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Analysis of *I36a* induction by C/EBP β via a half-CRE•C/EBP element in murine macrophages in dependence of its CpG methylation level

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Interleukin-36 α is a novel member of the IL-1 cytokine family that is highly expressed in epithelial tissues and several myeloid-derived cell types after induction. The transcription factor (TF) C/EBP β binds specifically to an essential half-CRE•C/EBP motif in the *I36a* promoter to induce *I36a* expression upon LPS stimulation. C/EBPs regulate gene expression by binding to recognition sequences that can contain 5'-cytosine-phosphate-guanine-3' dinucleotides (CpG), whose methylation can influence TF binding and gene expression. Herein we show that the half-CRE•C/EBP element in the *I36a* promoter is differentially methylated in the murine RAW264.7 macrophage cell line and in primary murine macrophages. We demonstrate that C/EBP β binding to the half-CRE•C/EBP element in the *I36a* promoter following LPS stimulation is insensitive to CpG methylation and that methylation of the CpG in the half-CRE•C/EBP element does not alter LPS-induced *I36a* promoter activity which correlated with similar *I36a* mRNA copy numbers and pro-IL-36 α protein amount in both cell types. Taken together, our data indicate that C/EBP β binding to the half-CRE•C/EBP element and subsequent gene activation occurs independently of the CpG methylation status of the half-CRE•C/EBP motif and underlines the potential of C/EBPs to recognize methylated as well as unmethylated motifs.

Genes & Immunity (2021) 22:313–321; <https://doi.org/10.1038/s41435-021-00153-5>

INTRODUCTION

The interleukin (IL)-36 cytokines constitute a subfamily of the IL-1 cytokine family and consist of three agonistic cytokines (IL-36 α , IL-36 β IL-36 γ), and the IL-36 receptor antagonist (IL-36RA) that play important roles in host immunity [1]. IL-36 cytokines are expressed by a variety of cells in different tissues, such as macrophages, dendritic cells, keratinocytes, and lung epithelial cells [2–7]. They bind to the IL-36 receptor (also referred to as IL-1R-like 2) that is widely expressed on many different cells, including murine bone marrow-derived dendritic cells, CD4⁺ T cells, mononuclear phagocytes, and various epithelial cells of skin, lung, and digestive tract tissues [4, 8, 9]. Similar to the receptor for IL-1, the IL-36 receptor recruits the transmembrane IL-1 receptor accessory protein upon ligand binding that subsequently activates intracellular signaling via mitogen-activated protein kinases and nuclear factor- κ B (NF- κ B) activated transcription leading to inflammatory responses [10–12]. At the transcriptional level IL-36 α and IL-36 γ themselves are regulated by NF- κ B, C/EBP β , and by the transcription factor T-box expressed in T cells, respectively [2, 3].

DNA methylation at cytosine residues by DNA methyltransferases is an epigenetic modification involved in mammalian development, lineage identity as well as transcriptional regulation [13]. Traditionally, DNA methylation of gene promoters was considered to silence transcription either directly by inhibiting binding of certain transcription factors (TF) to their binding sites [14–17] or indirectly by binding of TFs containing a methyl-CpG

(mCpG)-binding domain (MBD), that in turn recruits histone deacetylases which subsequently promote local chromatin condensation [18]. In recent years, a growing number of TFs lacking MBDs have been shown to bind to their transcription factor binding sites (TFBS) even when they are methylated, arguing against the paradigm that CpG methylation always represses transcription. Examples include transcriptional activators that contain a C2H4 zinc-finger domain like Kaiso and Krueppel-like factor 4 [19, 20], proteins with a helix-turn-helix DNA-binding domain like Zfx1/2 and Pax6 [21, 22], helix-loop-helix domain-containing TFs like c-MYC and ARNT2 [23, 24], and TFs belonging to the family of bZIP proteins like C/EBPs [25, 26].

C/EBP transcription factors belong to the bZIP family of transcription factors and are involved in tissue-specific gene expression, proliferation, differentiation, and inflammation [27]. In particular, C/EBP β has been shown to regulate inflammatory genes like the cytokines IL-6, IL-12 p40, and IL-36 α [3, 28, 29], the chemokines IL-8 and macrophage inflammatory protein-1 α [28, 30] as well as the proinflammatory genes for inducible NO synthase (NOS2) and cyclooxygenase-2 [31, 32] in macrophages. DNA binding and subsequent gene expression by C/EBP β requires the formation of homodimers or heterodimers with other C/EBP family members or members of the CREB/ATF family via the bZIP domain [33]. The consensus sequence as well as composite sites recognized by C/EBPs can contain a central CpG dinucleotide [27]. Several studies showed that C/EBPs are able to bind these

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Received: 13 May 2021 Revised: 5 October 2021 Accepted: 6 October 2021
Published online: 25 October 2021

sequences in vitro and in vivo with similar or even with increased affinities if the central CpG dinucleotide is methylated [17, 25, 26, 34]. As a consequence, such methylation can generate C/EBP binding sites at CRE-like sequences leading to activation of a subset of differentiation-specific genes by C/EBPs whilst inhibiting activation of these genes by CREB members [25]. The structural basis for this insensitivity to the methylation status of the bound DNA has been recently unravelled and was shown to be mediated via a so-called methyl-Arg-G triad [35].

Recently, we reported that C/EBP β confers transcriptional activation of *I136a* via an essential half-CRE-C/EBP site that contains a central CpG dinucleotide [3]. For IL-36 cytokines, there is to our knowledge, no information on epigenetic regulation, in particular on CpG methylation and its possible impact on transcription factor binding, available. Therefore, we herein analyzed the methylation level of this element in two different murine macrophage types and its impact on C/EBP β binding and transcriptional activation of *I136a*.

