF143 silencing resulted in a strong reduction of CEBPα transcript levels (Fig. 7, A–C). Because ZNF143 binds a motif conserved in CEBPA, CEBPD, and CEBPE (Fig. 2D), we examined the consequences of ZNF143 knockdown on CEBPα expression (as *CEBPD* is not expressed in U937 cells (29), the effects of *CEBPD* knockdown could not be analyzed). A significant reduction of *CEBP* transcripts was observed in U937 cells transduced with either of two ZNF143 shRNA constructs (Fig. 7D). Further, we analyzed the effect of ZNF143 silencing on other CEBP members and observed that *CEBPB* and *CEBPG* transcription was similarly reduced (Fig. 7D). *CEBPB* reduction was also detectable at the protein level (Fig. 7E). Although *CEBPB* and *CEBPG* do not present the binding motif identified...
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Figure 5. ZNF143 binds to the CCCAGCAG motif in the CEBPA promoter in vivo. A, ChIP-exo signal (fragment density, blue track) shows a strong enrichment in the proximal promoter of CEBPA compared with the input (gray track), indicating specific binding of ZNF143. Note the 10-fold difference in scale between input sequencing and ChIP-exo signal. Genomic positions on hg19 are indicated. B, zoom out of the ZNF143 peak in a 10-kb region around CEBPA. C, sequence logos of highly enriched ZNF143 binding motifs in the ChiP-exo peaks correspond to the conserved sequences found in the CEBPA proximal promoter. This demonstrates the preferential binding of ZNF143 to a motif containing the CCCAGCAG sequence, boxed in blue. Enrichment p values are indicated.

Figure 6. ZNF143, but not its homolog, ZNF76, transactivates the CEBPA promoter. A, no binding is observed to the ZNF143 probe (z1) in Schneider (S2) cells (lane 1). B, graph demonstrating that ZNF143, but not its homologue, ZNF76, activates transcription driven by the CEBPA promoter in luciferase reporter assays. Schneider cells, which do not express endogenous ZNF143 or ZNF76, were transiently transfected with luciferase reporter constructs containing the human CEBPA proximal promoter (−181 to +143) with either the wild-type or mutated octameric site alone (lane 1) or together with ZNF143 expression vector (lane 2), ZNF76 expression vector (lane 3), or an empty expression vector (PPAC; lane 4). Relative luciferase activity (RLU) normalized to Renilla luciferase is shown. Error bars, S.D. of three independent replicates.

Discussion

In this study, to gain insights into the transcriptional regulation of myeloid development, we investigated the regulatory control elements of the promoter of one of the master regulators of this process, C/EBPα. Because the programs driving hematopoietic development are largely driven by alterations in transcriptional activity, studying potential TF binding sites within such genes may prove to be key in defining the mechanisms that drive lineage development. Indeed, we demonstrate here that ZNF143 binds to the proximal promoter of CEBPA in vitro and that down-regulation of ZNF143 results in a marked decrease in C/EBPα mRNA and protein levels in myeloid cells.
Interestingly, mutational analyses showed that there is some flexibility in the 8-bp sequence for formation of a DNA–protein complex. Although, methylation interference assays defined three guanine residues as being in close proximity to a DNA–protein binding motif. A study of 100 mouse transcription factors (31), there is flexibility in the 8-bp sequence for formation of a DNA–protein complex. A ZNF143-specific antibody was employed. Actin was used as a loading control. Lanes 1–3, 52 cells overexpressing empty vector control (EV), ZNF76, and ZNF143, respectively. Lanes 4 and 5, U937 cells transduced with NSC, ZNF143 sh#1, and ZNF143 sh#2, respectively. F, quantitative ChIP-PCR using the U937 cells transduced with NSC (black bars), ZNF143 sh#1 (white bars), and ZNF143 sh#2 (gray bars). Immunoprecipitation was performed with antibodies against IgG control and ZNF143, and enrichment was determined in a CEBPA promoter region, intergenic region, and U6 promoter. Y axes indicate enrichment as a percentage of input. Error bars, S.D.

ZNF143 is a ubiquitously expressed transcriptional activator that belongs to the Kruppel family of zinc finger proteins. It has been shown to associate with CHD8 and contribute to the efficient transcription of the U6 RNA polymerase III (30). Furthermore, ZNF143 has been implicated in the transcriptional regulation of genes associated with cell cycle and DNA replication (34) and DNA repair genes following exposure to cisplatin (35). ZNF143 is also a key component of the three-dimensional chromatin structure (36) and is required for human embryonic stem cell pluripotency (37). However, until this study, ZNF143 had not been implicated in hematopoietic development.

