RESEARCH ARTICLE

REVISED Genome-wide regulation of CpG methylation by ecCEBP α in acute myeloid leukemia [version 2; peer review: 2 approved]

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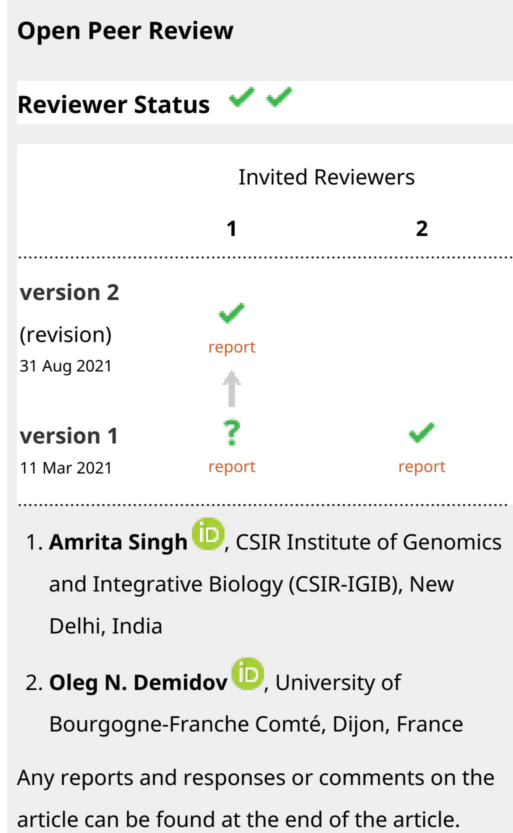
Abstract

Background: Acute myeloid leukemia (AML) is a hematopoietic malignancy characterized by genetic and epigenetic aberrations that alter the differentiation capacity of myeloid progenitor cells. The transcription factor *CEBPα* is frequently mutated in AML patients leading to an increase in DNA methylation in many genomic locations. Previously, it has been shown that *ecCEBPα* (extra coding CEBP*α*) - a lncRNA transcribed in the same direction as *CEBPα* gene - regulates DNA methylation of *CEBPα* promoter in *cis.* Here, we hypothesize that *ecCEBPα* could participate in the regulation of DNA methylation in *trans*.

Method: First, we retrieved the methylation profile of AML patients with mutated *CEBPα* locus from The Cancer Genome Atlas (TCGA). We then predicted the *ecCEBPα* secondary structure in order to check the potential of *ecCEBPα* to form triplexes around CpG loci and checked if triplex formation influenced CpG methylation, genome-wide.

Results: Using DNA methylation profiles of AML patients with a mutated *CEBPα* locus, we show that *ecCEBPα* could interact with DNA by forming DNA:RNA triple helices and protect regions near its binding sites from global DNA methylation. Further analysis revealed that triplex-forming oligonucleotides in *ecCEBPα* are structurally unpaired supporting the DNA-binding potential of these regions. *ecCEBPα* triplexes supported with the RNA-chromatin co-localization data are located in the promoters of leukemia-linked transcriptional factors such as MLF2.

Discussion: Overall, these results suggest a novel regulatory mechanism for *ecCEBPα* as a genome-wide epigenetic modulator through triple-helix formation which may provide a foundation for sequence-specific engineering of RNA for regulating methylation of specific genes.



Keywords
Acute myeloid leukemia, Triplex, DNA methylation, long non coding RNA, extra-coding CEBPα

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REVISED **Amendments from Version 1**

Figure 1b was updated. (Details: This was done to clarify the abundance of hypermethylated sites versus non-hypermethylated sites.)

Figure 1c was updated. (details: The x-axis was confusing and unclear, so we had to provide more explicit labels.)

A new image was added to Figure 1 as Figure 1d. (details: Using external data, we showed the relationship between ecCEBPA and CpG methylation on the predicted binding and non-binding sites.)

Since Figure 1d was not present before now, the previous Figure 1d and Figure 1e were updated to Figure 1E and Figure 1F respectively.

Figure 2a was updated: (details: we provided annotations on the image to show the single-stranded region that was rich in triplex-forming oligonucleotides.)

A new image was added to Figure 2 as Figure 2c. (Details: Through this, we showed the methylation difference in only the experimentally validated ecCEPBA contacts.)

Since Figure 2c was not present before now, the previous Figure 2c and Figure 2d were updated to Figure 2D and Figure 2E respectively.

We also provided a table of some manually curated functional annotations for genes that are close to the experimentally validated CpGs

Discussion:

We discussed the new findings presented in Figure 1d in the context of Di Ruscio's paper on ecCEBPA's expression and demethylation activities.

Underlying Data:

We provided the link to ENCODE data showing the normal methylome of HL-60 cells using RRBS.

Any further responses from the reviewers can be found at the end of the article

Introduction

Acute myeloid leukemia (AML) is a malignant tumor characterized by the proliferation of undifferentiated myeloblasts[1,2]. It is the most prevalent form of leukemia in older adults (>60 years) with an annual mortality rate of 50% and a 5-year survival rate of 24%[2,3]. With the combined effects of the global increase in average life expectancy and AML drug inefficiency, the number of patients is expected to significantly increase in the coming years[4,5].

The current understanding of the molecular interplay in AML has been defined under two distinct categories; (i) genetic abnormalities and (ii) non-random chromosomal rearrangements. Cases of AML with chromosomal rearrangements as t(15;17) [*PML-RARA*], t(9;22) [*BCR-ABL*], inv(16) [*CBFB-MYH11*], t(8;21) [*RUNX1-ETO*] are called cytogenetically abnormal (CA-AML), while cases with genetic abnormalities (including frequent mutations in *DNMT3A*, *NPM1*, *CEBPα*, *IDH1/2, TET2, FLT3-ITD*) are called cytogenetically normal (CN-AML)[1,4]. The former accounts for 50–55%, while the latter accounts for 45–50% of diagnosed AML cases[6,7]. Even though these mutations and chromosomal alterations are crucial for initiating AML, they are not sufficient to explain AML progression, heterogeneity, and relapse[8].