RESULTS

Differential DNA methylation of a half-CRE-C/EBP element in the *I136a* promoter in murine macrophages

We have previously shown that LPS-induced *I136a* mRNA expression in murine macrophages is essentially mediated by binding of C/EBP β to a half-CRE-C/EBP element within the *I136a* promoter even though classical C/EBP recognition sites are present in the promoter sequence [3]. In this study, we aimed to dissect the specificity of C/EBP β binding to the half-CRE-C/EBP element in relation to the level of methylation.

Detailed in silico analysis of the *I136a* promoter region comprising 1120 bp upstream of the transcriptional start site revealed a GC content of 43.21%, the absence of any CpG islands but the presence of nine CpG dinucleotides (Fig. 1A). Isolation of genomic DNA and subsequent bisulfite sequencing of the region containing the CpG in the half-CRE-C/EBP element showed low methylation of $9.70 \pm 10.01\%$ in RAW264.7 cells (Fig. 1B and D). In contrast, the methylation level of this site in BMDMs was $66.95 \pm 8.63\%$ (Fig. 1C and D), revealing a significant mean difference in methylation between both cell types (mean difference = 57.2%; 95% CI: 44.5, 67.5).

In vitro binding of C/EBP β to the methylated half-CRE-C/EBP element in the *I136a* promoter

Since it was demonstrated that CpG methylation of half-CRE elements enhances binding of C/EBP members and can activate tissue-specific gene expression, the significantly differential methylation status of the CpG in the half-CRE-C/EBP might lead to differential binding activities of C/EBP β in RAW264.7 macrophages and primary BMDMs. Therefore, we next analyzed the binding of C/EBP and CREB members to the unmethylated and methylated half-CRE-C/EBP element in nuclear extracts by EMSA using a radiolabeled oligonucleotide spanning the region from -314 to -290 relative to the TSS of the murine *I136a* gene.

We first studied the in vitro complex formation using nuclear extracts of RAW264.7 cells stimulated with LPS for 4 h. In EMSA with the unmethylated probe, DNA-protein interactions appeared as two major complexes in unstimulated and LPS-stimulated cells. Whereas constitutive DNA binding activity of the slower migrating complexes was nearly unchanged (arrowheads, Fig. 2A), the binding activity of the faster-migrating complexes increased in extracts from LPS-treated cells. Supershift experiments with antibodies against C/EBP β , C/EBP δ , CREB-1, and ATF-4 indicated that only C/EBP β and to a certain extent C/EBP δ are present in the inducible complex. Using the methylated probe, a similar inducible protein-DNA complex formation was observed. In comparison to the unmethylated probe, constitutive DNA binding activity was not detectable. The methylated probe also bound

C/EBP β and C/EBP δ but not CREB-1 nor ATF4 (Fig. 2A). Similar results were obtained with nuclear extracts from unstimulated and LPS-stimulated BMDMs. Irrespective of the use of unmethylated or methylated probe, LPS treatment induced a protein-DNA complex that supershifted with antibodies against C/EBP β and to a minor extent by anti-C/EBP δ antibodies. Again, the formed complexes did not contain a detectable amount of CREB-1 nor ATF4 (Fig. 2B). However, in the extracts of both cell types, the complexes formed with the methylated probe seemed to form more specific complexes with less background as compared to the unmethylated probe which is best emphasized by the reduced constitutive protein binding in the extracts from RAW264.7 macrophages using the methylated probe.

This suggests that C/EBP β is able to bind unmethylated as well as methylated half-CRE-C/EBP sequences and that DNA methylation of the half-CRE-C/EBP element enhances specificity of protein binding.

DNA binding of C/EBP β is not enhanced by methylation

To test whether CpG methylation enhances binding of C/EBPs to DNA probes in vitro and to further analyze if there is any preferential binding to the unmethylated or methylated half-CRE-C/EBP oligonucleotides the protein-DNA complex formation was assessed with different approaches. First, we performed competition EMSA. For this, a radiolabeled oligonucleotide containing the C/EBP consensus motif (5'-TTGCGCAA-3') was incubated with nuclear extracts of unstimulated and LPS-stimulated RAW264.7 cells in the presence of increasing amounts of unlabeled unmethylated or unlabeled methylated half-CRE-C/EBP-*I136a* oligonucleotides used before. As shown in Fig. 3A both oligonucleotides concentration-dependently decreased protein-DNA complex formation with comparable inhibitory effects.

Next, we determined dissociation constants (K_D) of the murine C/EBP β -bZIP domain (Fig. 3B) to double-stranded half-CRE-C/EBP-*I136a* oligonucleotides with unmethylated or methylated CpG using fluorescence polarization assays (Fig. 3C). The C/EBP β DNA binding domain bound the unmodified oligonucleotides with a K_D of $0.08 \pm 0.03 \mu\text{M}$ (Fig. 3D). Under the same conditions, the methylated oligonucleotides were bound with a K_D of $0.10 \pm 0.03 \mu\text{M}$ (Fig. 3D), revealing a nonsignificant mean difference between unmethylated and methylated oligonucleotides (mean difference = $0.0157 \mu\text{M}$; 95% CI: -0.0213, 0.0438).

Together, these data further indicate, that C/EBP β can recognize the half-CRE-C/EBP site located in the *I136a* promoter irrespectively of the methylation status of central CpG in vitro.