Figure 7. ZNF143 regulates C/EBPα expression. A, Western blots showing protein expression of ZNF143, both C/EBPα isoforms and HSP90 loading control in U937 cells that were transfected with NSC shRNA or two independent shRNA vectors targeting ZNF143 (sh#1 and sh#2). B, bar graphs quantifying the C/EBPα protein levels from A demonstrate that knockdown of ZNF143 in U937 cells leads to down-regulation of C/EBPα protein levels using two independent shRNA-targeting constructs. C, bar graphs demonstrating levels of ZNF143 mRNA in cells expressing NSC, sh#1, or sh#2. D, CEBPA, CEBPB, CEBPE, CEBPG, and FOS mRNA levels in cells expressing NSC, sh#1, or sh#2. E, Western blots demonstrating that ZNF143 knockdown in U937 cells decreases C/EBPα and C/EBPβ levels. A ZNF143-specific antibody was employed. Actin was used as a loading control. Lanes 4 and 5, U937 cells transduced with NSC, ZNF143 sh#1, and ZNF143 sh#2, respectively. F, quantitative ChIP-PCR using the U937 cells transduced with NSC (black bars), ZNF143 sh#1 (white bars), and ZNF143 sh#2 (gray bars). Immunoprecipitation was performed with antibodies against IgG control and ZNF143, and enrichment was determined in a CEBPA promoter region, intergenic region, and U6 promoter. Y axes indicate enrichment as a percentage of input. Error bars, S.D.
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although its knockdown in Zebrafish causes reduced blood cell formation (38).

Whereas the expression of ZNF143 is ubiquitous (Fig. 2C), at the same time, it appears to be important for expression of C/EBPα in myeloid cells. Furthermore, the regions of the CEBPA promoter required for activity in myeloid cells appear to be distinct from those that drive its expression in hepatic cells (16). We hypothesize that ZNF143, rather than being a sole regulator of CEBPA expression in myeloid cells, might interact with other cell type–specific regulatory factor(s) that are yet to be identified. Therefore, it will be important in future studies to identify the component(s) that might further contribute to myeloid transcriptional activity of the CEBPA promoter. A number of ubiquitously expressed transcription factors can contribute to myeloid specificity of promoters, including Sp1 (39–42). One possibility is that other transcription factors cooperate with ZNF143 to provide specificity. Among various candidates, the transcriptional activator Notch1 and its associated DNA-binding repressor, RBPJ, strongly overlap with ZNF143-binding sites in other cell types (21, 28). Interestingly, the Notch target, Hes-1, represses the Cebpα promoter in myeloid cells, with a Hes-1 site at ∼170 bp (43). In addition, a number of binding sites for the transcriptional repressor THAP11 were observed to coincide with ZNF143 sites (21, 44). Notch1 signaling is aberrant in many human cancers and in myeloid leukemia (45) and has been implicated in regulating self-renewal of tumor stem cells (46). Similarly, THAP11 is involved in leukemia (45) and has been implicated in regulating self-renewal of embryonic stem cells (47). C/EBPα is involved in signaling is aberrant in many human cancers and in myeloid cells (15) and solid tumors (48). Taken together, it appears that ZNF143 may modulate genes involved in biological processes related to cell growth in rapidly dividing cells, such as stem cells and tumors, via potential cofactors THAP11 and Notch1.

A second possibility is the recent observation that ZNF143 can contribute to specificity by contributing to interactions between promoters and distal regulatory elements (49). Recently, two groups have identified an important enhancer at +42 kb relative to the transcription start site that interacts with the promoter to mediate myeloid-specific expression of C/EBPα (50, 51). The effect of competitive or combinatorial binding of ZNF143 and other potential partners on the transcriptional activity of target genes, such as C/EBPα, as well as the potential interaction of ZNF143 binding to the CEBPA promoter and distal regulatory elements (18, 50, 51) merit further investigation.