Recently, studies have identified the role of non-coding RNAs, especially long non-coding RNAs (lncRNA) in the initiation and progression of cancers[9–11]. LncRNAs are emerging functional transcriptional products of at least 200 nucleotides lacking an open reading frame. Although they account for a large proportion of transcriptional products in mammals (about 58,000 loci)[12], only a small number of lncRNAs have been well characterized. Although lncRNAs are mostly not conserved evolutionarily, they are heavily regulated suggesting their functional role[12,13]. They may function either as signal transducers, protein guides, or molecular scaffolds to regulate transcriptional and epigenetic events[14–16]. Some lncRNAs perform these functions in *cis*- by modulating transcription of nearby genes (*Dum*)[17] or act in *trans*-, by modulating genes at multiple distant loci (*MALAT1*)[18], while some can do both (*HOTAIR*)[9,19].

Recent studies have identified lncRNAs for their remarkable role in regulating major epigenetic processes such as DNA methylation and chromatin remodeling. DNA methylation in mammals is coordinated by one of the three DNA methyltransferases (DNMT); *DNMT1, DNMT3A,* and *DNMT3B*[7,17,20]. LncRNAs have been identified in recent studies as important agents that can modulate DNA methylation, either by activating or repressing *DNMTs*. For example, the lncRNA *Dum* was discovered to repress a nearby gene *Dppa2* by recruiting multiple *DNMTs* leading to methylation of a promoter region, thus promoting myoblast differentiation[17]. Conversely, the lncRNA *H19* represses the activity of *DNMT3B* by interacting with *SAHH* which hydrolyzes *SAH*, a step required for *DNMT3B* activation[21]. *ecCEBPα,* which is transcribed from the *CEBPα* locus, directly blocks *DNMT1* to prevent methylation of proximal and distal located promoters, thus promoting *CEBPα*-mediated granulocyte differentiation[20]. Mechanistic studies via reduced representation bisulfite sequencing and RNA immunoprecipitation sequencing shows that *ecCEBPα* suppresses DNA methylation in *cis*- by acting as a shield that sequesters *DNMT1* from the *CEBPα* promoter. We speculate that *ecCEBPα* could regulate *DNMT1* activities in distant DNA regions (*in trans-*) as well. The mechanism of this potential interaction is to be determined.

Methods

ecCEBPα sequence

In the recent version of GENCODE, *ecCEBPα* is not annotated most likely due to an overlap with a protein-coding gene *CEBPα* on the same strand. We retrieved the complete *ecCEBPα* sequence from the human genome (hg19, chr19: 33298573-33303358) based on information reported in the work of Di Ruscio *et al.*[20]. *ecCEBPα* is approximately 4.8kb and it overlaps with the intronless *CEBPα* gene (~2.6kb) on the same strand. *ecCEBPα* does not share either the same transcription start site (TSS) or transcription end site (TES) with *CEBPα*, starting ~0.89kb upstream and ending ~1.46kb downstream of the *CEBPα* gene.

DNA methylation data processing

CpG methylation (Illumina 450K array) and *CEBPα* mutation data for 186 AML patients were retrieved from the Cancer Genome Atlas (TCGA: http://firebrowse.org). CpG methylation levels were measured in 307796 unique loci. We split all the AML patients into two groups based on CEBPα mutation status (13 patients with a CEBPα mutation and 173 patients without a mutation). We classified a CpG position as hypermethylated (HM) in patients with a CEBPα locus mutation if DNA methylation level was significantly increased in the case of a CEBPα mutation (t-test, FDR ≤ 0.05 and Δ-value ≥ 0.1) and all non-hypermethylated (NHM) CpG in the case of a CEBPα mutation were classified as non-hypermethylated CpGs (t-test, FDR >0.05 and $|\Delta\text{-value}| < 0.1$). As a result, we obtained 11955 HM and 261433 NHM CpGs.

Secondary structure and triplex prediction

As suggested in a previous study[22], unpaired RNA nucleotides are more likely to form triplexes with DNA. We predicted RNA secondary structure using RNAplfold (V 2.4.14), from the Vienna suite using a cut-off for pairing probability (-c) of 0.1[23,24]. To search for potential interactions between ecCEBPα and DNA target regions we used only unpaired nucleotides, while the nucleotides predicted to pair were replaced with 'N'.

DNA target regions were defined as 100 nucleotides centered at each CpG. To predict *ecCEBPα* triplex formation with DNA target regions, we used Triplexator (V 1.3.2)[25], since it has higher accuracy of prediction[14], with the following optimization parameters suggested in 22: minimum length = 10 nucleotides, error rate = 20%, G-C content = 70%, and filter-repeats = off. Using these parameters out of 307796 unique CpG loci, we predicted 272131 loci with at least one triplex and 35715 without any. Among them, 10351 and 222105 potential triplex targets were predicted in the HM and NHM regions respectively.

To estimate the statistical significance of predicted triplexes we used Triplex domain finder (TDF v 0.12.3), which clusters RNA triplex-forming oligonucleotide (TFO) into DNA binding domains (DBD)[26]. Briefly, all 272131 CpG loci with at least one predicted triplex were taken as input target regions. By predicting triplexes in the background regions, TDF is capable of estimating the statistical significance of *ecCEBPα* binding between target regions and other non-target CpGs regions. Since TDF allows only to mask regions in the genomic background rather than to select the background explicitly we had to prepare a special mask for the non-target regions. To do so we removed 35715 CpG loci with zero triplex predictions from the human genome using BEDtools subtract (BEDTools v2.29.2). TDF was implemented with a minimum triplex length (-l) of 10 nucleotides, an error rate (-e) of 20%, and (-f) to mask background loci in 100 random samplings (-n).