Methylation of the CpG in the half-CRE-C/EBP element does not inhibit *I136a* promoter activity

Next, we assayed the relevance of CpG methylation of the half-CRE-C/EBP element in a system more resembling the in vivo situation in the nucleus. To evaluate whether the binding of C/EBP β to the methylated half-CRE-C/EBP element is biologically relevant we performed transient transfections using RAW264.7 cells. The *I136a* promoter region, comprising the essential regulatory elements for gene induction between -357 and -45 relative to the TSS, was cloned into a reporter plasmid where all CpG dinucleotides have been deleted from the plasmid backbone (pCpGL) [36], (Fig. 4A). The CpG in the half-CRE-C/EBP site in the pCpGL-*I136a*^{-357/-45} plasmid was enzymatically methylated in vitro using SssI (CpG) methylase and S-adenosylmethionine. We confirmed methylation efficacy by restriction analysis. *Hpy*CH4IV linearized the unmethylated plasmid completely (asterisk, Fig. 4B) whereas the methylated plasmid was not linearized, indicating complete methylation (Fig. 4B).

Next, we transfected the methylated and unmethylated plasmids into RAW264.7 cells and determined luciferase activity in untreated cells or cells stimulated with 100 ng/ml LPS after 8 h. As shown in Fig. 4C, stimulation with LPS led to an increased

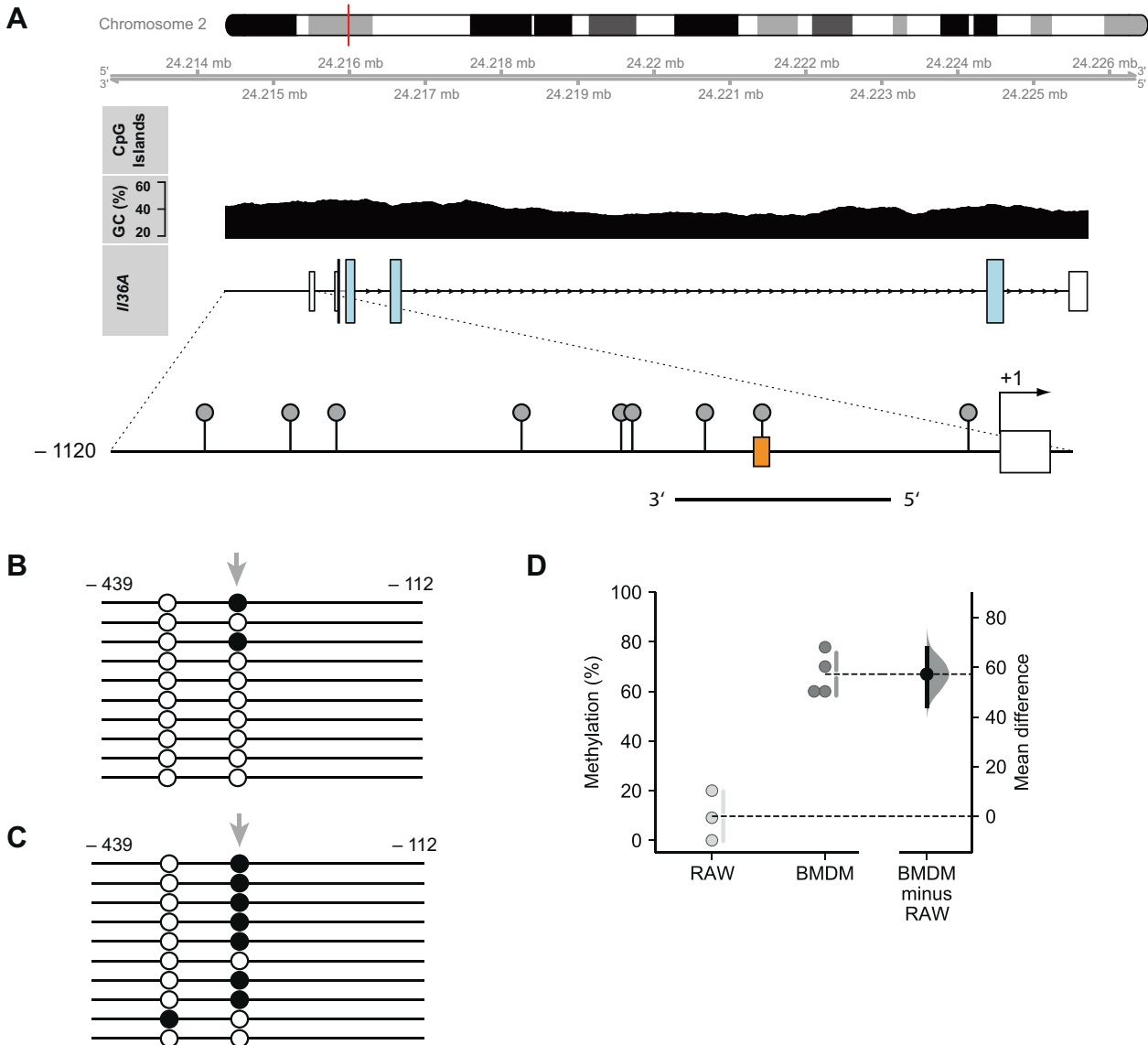


Fig. 1 DNA methylation status of the half-CRE-C/EBP element in the *I136a* promoter. **A** Schematic representation of the *I136a* gene structure (Genebank entry KM205447.1), CpG islands, GC content (upper part), and the promoter region indicating CpG sites (lollipop, lower part). The position of *I136a* on mouse chromosome 2 (red line), the half-CRE-C/EBP element (orange box) and the amplified region for bisulfite sequencing analysis on the (–) strand are indicated. **B, C** The DNA methylation status for the region indicated in (A) was determined from RAW264.7 (B) or BMDMs (C) by bisulfite sequencing analysis. Each line represents sequencing results of an individual clone (open circle, unmethylated CpG; filled circle, methylated CpG). The CpG in half-CRE-C/EBP element is indicated by grey arrows. **D** Quantification of DNA methylation in RAW264.7 (light gray dots) and BMDMs (dark gray dots) from $n = 3$ (RAW264.7) or $n = 4$ (BMDM) experiments. Individual data points and summary measurements (mean \pm SD) are plotted on the left-hand side of the panel; effect size (mean differences, black dot) with bootstrapped 95% confidence intervals and resampling distribution are shown on the right-hand side of the panel.