Experimental procedures

Constructs

We used the GRCh37/hg19 reference genome to determine the sequence of the CEBPA proximal promoter region. All CEBPA constructs used in this study are numbered here relative to the TSS determined previously (16), as illustrated in supplemental Fig. S1. Luciferase constructs were kindly provided by Dr. Gretchen Darlington and were previously used in a study involving Hep3B2 cells (16). These constructs were generated by first subcloning a 2.2-kb EcoRI/NruI fragment into PXPI. Further deletions were generated using BamHI, KpnI, Stul, EagI, AvrII, and SmaI sites. Deletion constructs starting from position −110 or −95 were generated by PCR, subcloned into the pGEM-T vector, and subsequently cloned into the PXPI vector (52). These constructs were sequenced using the dideoxy method. We then used PCR to generate another series of deletions within 443 bp of the TSS and extending to the initiating ATG (+124). Mutant constructs were generated, using the method of Zaret et al. (53). Two internal primers containing a 9-base mutation, from −117 to −108, were synthesized, primers C (5'-CTAGTTTCACCAAGCAGCGCGCGCCG-3') and B (5’-CGGGCGCGGTTCGTTGAGTAACCT-3’). Two external BamHI-containing primers, A (5’-CCGGGATCCCCAGGCTCTGTCTTCGCT-3’) and D (5’-GGGTATACCCCGGGGGGAGTGTTCT-3’), were synthesized. 40 PCR cycles (94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min) were performed using primers A and B in one reaction and primers C and D in the other. PCR products were gel-purified. 1 μl of each purified product was added to a 48-μl PCR for 3 cycles of the following: 94 °C, 1 min; 55 °C, 1 min; 72 °C 1.5 min. 1 μl of primers A and D (10 μM) was added, and 40 PCR cycles were performed: 94 °C, 1 min; 55 °C, 1 min; 72 °C 1.5 min. The PCR fragment was size-fractionated on a 1.2% agarose gel and purified using Gene-Clean (BIO 101). The fragment was digested with BamHI and KpnI (New England Biolabs) and subcloned into PXPI. Subsequent deletions were made using internal primers. shRNA sequences specifically targeting and consequently down-regulating ZNF143 were cloned into the retroviral vector pRSeG (54). sh#1 (5’-GGGCGTACCCCGGGGGGAGTGTTCT-3’) targets a sequence from exon 3 that was the human version of an RNAi sequence used previously to target murine ZNF143 in stem cells (55). sh#2 (5’-GGGCGCATCTACAGGAGTGTTCT-3’) targets a sequence located in the 3’-UTR and was found using Dharmacon’s design program for shRNA. ZNF143 and ZNF76 expression constructs were generously donated by Dr. Carbon (Université de Strasbourg, France) and Dr. Kunkel (Texas A&M University, College Station, TX).

Cell culture, transient transfections, and luciferase assays

U937 cells were maintained in RPMI 1640 medium supplemented with 10% FBS and transiently transfected with the indicated constructs (see above, and see Pahl et al. (56)). Briefly, 107 cells were electroporated at 220 V at 960 microfarads with a Bio-Rad Gene pulser with 20 μg of supercoiled luciferase reporter construct and 1 μg of CMV-hGH, a plasmid expressing human growth hormone from a CMV promoter. Cells were incubated at 37 °C for 6 h, harvested by centrifugation at 1000 rpm for 5 min, and lysed. Luciferase activity was measured on a Monolight 2010 Luminometer (Analytical Luminescence Laboratories). Transfection efficiencies were controlled by measuring human growth hormone levels by radioimmunoassay (Nichols Institute). Schneider (S2) cells were obtained from ATCC and cultured in insect medium (Sigma) supplemented with 10% FBS at 25 °C and transfected with Transfectin (Bio-Rad).
**Preparation of nuclear extracts**

Nuclear extracts were prepared as follows: cells were spun down and washed with cold PBS and resuspended in isotonnic buffer A (10 mM Heps, 1.5 mM MgCl₂, pH 7.9) for 30 min. Cells were lysed with a 1-ml tuberculin syringe (18-gauge) by rapidly pumping the sample through at least five times. Nuclei were then lysed and resuspended in buffer B (10 mM Heps, 1.5 mM MgCl₂, 400 mM NaCl, pH 7.9). Protein concentration was determined using the method of Bradford (57) (Bio-Rad), and extracts were aliquoted and frozen at −80 °C.

**EMSA**

Gel shifts assays were performed by incubating 20 μg of nuclear extract with 5 μl of 5× buffer (50 mM Heps, pH 7.9, 250 mM KCl, 10 mM MgCl₂, 1 mM DTT), 2 μg of poly(dI-dC), and 50,000 cpm of ³²P-labeled double-stranded probe. Samples were loaded on a 6% polyacrylamide non-denaturing gel and run at 200 V for 3 h in 1× TBE (89 mM Tris, pH 7.6, 89 mM boric acid, 2 mM EDTA). Gels were dried and exposed to film for 12 h or less. ZNF143 antibodies were generously donated by Dr. Philippe Carbon (58). ZNF76 antibodies were generously donated by Dr. Yu-Chung Yang (Case Western University) (33). The sequences of the probes used in EMSA are provided in Table 3.