RNA:chromatin colocalization analysis

To validate the predicted interactions we used RNA:chromatin interactome obtained with iMARGI method capturing chromatin-associated RNA (caRNA) and their genomic interaction loci[27]. The data was downloaded from GEO (GSM3478205). The iMARGI dataset was mapped to the hg38 genome assembly. We used UCSC Liftover to convert *ecCEBPα* sequence coordinates from hg19 to hg38 sequences[28]. We expanded the DNA coordinates of CpGs by 3.0kb nucleotides upstream and downstream. IntersectBed from BEDTools was used to check the co-location of predicted triplexes and experimentally validated interactions of *ecCEBPα*[29]. Fisher's exact test was calculated for the number of confirmed *ecCEBPα* interactions between TDF and iMARGI data.

GO enrichment analysis

Finally, since Illumina 450K array probes are located close to genes, we performed functional enrichment using BiNGO (v 3.0.3) (binomial test)[30] to infer the biological significance of the genes potentially affected by *ecCEBPα* binding.

All statistical analyses were performed using R 4.0 or SciPy v1.5.1 library. Visualization was done in Cytoscape 3.2.0[31] and Python 3.7. Code is available at https://zenodo.org/record/4385259[32].

Results

ecCEBPα forms triplexes with promoter regions and affects promoter methylation

The current study explores the potential of the lncRNA *ecCEBPα* in the modulation of global CpG methylation status in *trans* via direct interaction with DNA regions. *ecCEBPα* (extra coding *CEBPα*), reported in work by Di Russio *et al.*[20], is located on chromosome 19 and transcribed from the *CEBPα* locus (Figure 1a).

Mutations in *CEBPα* locus are a common feature of AML leading to whole genome hypermethylation (Figure 1b). Since TCGA is focused on protein-coding genes, all reported mutations are located within the *CEBPα* gene and could affect both *CEBPα* and *ecCEBPα*. To investigate if *ecCEBPα* could affect DNA methylation in *trans,* first we checked if it is capable of interaction via forming triple helices (triplexes) with its binding sites and if such interactions affect DNA methylation. We observed that regions capable of forming triplexes with *ecCEBPα* remain protected from global DNA hypermethylation observed in case of a mutation in a *CEBPα* locus (Figure 1c, Fisher's exact test, p-value <0.001). Furthermore, the overall methylation profile of HL-60 cells with overexpressed ecCEBPα (Wang *et al.*) also have a protective effect on ecCEBPA's binding sites in comparison to the rest of the genomic CpGs (Figure 1d, Fisher Test: p-val = 1.24E-09). This result suggests a negative relationship between DNMT access to promoter sites and *ecCEBPα* binding.

ecCEBPα binding is not affected by the mutation in the *CEBPα* locus

To investigate deeper the potential of ecCEBPα to form triplexes we used Triplex Domain Finder (TDF) - a triplex prediction tool that refines the resolution of predicted TFOs in RNA into DNA binding domains (DBD) and calculates the significance of the number of predicted triplexes for each DBD. Overall, 17 significant DBDs were identified within *ecCEBPα*, interspaced