luciferase induction (fold change LPS vs. ctrl) in cells transfected with unmethylated and methylated reporter plasmid, respectively. We did not observe a significant difference in luciferase induction between cells transfected with the unmethylated or methylated plasmid (mean difference = 6.94 RLU; 95% CI: –7.47, 19.7).

These data show that CpG methylation of the half-CRE-C/EBP site does not significantly influence *I136a* promoter activity in LPS-stimulated RAW264.7 cells.

Methylation of the CpG in the half-CRE-C/EBP element does not influence IL-36 α expression

Next, we analyzed whether the marginal expression differences in cells transfected with the methylated reporter plasmid were reflected by *I136a* promoter activation in its ‘in vivo’ context in the

nucleus. For this we directly compared expression of *I136a* induced by LPS in RAW264.7 cells and BMDMs, which have significantly different methylation level of the half-CRE-C/EBP site (Fig. 1). Cells were stimulated with LPS for 8 h and isolated total RNA was subjected to absolute quantification by qRT-PCR. As shown in Fig. 5A, *I136a* induction was detectable after stimulation of RAW264.7 cells as well as BMDMs with LPS. We did not observe a significant difference in the fold change induction of *I136a* mRNA between LPS-stimulated RAW264.7 cells and BMDMs (mean difference = –101.4; 95% CI: –2276.3, 1748.2). Furthermore, Western blot analysis of pro-IL-36 α in cell lysates of RAW264.7 cells and BMDMs also revealed similar levels of the cytokine after stimulation with LPS (Fig. 5B) supporting the hypothesis that DNA methylation of the half-CRE-C/EBP site does not influence *I136a* expression.