**ZNF143 knockdown experiments**

Two shRNA constructs were used to generate virus in Phoenix A cells. A mixture of plasmid DNA and Lipofectamine 2000 was added to 150-mm plates of 80% confluent Phoenix A cells containing DMEM without FBS. 4 h later, 10% FBS was added. 60 h later, the medium was collected, concentrated with a Cencontaining DMEM without FBS. 4 h later, 10% FBS was added. was added to 150-mm plates of 80% confluent Phoenix A cells

**Table 3**

Probes used in EMSA

| Probe | Sequence (5’–3’) |
|-------|-----------------|
| Probe I | GCCGGCCGCCGCGCTAGAGCCAG |
| Probe II | CTTAGGGCGCGCGGCGGCGC |
| Probe III | GCCGGCGCGCGCGGGGAGAG |
| Probe M | CTTAGG [b]TCCACAAGC CGCGCGCG |
| Sp1 site, PU.1 promoter | AAYCCGCGTCTGCGGAGTAC |
| z1: optimal STAF/ZNF143 binding probe (25) | ATTTCCCATGATCTCAGCCG |
| z2: ZNF143 probe (26) | CCGGGGGGCATTGTGGGCCGT |
| z3: ZNF143 probe (26) | ACCGCGCGTATTGGCCGT |

**Generation of antibodies against human ZNF143**

Rabbit polyclonal ZNF143 antibody was raised against a C-terminal epitope of human full-length ZNF143 (from amino acid 572 to 594, AFHTASSEMGHQQHSHHLVT). Briefly, preimmunized blood was taken 2 weeks before first antigen injection. Antigen was injected into rabbits on days 0, 21, and 42. Blood samples were taken on days 28 and 49. Rabbits were euthanized, and blood was collected for further antibody purification on day 56.

**UV cross-linking**

EMSA was performed with a double-stranded oligonucleotide containing a halogenated analog of thymidine (BrdU). The sequence of the probe was 5’-CTAGGACCCAGCAGCGGCG-3’ and 5’-TGGGCGAAGGTATGTCGA-3’; CEBPG, 5’-CCCTGCTC-TCATTTCTACTTG-3’ and 5’-ACACTAATTCCGTTCACCC-3’; FOS, 5’-CCGCGGATACCTCCTCTTAC-3’ and 5’-GTGGGAATCGTTGCGACT-3’.

**Methylation interference**

50 ng of a single-stranded oligonucleotide spanning from −87 to −111 bp (5’-GCGGCGGGGCGGCTGCTGGG-TCCA-3’) was end-labeled with 50 μCi of [γ-³²P]ATP and T4 polynucleotide kinase. This was then gel-purified and annealed to 50 ng of complementary oligonucleotide. 10⁶ dpm of this fragment was then methylated under limiting conditions by adding 1 μl of DMSO to 200 μl of DMS reaction buffer (50 mM sodium cacodylate, pH 8.0, 1 mM EDTA, pH 8.0) for 5 min at room temperature. Stop buffer (1.5 M sodium acetate, pH 7.0, 1 M

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β-mercaptoethanol was added, and the partially methylated fragment was ethanol-precipitated. 50,000 cpn of this methylated probe was incubated with 20 μg of U937 nuclear extract. This sample was then run on a 6% acrylamide gel in 1× TBE. The gel was exposed to film, and the complexes of interest were located by superimposing the X-ray film over the gel. The fragments of interest were isolated and extracted from the gel using 0.3 M ammonium acetate. The pellet was resuspended in 100 μl of 1 M piperidine and heated to 90 °C to cleave the DNA–protein complex. Samples were then lyophilized and run on a 4% denaturing urea gel. Chemical sequencing of unmethylated oligonucleotides was performed by the Maxim–Gilbert method (59), and samples were run along with test samples for comparison.