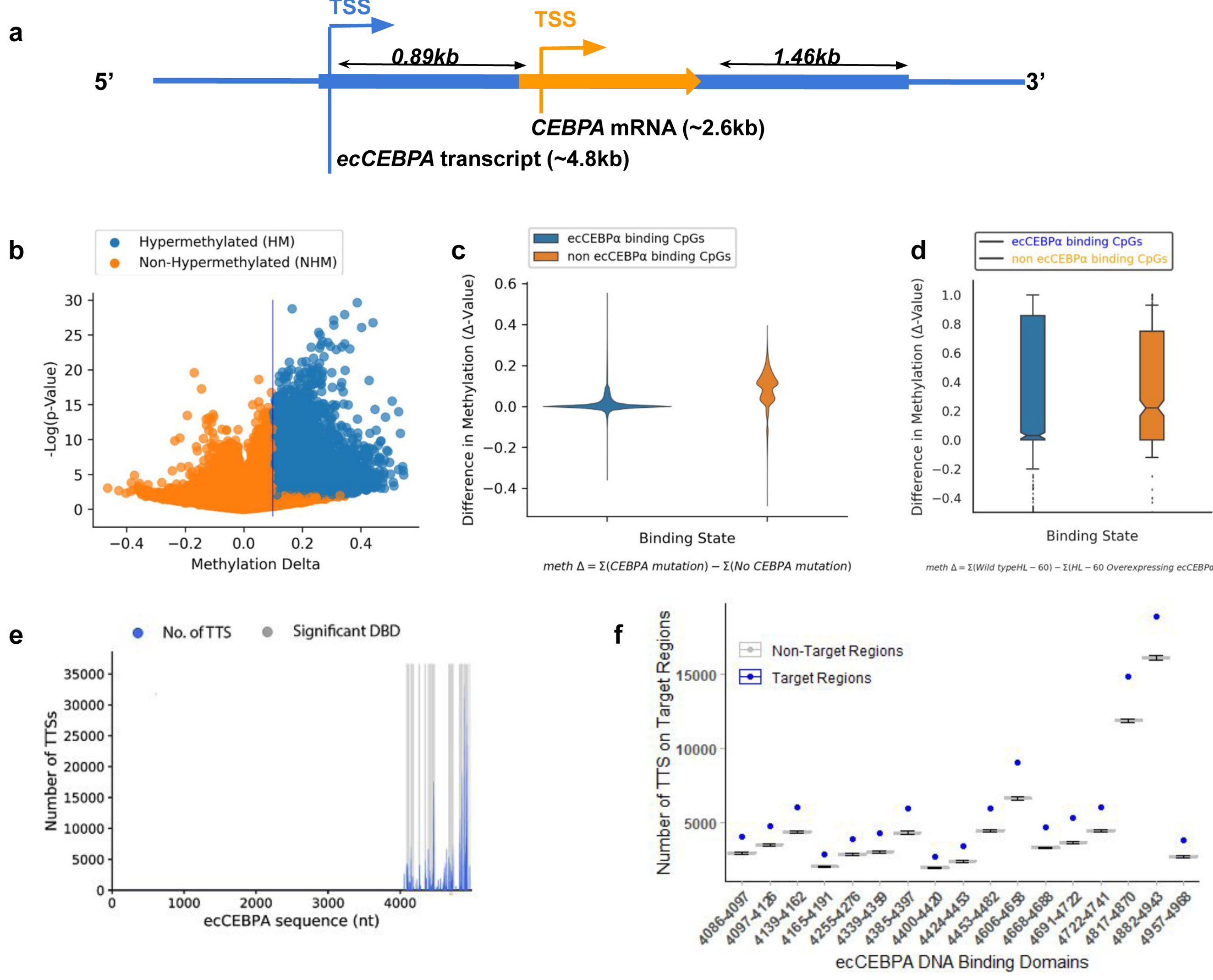

Figure 1. (**a**) Schematic diagram for transcriptional products in the CEBPα locus; CEBPα is represented by an orange arrow and ecCEBPα is represented by the blue line. (**b**) Global change in DNA methylation. (**c**) Difference in DNA methylation levels between patients with and without CEBPα mutation in the regions of ecCEBPα predicted binding (non parametric t-test, p-value >1E-10). (**d**) Number of DNA Triplex Target Site (y-axis) and a location of the corresponding TFO on the ecCEBPα (TFO: RNA; x-axis). (**e**) Number of Triplex Target sites per TFO predicted for NHM and HM CpG regions.

between sequences 4086 and 4968 towards the 3' end of the lncRNA (Figure 1e). These DBDs form triplexes with the majority of regions protected from hypermethylation in patients with a *CEBPα* mutation (Figure 1f, *Extended data:* Supplementary Table 1). The DBD region is located downstream from the *CEBPα* gene suggesting that *ecCEBPα* binding region is not affected by the mutation in the *CEBPα* locus. The predicted secondary structure of the *ecCEBPα* sequence showed that more than 95% of sequence positions from 4087-4987 (~0.5kb from *CEBPα* TES) (Figure 1e) were unpaired and potentially capable of forming triple helices with the target DNA region (Figure 2a).

ecCEBPα interacts with predicted binding sites

Since we use relatively relaxed thresholds for triplex prediction, we decided to validate the predicted RNA:DNA triplexes using experimental data obtained with the iMARGI method, allowing detection of RNA-chromatin interactions. We identified 157 *ecCEBPα* contacts within the iMARGI dataset and 29 of them contained predicted triplexes. Altogether, these 29 iMARGI interactions were made up of 182 predicted TTS (Fisher's exact test, p-value < 2.2E-16) located *in cis and in trans* on 14 chromosomes (Figure 2b). Chromosomes 19 (the native chromosome for *ecCEBPα*) are accounted for by all predictions. Since these ecCEBPA contacts have been