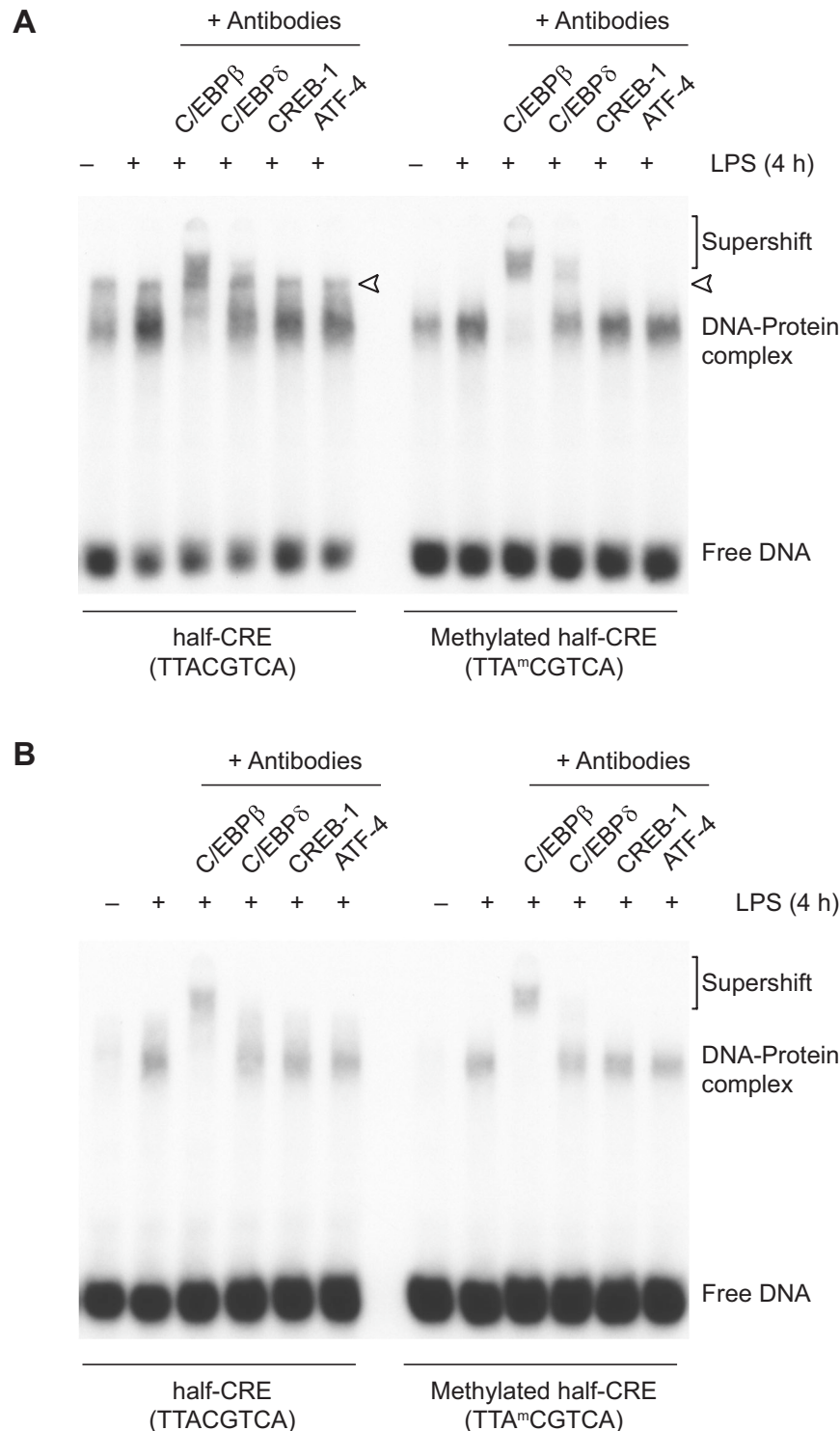


Fig. 2 Transcription factor binding to the methylated half-CRE-C/EBP site of the *I/36a* promoter. **A** EMSAs were performed with nuclear extracts from untreated and LPS-treated (4 h, 5 μ g/ml) RAW264.7 cells using radiolabeled oligonucleotides with unmethylated and methylated half-CRE-C/EBP element as probes. Supershift experiments were performed with specific antibodies against C/EBP β , C/EBP δ , CREB-1/ATF-1, and ATF4. The arrowhead indicates a constitutively formed complex. One representative experiment of two independent experiments is shown. **B** EMSAs using the same probes and antibodies as in (A) performed with nuclear extracts from untreated and LPS-treated (4 h, 2.5 μ g/ml) BMDMs. One representative experiment of two independent experiments is shown.

DISCUSSION

I/36a is expressed in a variety of cell types and in different tissues at different levels [11]. Yet information on epigenetic regulation, in particular on CpG methylation and its possible impact on

transcription factor binding are missing. We have previously shown that in murine macrophages LPS-induced *I/36a* mRNA expression requires binding of C/EBP β to a half-CRE-C/EBP element within the *I/36a* promoter [3]. Here we observed

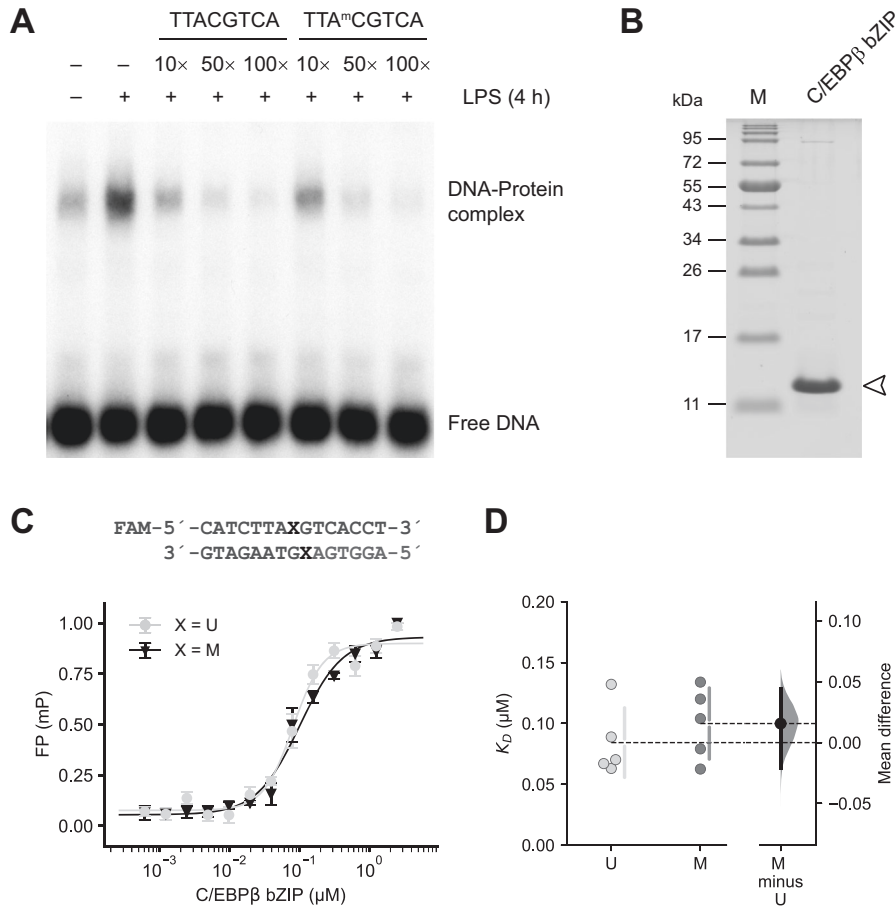


Fig. 3 The effects of CpG methylation on DNA binding by C/EBP β . **A** Competitive EMSAs were performed with a radiolabeled C/EBP consensus oligonucleotide probe and nuclear extracts from unstimulated and LPS-stimulated (4 h, 5 μ g/ml) RAW264.7 cells. A 10-, 50-, and 100-fold molar excess of the unmethylated or methylated half-CRE-C/EBP-*I36a* oligonucleotide was used in the competition reactions containing LPS-stimulated RAW264.7 nuclear extracts. One representative experiment of two independent experiments is shown. **B** 15% Bis-Tris PAGE gel showing the purified C/EBP β -bZIP domain (2.5 μ g, arrowhead) used in the fluorescence polarization (FP) assays. **C** DNA binding affinities measured by FP of the C/EBP β -bZIP domain with oligonucleotides of the *I36a* half-CRE-C/EBP element containing an unmethylated (U, light gray) or methylated (M, dark gray) central CpG. Data represent mean \pm SD from one representative experiment performed in triplicate. **D** Determination K_D values using FP assays shown in (C) based on $n = 5$ experiments. Individual data points and summary measurements (mean \pm SD) are plotted on the left-hand side of the panel; effect size (mean differences, black dot) with bootstrapped 95% confidence intervals and resampling distribution are shown on the right-hand side of the panel.

differential methylation of this element in RAW264.7 cells and primary murine macrophages, leading to the question of whether this difference in methylation points to epigenetic regulation of *I36a* by affecting TF binding and subsequent *I36a* gene expression.