**Protein purification**

A 10-liter preparation of HeLa cells was obtained from the National Culture Facility. After centrifugation of this culture, the 10-ml pellet of cells was resuspended in 10 ml of isotonic buffer A (10 mM Hepes, 1.5 mM MgCl₂, pH 7.9) for 30 min. Cells were lysed with a 0.1-ml tuberculin syringe (18-gauge) by rapidly pumping the sample through at least five times. Nuclei were then lysed and resuspended in buffer B (10 mM Hepes, 1.5 mM MgCl₂, 400 mM NaCl, pH 7.9). Protein concentration was determined using the method of Bradford (57) (Bio-Rad). Ultimately, we obtained 7.5 ml of protein at a concentration of 10 μg/μl. This sample was then desalted into 10 μl Tris, pH 8.0, using a 5-ml HiTrap desalting column, containing Sephadex G25 (GE Healthcare). The sample was then passed over a 5-ml HiTrap-Q HP column (GE Healthcare). The salt concentration was increased in a step gradient from 100 mM salt to 1 M salt in 100 mM increments. 5 ml/step at a flow rate of 1 ml/min were collected. Fractions were collected and tested for the presence of the DNA-binding activity of interest using EMSAs. As 95% of the activity was found in the 200 mM salt fraction, we concentrated this fraction using a VivaSpin protein concentrator (GE Healthcare), resuspended it in 4× SDS sample buffer (40% glycerol, 240 mM Tris, pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% β-mercaptoethanol), and boiled it for 10 min. We subsequently ran this sample on a 10% polyacrylamide SDS gel and excised 20 different fractions around the 100-kDa marker. Each sample was ground up, resuspended in 300 μl of renaturation buffer (100 mM NaCl, 10 mM Hepes, pH 7.5, 1 mM EDTA), and stored at 4 °C overnight. We performed EMSA on the fractions with probe II to determine which samples contained proteins that were capable of binding to 32P-labeled probe II. Those samples, as well as a sample that did not show any DNA binding activity, were sent to the Biopolymer facility at Harvard Medical School.

**ChIP-seq input library preparation**

8.5 × 10⁷ U937 cells were cross-linked in 1% formaldehyde (Sigma) for 10 min at room temperature. After cross-linking, cells were washed once with ice-cold PBS. Cell lysis was carried out for 10 min on ice using 350 μl of lysis buffer containing 1% SDS (1st Base), 10 mM EDTA (1st Base), 50 mM Tris, pH 8 (1st Base), and Complete protease inhibitors (Roche Applied Science). Genomic DNA was sheared in a Bioruptor™ Next Gen water bath sonicator (Diagenode) to ~200–500 bp by 10 cycles of 30 s ON, 30 s OFF, on High. DNA fragment size was verified via agarose gel electrophoresis. Input chromatin was reverse–cross-linked in 125 μl of 1% SDS and 0.1 μl NaHCO₃ (Sigma) for 6–7 h at 65 °C. The chromatin was purified using the QIAquick PCR spin kit (Qiagen), and at least 5 ng of input DNA was processed for deep sequencing using TruPlex-FD kits (Rubicon Genomics) according to the manufacturer’s instructions. The input library was sequenced using Illumina HiSeq2000 at the Duke–NUS Genome Biology Facility, and tags were mapped to human genome build hg19.

**Quantitative ChIP followed by PCR**

60 × 10⁶ U937 cells transduced with ZNF143 targeting shRNA constructs (sh#1 and sh#2) or NSC shRNA were used for quantitative ChIP-PCR as described previously (61). Briefly, cells were fixed with 1% formaldehyde and then neutralized with glycine at a final concentration of 0.125 M. After washing with ice-cold PBS three times, cells were lysed in lysis buffer and sonicated to obtain 500-bp genomic DNA fragments. Samples were divided into three equal aliquots, and each was incubated overnight with IgG or antibodies to ZNF143 or CEBPα. The next day, magnetic beads were added for 2 h and subsequently washed (61). After reverse–cross-linking and purifying chromatin, quantitative PCR was performed using the following oligonucleotides: CEBPA promoter region, 5′-GTCGAAAATATGGGCCG-3′ and 5′-GTCGAAAAATATGGGCCG-TCC-3′; L6 promoter region, 5′-GCAAAACGCCACAGTGG-AAGC-3′ and 5′-CTATGTCCGCCAACTTCCTC-3′; and intergenic region, 5′-CAGCAGTTCTGTCATCTC-3′ and 5′-GTTGCTGAGAACCAGCAC-3′.
Author contributions—D. G. T. and A. D. F. conceptualized the work. D. G., A. L., B. B., M. A.-J., Q. Z., M. K., A. E., K. D. S., H. R., P. Z., S. K., R. S. W., E. L., U. S., G. C., S. C., L. M. S., and M. H. C. were responsible for methodology, formal analysis, and investigation. D. G., A. L., M. A.-J., L. M. S., and D. G. T. wrote the manuscript. D. G., A. L., M. A.-J., and L. M. S. were responsible for visualization. A. L. and D. G. T. supervised the work. D. G. T. acquired funding.

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