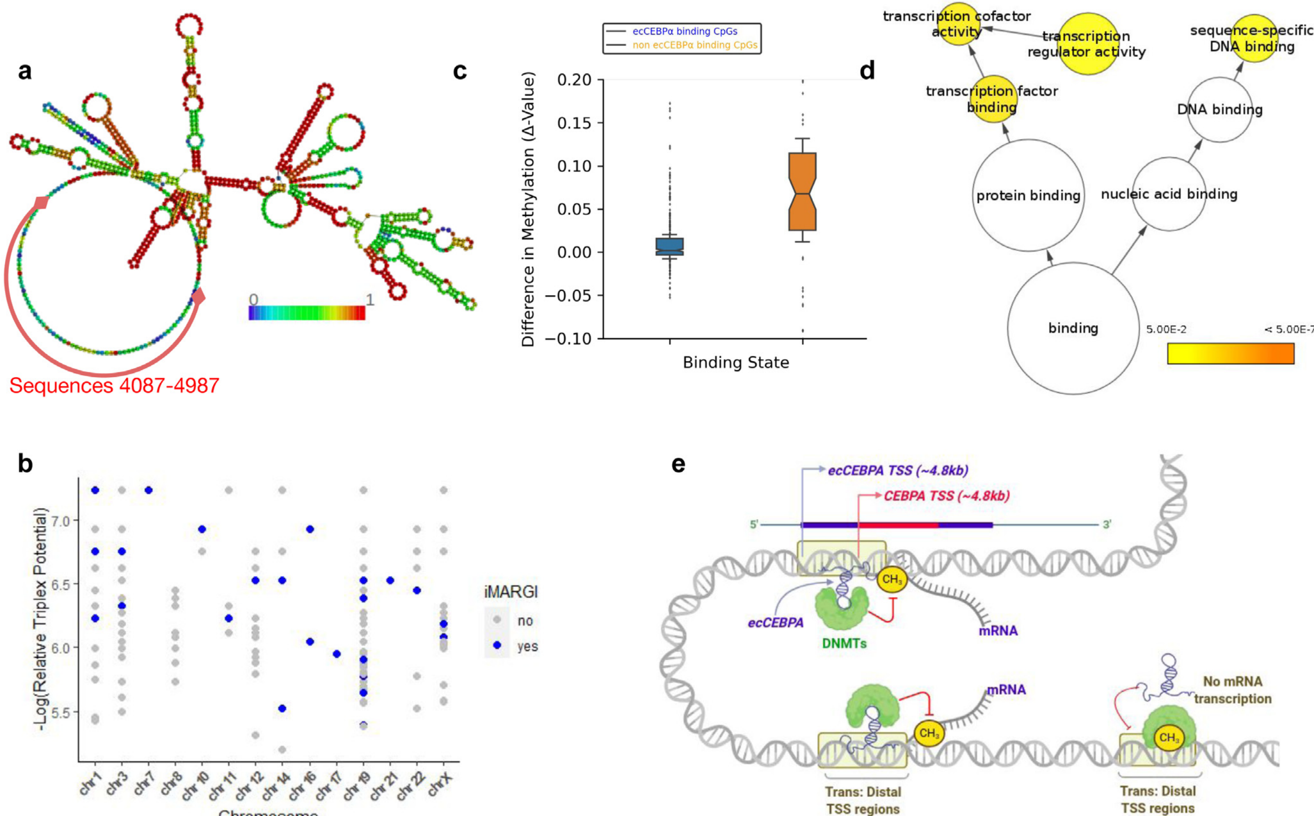

Figure 2. (**a**) Predicted secondary structure of ecCEBPα. Nucleotide color represents base pairing probabilities as predicted by RNAplfold. (**b**) Experimentally validated ecCEBPα triplexes per chromosome. The x-axis represents the chromosome and the y-axis represents the triplex potential relative to TTS. Blue points represent all TDF predictions that are present in the iMARGI dataset. (**c**) Functional enrichment of genes located nearby CpG with predicted triplexes. (**d**) Schematic representation of ecCEBPα:DNA interactions in trans and its implication on DNA methylation. The presence of ecCEBPα inhibits DNA methylation process.

experimentally validated, we suggested that they are the most reliable regions where ecCEBPA might have a protective effect from DNA methylation. Overall, a mean methylation delta score of experimentally validated binding sites is significantly lower than that of the non-binding sites (p-value < 1E-09) (Figure 2c).

Genes protected from methylation by *ecCEBPα* are involved in transcription factor activities

We performed gene ontology analysis on the genes located nearby 182 ecCEBPα triplexes supported by iMARGI. Key gene ontology categories such as nucleic acid binding and transcription factor activities were enriched among putative *ecCEBPα* targets (Figure 2d). Representative genes among transcription factors include *MLF2, SUV39H2, RBM5, UBTF,* and among sequence-specific DNA binding proteins include *POU2F2, MED12L, and DNASE1L1*. The enrichment in transcription factors (TF) suggests that triplex formation may represent a possible mechanism employed by *ecCEBPα* to regulate TF methylation and as a consequence, their expression. Furthermore, out of the 33 genes we identified to be targets of ecCEBPA, 16 genes (ICAM1, PDXK, etc.) are clearly related to hematopoiesis and various leukemias. A summary of the genes and their function is provided in Table 1.

Discussion

Unlike other forms of cancers, AML progression is often mutation-independent but may be explained by altered epigenetic regulation, DNA methylation specifically[1,8]. In this study, we elucidate a putative mechanism for the regulation of DNA methylation by *ecCEBPα*. In a previous study, *ecCEBPα,* which accompanies the transcription of *CEBPα* on the same locus, was shown to protect the promoter of *CEBPα* from DNA methylation leading to active expression[20]. We speculated that *ecCEBPα* might perform a similar function in trans. We demonstrated that *ecCEBPα*-DNA triplex formation might provide the molecular basis of this interaction. *ecCEBPα* binding presumably protects the region from genome-wide hypermethylation induced by *CEBPα* mutation in AML patients. Diruscio *et al.* confirmed that the most mutations in CEBPα do not influence the expression of ecCEBPα but rather, its ability of the RNA to fold properly. We suggest that the inability to fold

Table 1. AML specific function of nearby genes that are protected by ecCEBPA.