Regulation of gene expression by methylation of CpG motifs is one of several universal epigenetic mechanisms [37]. The majority of data linking CpG methylation of the promoter regions and gene expression is derived from studies of genes with CpG islands (a region longer than 500 bp with an observed CpG/expected CpG ratio of 0.65) in the promoter region [38]. CpG islands are not present in the *I36a* promoter. However, the promoter harbors a number of CpGs proximal to the transcriptional start site, including one located within the essential half-CRE-C/EBP element. Such proximal, non-island CpGs are nowadays considered important for regulation of gene expression and examples of promoters affected by methylation of these non-island CpGs include e.g., *Il2*, *NOS2*, *MMP13*, *Il1B*, and *Il18BP* [39–43]. While in most cases, methylation of the CpG sites results in transcriptional inactivation, for other genes methylation increased binding of TFs and thus resulted in stronger gene expression. Examples include *Bglap-rs1* and *MMP9* [25] as well as *PAX2* [44]. This regulation at the epigenetic level may serve and control cell/tissue type-specific

physiological functions or lead to the reactivation of early developmental genes in malignancy.

Several lines of evidence presented herein suggest that methylation of the half-CRE-C/EBP element in the *I36a* promoter has no influence on *I36a* mRNA expression. Gel shift experiments and fluorescence polarization assays demonstrated that C/EBP β is able to bind the unmethylated as well as methylated form of the CRE-C/EBP element in vitro, which is in agreement with earlier studies demonstrating binding of C/EBP β to methylated consensus sequences [25]. Interestingly, we also observed the binding of C/EBP δ to both probes although overall binding was lower compared to C/EBP β . This is in contrast to studies using methylation binding arrays showing that methylation inhibits binding of C/EBP δ [26]. Furthermore, we did not detect the formation of C/EBP β /ATF4 heterodimers indicating that the heterodimers are not formed after LPS stimulation because ATF4 is presumable not induced in sufficient amounts. However, for lysates from both cell types we detected less unspecific binding when the methylated probe was used, indicating that methylation interferes with the binding of some unidentified TFs recognizing the unmethylated probe. Therefore, one could speculate that methylation modulates specificity of *I36a* mRNA expression under certain circumstances. Indeed, Yin et al. demonstrated reduced

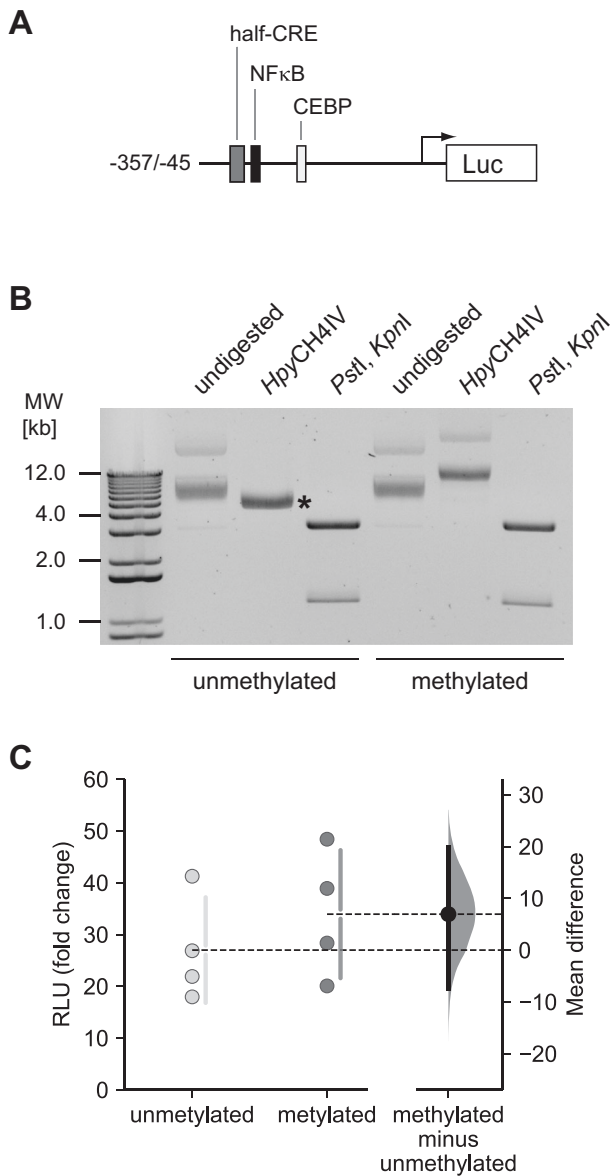


Fig. 4 Inhibition of the *I36a* promoter activity by methylation of the CpG sites in the half-CRE-C/EBP element. **A** Schematic representation of the pCpGL-*I36a*^{-357/-45}-luciferase reporter construct. Transcription factor binding sites are indicated. **B** In vitro methylation of pCpGL-*I36a*^{-357/-45} using *SssI* (CpG) methylase and *S*-adenosylmethionine. Methylation was confirmed by enzymatic digestion of the plasmid followed by gel electrophoresis. The unmethylated plasmid (lanes 1-3) is completely linearized by *HpyCH4IV* (asterisk) whereas *HpyCH4IV* does not linearize the methylated plasmid (lanes 4-6). Digestion with *PstI* and *KpnI* releases the *I36a*^{-357/-45} insert from the plasmid **C** RAW 264.7 cells were co-transfected with the methylated/non-methylated pCpGL-*I36a*^{-357/-45} construct along with the pRL-TK vector. 24 h after transfection cells were stimulated with LPS (100 ng/ml) for 8 h or left untreated. Firefly luciferase activity was normalized to that of *Renilla* luciferase and is expressed as fold change in luciferase induction (ratio LPS vs. ctrl) from $n = 4$ experiments. Individual data points and summary measurements (mean \pm SD) are plotted on the left-hand side of the panel; effect size (mean differences, black dot) with bootstrapped 95% confidence intervals and resampling distribution are shown on the right-hand side of the panel.

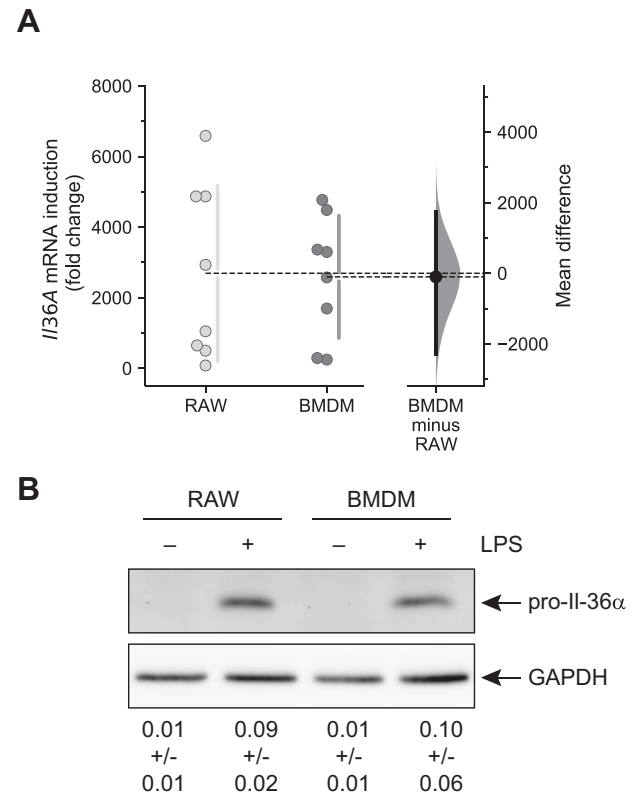


Fig. 5 Induction of *I36a* expression in LPS-stimulated macrophages. **A** RAW264.7 cells and BMDMs were stimulated with 1 μ g/ml LPS for 8 h or left untreated and *I36a* mRNA copy numbers were determined by qRT-PCR from $n = 8$ experiments and are expressed fold change in *I36a* induction (ratio LPS vs. ctrl). Individual data points and summary measurements (mean \pm SD) are plotted on the left-hand side of the panel; effect size (mean differences, black dot) with bootstrapped 95% confidence intervals and resampling distribution are shown on the right-hand side of the panel. **B** Western Blot analysis of pro-IL36 α expression in RAW264.7 cells and BMDMs stimulated with 1 μ g/ml LPS for 8 h or left untreated. One representative Western blot out of 3 independent experiments is shown. Ratio of pro-IL-36 α to GAPDH determined by densitometry is shown below (arbitrary units, $n = 3$ experiments, mean \pm SD).

binding for the majority of bZIP family members to their motifs upon methylation (Fig. 5C in [17]). This supports the hypothesis that methylation of the half-CRE-C/EBP element in BMDMs may increase the specificity for C/EBP β , one of the TFs in this family that tolerates methylation within the central CpG.

In contrast to the study by Rishi et al., we did not observe enhanced C/EBP β binding to the methylated motif in vitro [25]. Since *I36a* activation is C/EBP β -dependent our data suggest that tolerance of a transcriptional regulator for CpG methylation might help to overcome otherwise inhibitory epigenetic modifications. In line with this, our findings are consistent with previous reports showing that in vivo binding by C/EBP β tolerates CpG methylation [34]. Furthermore, it is also in accordance with recently published data that compared relative affinities for C/EBP β in vitro binding using methylated and unmethylated libraries [45]. Accordingly, we analyzed the specific sequence context of the *I36a* half-CRE-C/EBP element by comparing methylated and unmethylated relative affinities with those of a consensus C/EBP motif and a consensus CRE motif, respectively, based on published data for binding of C/

EBP β homodimers [45]. Such a comparison revealed a relatively low binding affinity (compared to a consensus C/EBP motif) for the half-CRE-C/EBP motif that, however, did not differ between methylated and unmethylated sequences (Supplementary Fig. 1A). In contrast, *in vitro* binding of C/EBP β to a consensus CRE motif was enhanced when methylated, confirming previous data [25]. This methylation insensitivity is also reflected in the energy logos that are derived from the oligomer enrichment tables (Supplementary Fig. 1B). In agreement, induction of luciferase expression upon LPS stimulation, the *Il36a* mRNA expression, and the amount of pro-*Il-36a* at the protein levels did not differ significantly in the two cell types despite the observed difference in methylation. However, since only 66% of the cells in the BMDM population had a methylated half-CRE-C/EBP element, we cannot exclude that incomplete methylation influenced the effect size of the qRT-PCR and Western Blot assays. Taken together, our data suggest that methylation of this motif does not influence endogenous promoter activity. Therefore, we speculate that asymmetry of the half-CRE-C/EBP element and/or the nucleotides flanking the central CpG in the different binding motifs might determine whether there is no effect or enhanced binding if the CpG is methylated.