Gene	Role	Reference
FLVCR1	Alternative splicing disrupts of FLVCR1 disrupts erythropoiesis in Diamond-Blackfan anemia. (Rey *et al.* 2008)	https://haematologica.org/article/view/5065
PSMC6	Identified as part of a novel cluster for classifying AML risk and predicting outcomes. (Wilson *et al.* 2006)	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1895492/
CMIP	Identified as one of the top genes expressed after rhIGFBP7 treatment (to suppress clonal growth) of AML cases. (Verhagen *et al.* 2018)	https://www.cell.com/cell-reports/pdfExtended/S2211-1247(18)31838-2
GNA13	A crucial signalling component of the GPR84/Beta-Catenin Signaling Axis in AML Stem Cells. (Dietrich *et al.* 2014)	https://ashpublications.org/blood/article/124/21/3577/97803/GNA13-a-Novel-Component-of-the-GPR84-Beta-Catenin
ICAM1	Responsible for migration and adhesion of myeloid cells in hyperleukocytic AML. (Zhang *et al.* 2006)	https://onlinelibrary.wiley.com/doi/full/10.1111/j.1365-2257.2006.00784.x
PDE4C	Its mutation/absence leads to suppression of apoptotitc response in myelogenous symptoms. (Lerner and Epstein 2006)	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1383661/
ANKRD27	Identified as part of a novel cluster of competing endogenous RNA that predict AML survival and prognosis. (Wang *et al.* 2020)	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7432272/
PDXK	Responsible for Vitamin B6 addiction of AML cells. (Chen *et al.* 2020)	https://pubmed.ncbi.nlm.nih.gov/31935373/
NLRP3	The NLRP3 inflammasome is upregulated as part of the stress response in AML cells. (Jia *et al.* 2017)	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5754918/
PNPLA3	Responsible for elevated transaminases in lymphoblastic anaemia. (Bruschi *et al.* 2017)	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5683790/
CELSR1	Potentially contains mutations that contribute to patient specific mutations that are responsible for the origination of AML.(Skoczen *et al.* 2019)	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3407563/
PFKFB4	Responsible for survival of acute monocytic myeloid leukemia by suppression of apoptosis. (Wang *et al.* 2020)	https://pubmed.ncbi.nlm.nih.gov/32299611/
COL7A1	Contains somatic variations that are described to be potentially pathogenic in ALL patients. (Skoczen *et al.* 2019)	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6978700/
MED12L	Regulates hematopoietic stem cell (HSC) specific enhancers. It loss leads to loss of HSC stemness. (Aranda-Orgilles *et al.* 2016)	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5268820/
GALNTL5	Contains deleterious deletions that lead to copy number alteration in core binding factors of both adult and pediatric AMLs. (Kühn *et al.* 2012)	https://ashpublications.org/blood/article/119/10/e67/29576/High-resolution-genomic-profiling-of-adult-and
TAZ	Controls AML stemness and differentiation by modulating toll-like receptor (TLR) signaling. (Seneviratne *et al.* 2019)	https://pubmed.ncbi.nlm.nih.gov/30930145/

properly even in overexpressed cases may affect its structural ability to bind its targets. *ecCEBPα* contains a TFO/DBD-rich region at its 3' end, with low pairing probability, suggesting that it is capable of triplex formation. Several of the predicted *ecCEBPα* binding sites, which include transcription factors such as MLF2, SUV39H2, RBM5, UBTF, and sequence-specific DNA binding proteins include *POU2F2, MED12L, and DNASE1L1,* were supported by experimental iMARGI RNA:chromatin interactions data.

Currently, the understanding of lncRNA function and mechanisms of action is limited to a few dozen well-annotated lncRNA transcripts. A few functional characterization attempts are based on the 'guilt by association' hypothesis, which may not resonate well with the ability of lncRNA to interact in trans[33]. As thoroughly reviewed previously, lncRNAs such as *ecCEBPα, Dum, Dali, Dacor1, and LINCRNA-P21* interact with DNA in trans to regulate DNA methylation[34]. The results presented herein further demonstrate that triplex formation between *ecCEBPα* and CpG containing DNA regions could indeed be regulatory and protect CpG sites from DNMT activity.

Unfortunately, RNA:chromatin interaction protocols are relatively new and the data is available only for a few cell types. Since RNA:chromatin interactions are highly cell-type specific[35] and lowly expressed, it is not surprising that we could validate only a few of the predicted interactions. Nevertheless, based on our results, we suggest a model of potential *ecCEBPα* chromatin interaction *in trans* (Figure 2e). In this model, *ecCEBPα* uses its unpaired regions to directly bind to specific DNA sequences by forming triplexes and in this way prevents DNA methylation in the region of binding. *ecCEBPα* binding to distant regions could be mediated either by 3-dimensional chromatin organization[17] which brings them close to *ecCEBPα*.

Recent studies have observed that promoter or transcription start sites (TSS) regions, which tend to be rich in CpG dinucleotides, are TTS-rich and potential triplex-forming hotspots[36,37]. Through functional enrichment analysis, we observed that transcription factors might be preferential targets of *ecCEBPα*. Interestingly, previous studies have shown that the suppression of a myeloid leukemia factor (*MLF2*), an oncogene in breast cancer and myeloid leukemia[38,39] as well as UBTF which controls rDNA expression[40,41] contributes significantly to cancers upon promoter hypermethylation[40,42]. The suppressor of variegation 3-9 homolog 2 (*SUV39H2*), a histone-lysine-N-methyltransferase which regulates the hypermethylation H3K9 has also been reported to indirectly influence over 450 promoters in AML[43]. Having in mind that *ecCEBPα* is transcribed from CEBPα locus - a key transcription factor of hematopoiesis - this lncRNA could participate in the formation of a hub in the hematopoiesis regulatory network.

Conclusion

In conclusion, we have shown that ecCEBPα could serve as a trans-acting regulatory agent protecting its binding sites from genome-wide CpG methylation, and its dysregulation could contribute to aberrant methylation profile in AML patients. These results suggest a novel regulatory mechanism for ecCEBPα as a modulator of DNA methylation through triplex formation providing a foundation for sequence-specific engineering of RNA for regulating methylation of specific genes.

Data availability

Underlying data

Complete ecCEBPα sequence retrieved from the human genome (hg19, chr19: 33298573-33303358): https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.13/

CEBPα mutation data for 186 AML patients retrieved from the Cancer Genome Atlas (TCGA): http://firebrowse.org.

GEO: Embryonic kidney that expresses SV40 large T antigen, Accession number GSM3478205: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM3478205

GEO: DNA Methylation by Reduced Representation Bisulfite Seq from ENCODE/HudsonAlpha GSM980576: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM980576

Zenodo: josoga2/eccebp-alpha-project: F1000 Code Release, https://doi.org/10.5281/zenodo.4385259[32]

This project contains the following underlying data:

- DNA_BINDING_DOMAINS_ID.tsv
- predicted_secondary_structure_of_ecCEBPA.fa
- probes.csv (main data)

Code used for analysis available from: https://github.com/josoga2/eccebp-alpha-project/tree/f1000

Archived code as at time of publication: https://doi.org/10.5281/zenodo.4385259[32]

Extended data

Zenodo: Supplementary Data for Secondary structure and DNA binding domain prediction, http://doi.org/10.5281/zenodo.4433222[44].

This project contains the following extended data:

- Supplementary Table 1: Summary table of DNA binding domains (DBD), the counts of target regions within the genome and statistical analysis.
- ecCEBPα secondary structure prediction with RNAplfold

Data and code are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

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Version 2

Reviewer Report 15 September 2021

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Amrita Singh
CSIR Institute of Genomics and Integrative Biology (CSIR-IGIB), New Delhi, Delhi, India

All the major comments raised by the reviewer have been answered thoroughly by the authors. The additional methylation analysis of HL-60 datasets in both normal and overexpressed ecCEPBα is highly appreciated. Moreover, re-labelling of the plots as highlighted previously has made it easier to comprehend them. However, there are few minor technical remarks, both figures 1 and 2 do not have the legend for the updated figure 1(d) and 2 (c), and accordingly, the subsequent legends labelling needs to be modified.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Structure function relationship of noncoding RNA, with major focus on LncRNA function via triple helical structures.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 10 June 2021

https://doi.org/10.5256/f1000research.31133.r81320

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Oleg N. Demidov

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The manuscript by Ogunleye AF and co-authors describes a novel phenomenon of lncRNA ecCEBP α -dependent regulation of methylation in several genes. The authors analyzed the open datasets and showed that mutations in CEBPA gene (coding both, CEBPA transcriptional factor and lncRNA ecCEBPα) changed the global CpG methylation status in AML cells. The manuscript is nicely written, conclusions are logically justified. This work will be interesting to the readers in the epigenetic regulation and cancer research field.

Still, I have several concerns about the analysis. There are a lot of predicted triplex target regions for ecCEBPA genome-wide. Some of them could be false-positive predictions. Is there any way to confirm the observed effect only of experimentally validated contacts? Or in regions where predictions are most reliable?

Also, it is unclear other genes, not only transcription factors, predicted to be regulated by ecCEBPA are related to hematopoiesis. It would be helpful to have at least some hypotheses in the discussion. If there is some link found between ecCEBPA targets and regulation of hematopoiesis, it would really strengthen the conclusions of the work. Probably, KEGG pathway enrichment analysis may help with that.

Minor comments:

It is unclear on the figure with a secondary structure of ecCEBPA where the triplex-forming region is located.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Models of human diseases, cancer research, fibrosis, paging

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 05 Aug 2021

ADEWALE OGUNLEYE, Moscow Institute of Physics and Technology, Moscow, Russian Federation

Response to Reviewer Two

Comment one: There are a lot of predicted triplex target regions for ecCEBPA genome-wide. Some of them could be false-positive predictions. Is there any way to confirm the observed effect only of experimentally validated contacts? Or in regions where predictions are most reliable?

Response one: We are grateful to the reviewer for this suggestion. We performed a validation for the most reliable predicted triplexes using only experimentally validated ecCEBPA:DNA contacts. Overall, a mean methylation delta score of only 0.01 (p-value < 1E-09) was observed which further emphasizes the protective effect of ecCEBPA.
The following text and images were added to the main body of the paper:
"Since these ecCEBPA contacts have been experimentally validated, we suggested that they are the most reliable regions where ecCEBPA might have a protective effect from DNA methylation. Overall, a mean methylation delta score of experimentally validated binding sites is significantly lower than that of the non-binding sites (p-value < 1E-09) (Figure 2C)."

New figure panels (1&2) here: https://drive.google.com/file/d/1iynm-t0DfGkxLF5vsumG4iCcfo2u2xCJ/view?usp=sharing

Comment two: Also, it is unclear if other genes, not only transcription factors, predicted to be regulated by ecCEBPA are related to hematopoiesis. It would be helpful to have at least some hypotheses in the discussion. If there is some link found between ecCEBPA targets and regulation of hematopoiesis, it would really strengthen the conclusions of the work. Probably, KEGG pathway enrichment analysis may help with that.

Response two: We thank the reviewer for this important comment. Indeed, we discovered that other non-transcription factor genes have links to acute myeloid leukemia via literature mining. Unfortunately, this could not be enriched through KEGG. We suggest including a supplementary table that details the different roles of the identified genes.

In the main text, we added:
"Out of the 33 genes we identified to be targets of ecCEBPA, 16 genes (ICAM1, PDXK, etc.) are clearly related to hematopoiesis and various leukemias. A summary of the genes and their function is provided in Table 1."

Table 1: AML specific function of nearby genes that are protected by ecCEBPA (added to the paper).

Competing Interests: No competing interests were disclosed.

Reviewer Report 15 April 2021

https://doi.org/10.5256/f1000research.31133.r81314

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Amrita Singh
CSIR Institute of Genomics and Integrative Biology (CSIR-IGIB), New Delhi, Delhi, India

The article has addressed an important question of the ability of a lncRNA to act in trans and regulate a multitude of genes, which might be affecting the progression of acute myeloid leukemia. The authors have also highlighted the possibility of a triplex structure for defining the binding of lncRNA at its target gene CpG region. However, there are certain parts wherein the authors haven't been able to convey their points properly.

1. In Figure 1(b), a global DNA hypermethylation has been represented in AML patients with CEBPα mutation, but the numbers of the HM and NHM sites in the methods section do not reflect the same. It shows higher sites for NHM, than the HM sites, kindly check if some mislabeling is there at the authors part.

2. Also, the reviewer has failed to understand Figure 1(c). In both the x-axis and the side legend, binding and non-binding have been depicted, but which one is the AML patients with and without mutation isn't clear from the figure.

3. There are few other key points that were not clear;

   (a) what is the level of ecCEBPα expression in AML patients with and without mutation.

   (b) Additionally, if the expression of the lncRNA remains the same in both cases, how does one explain the increase in binding of the lncRNA and subsequent higher NHM sites, in the case of AML patients with CEBPα mutation?

   (c) Moreover, a previous report (Di Ruscio *et.al*[1]) on ecCEBPα had also analyzed genome-wide methylation, in which they report that methylation levels remain unchanged even when ecCEBPα was overexpressed. This is in contrast with the major theme of the paper i.e., methylation of genes in trans is affected by the ecCEBPα. The authors should comment on this in the discussion part.

References

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Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Structure function relationship of noncoding RNA, with major focus on LncRNA function via triple helical structures.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 05 Aug 2021

ADEWALE OGUNLEYE, Moscow Institute of Physics and Technology, Moscow, Russian Federation

Response to Reviewer One
Comment One: In Figure 1(b), a global DNA hypermethylation has been represented in AML patients with CEBPα mutation, but the numbers of the HM and NHM sites in the methods section do not reflect the same. It shows higher sites for NHM, than the HM sites, kindly check if some mislabeling is there at the authors part.

Response Two: We believe that there is a confusion here. The plot was correct and reflected the change in DNA methylation in all CpG positions including those that were not statistically differentially methylated. We counted hypermethylated CpG (t-test, FDR $\leq$0.05 and Δ-value $\geq$0.1) and those that were not hypermethylated (t-test, FDR >0.05 and |Δ-value| <0.1). In this way, we refer to non-hypermethylated (NHM) to any CpG with DNA methylation change less than 0.1, which includes either not changing or hypomethylated and not only hypomethylated. To avoid this confusion we suggest replacing the plot with the one below. We specified a 0.1 mid-point to aid visualization. (Figure 1b).

Comment Two: Also, the reviewer has failed to understand Figure 1(c). In both the x-axis and the side legend, binding and non-binding have been depicted, but which one is the AML patients with and without mutation isn't clear from the figure.

Response Two: Each violin in Figure 1(c) is a representation of the differential methylation score between CpGs of AML patients with and without CEBPa mutation. The blue violin represents all CpGs that are located in ecCEBPA binding sites, while the orange violin represents CpGs that do not bind with ecCEBPA. To provide clarity, we corrected the labels on the X and Y axes and added explicit explanation in the legend of the figure (Figure 1c). Difference in methylation = Mean methylation of CpGs in AML patients with CEBPA mutation - Mean methylation of CpGs in AML patients without CEBPA mutation.

Comment 3: There are few other key points that were not clear;
(a) what is the level of ecCEBPα expression in AML patients with and without mutation.
(b) Additionally, if the expression of the lncRNA remains the same in both cases, how does one explain the increase in binding of the lncRNA and subsequent higher NHM sites, in the case of AML patients with CEBPα mutation?
(c) Moreover, a previous report (Di Ruscio et.al1) on ecCEBPα had also analyzed genome-wide methylation, in which they report that methylation levels remain unchanged even when ecCEBPα was overexpressed. This is in contrast with the major theme of the paper i.e., methylation of genes in trans is affected by the ecCEBPα. The authors should comment on this in the discussion part.

Response:
(a&b): It is relatively difficult to estimate the expression level of ecCEBPA since this gene is not in the annotation used by TCGA. The raw data is not freely available in TCGA. ecCEBPA gene also overlaps with the CEBPA gene making the estimation of expression of each of the genes even more complicated. Since the mutation in AML patients happens in the body of CEBPA gene, we do not expect a significant change in the expression of either CEBPA or ecCEBPA.

We explained the relationship between ecCEBPA binding and methylation in the first result section (Figures 1c,d&e). We suggested that the mutation(s) in CEBPA does not affect the expression of the lncRNA, but rather the ability to bind its targets.

We further emphasize this point in the paper with this line (discussion):
"Di Ruscio et al. confirmed that most mutations in CEBPα do not influence the expression of ecCEBPα but rather, its ability of the RNA to fold properly. We suggest that the inability to fold properly even in overexpressed cases may affect its structural ability to bind its targets."

(c): We are very grateful to the reviewer for this valuable comment. In response to this, we retrieved the DNA methylation data (RRBS) for wild-type HL-60 cells (ENCODE Dataset) and overexpressed HL-60 cells (Di Ruscio *et al*). We then calculated the methylation difference between the two cell states and compared it between the ecCEBPA binding sites versus the non-binding sites. Non ecCEBPA binding sites were more methylated in comparison to ecCEBPA binding sites (Fisher Test: p-val = 1.24E-09). Bearing in mind that ecCEBPA targets are located genome-wide, it is tempting to suggest that "enforced overexpression" (which was achieved with the R1 variant of ecCEBPA; comprise of downstream ecCEBPA sequences) of ecCEBPA strongly protects local CpGs from methylation while the rest of the genome gain some methylation.

We added the following statement to the results in the main text:
"Furthermore, the overexpression of ecCEBPA in HL-60 cells (Wang et al) lead to ecCEBPA binding sites stay unmethylated while non ecCEBPA binding sites gain methylation (Fisher Test: p-val = 1.24E-09) , suggesting a protective effect on ecCEBPA's binding to its target locations (Fig 1d)."

Competing Interests: No competing interests were disclosed.

Comments on this article

Version 1

Reader Comment 14 Apr 2021

Olumide Inyang, moscow institute of physics and technology (MIPT), Russian Federation

This report gave a clear understanding of ecCEBPα as a genome-wide epigenetic modulator via triple-helix stacks for RNA(engineered) in regulating methylation of specific genes...great work from the authors.

Competing Interests: No competing interests were disclosed.

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