Altogether, the data presented herein emphasize the potential of C/EBP β to recognize methylated as well as unmethylated binding sites. Structural analysis or *in silico* modelling need to be performed in the future to unequivocally elucidate the binding mechanism of C/EBP to the half-CRE-C/EBP element present in the *Il36a* promoter. Furthermore, our data indicate that *Il36a* is most likely not regulated epigenetically by CpG methylation of the half-CRE-C/EBP element in the proximal promoter region of the gene in murine macrophages.

MATERIALS AND METHODS

Reagents

Media used for macrophage cell culture were obtained from ThermoFisher Scientific (Darmstadt, Germany). Purified ultra-pure LPS from *Escherichia coli* 0111:B4 and chemically competent *E. coli* GT115 were purchased from Invivogen (Toulouse, France). If not stated otherwise all other reagents were from Sigma (Taufkirchen, Germany). Antibodies against ATF/CREB-1 (Cat# sc-270, RRID:AB_2290030), CREB-2/ATF-4 (Cat# sc-200, RRID:AB_2058752), C/EBP β (Cat# sc-150, RRID:AB_2260363), and C/EBP δ (Cat# sc-151, RRID:AB_2078200) were purchased from St. Cruz (Heidelberg, Germany). The antibody against GAPDH (D16H11, RRID:AB_11129865) was obtained from Cell Signaling (Frankfurt am Main, Germany) and goat anti-mouse *Il-36a* (AF2297, RRID:AB_355216) was from R&D Systems/Bio-Techne (Wiesbaden-Nordenstadt, Germany). Secondary goat anti-rabbit IgG, HRP-linked (#7074, RRID:AB_2099233) was obtained from Cell Signaling and donkey anti-goat IgG AP-linked (Cat# sc-2022, RRID:AB_631723) was from St. Cruz.

Plasmid construction and *in vitro* methylation of plasmid DNA

The plasmid pCpGL-TA-*Il36a*^{-357/-45} was generated by cloning a synthetic DNA fragment containing the TATA box of the human EF1 promoter (5'-GATCTCAGGGTGGGGGAGAACCATATATAAGTGCAGTAGTCTCTGTGAA-CATTCA-3') into pCpGL-basic (kindly provided by M. Rehli). Subsequently the *Il36a* promoter region from -357 to -45 relative to the transcription start site was amplified using the oligonucleotides 5'-GCCTGCAGTTGC ACTTCTGTAGGTTTC-3' and 5'-GCAGATCTAGAGGAGGTTATGCCTCAG-3' and the plasmid pGL3-*Il36a*-1120-Luc [3] as template. All pCpGL plasmids were maintained in *E. coli* GT115. Plasmid pJet-*Il36a* was generated by amplifying a fragment of *Il36a* from cDNA using the qRT-PCR primers *Il36a*_{for} (5'-AAGGAACCTGTAAAGCCTCTCT-3') and *Il36a*_{rev} (5'-CAGTTCTGGGTCAGAATGAGTG-3') and subsequent cloning into pJET1.2/blunt vector as described by the manufacturer (ThermoFisher Scientific). The DNA fragment encoding mouse C/EBP β residues 221–296 (bZIP domain) was cloned into pET-45b(+) vector (Novagen/Merck, Darmstadt, Germany) *via* 'in vivo assembly' [46] to generate a His-tagged version of the bZIP domain. The bZIP domain was amplified using the primers pET-45b_f (5'-CTGGTAAAGAAACCGCTGCTG-3') and pET-45b_r (5'-GTGATGGTGGTG GTGATGTG-3') and the plasmid pCpDNA-C/EBP β -wt [3] as template. The

vector backbone was amplified with the primers CEBPb_bZIP_f (5'-CACCACCACCATCACCTGTCGATGAATACAAGATG-3') and CEBPb_bZIP_r (5'-CGGTTTCTTTACCAGTCAGCAGTGGCCCGCCGAGG-3'). Both fragments were combined, digested with *DpnI* and transformed into *E. coli* BL21(DE3).

All constructs were sequenced before use and plasmids used for transfection experiments were purified with the EndoFree[®] plasmid maxi kit (Qiagen, Hilden, Germany).

Plasmids were methylated using *SssI* (New England Biolabs, Frankfurt am Main, Germany) as described previously [36]. Following methylation DNA was purified and quantified spectrophotometrically. The completeness of methylation was controlled by digesting both methylated and unmethylated DNA with the methylation sensitive restriction enzyme *HpyCH4IV* (New England Biolabs).

Macrophage cell culture

The mouse macrophage cell line RAW264.7 (ATCC, TIB-71) was maintained in DMEM supplemented with 10% FCS, 1% glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin (referred to as complete medium) at 37 °C and 8% CO₂. Isolation and culture of primary BMDMs were done essentially as described before [3]. Cells were harvested on day eight, seeded in appropriate cell culture dishes, and used for experiments on day nine. All animal experiments were approved by the appropriate ethical board (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg, Germany).

RNA isolation and absolute qRT-PCR

For absolute quantification of copy numbers by real-time PCR (qRT-PCR), total RNA was isolated using RNeasy[®] Mini Kit (Qiagen) and reverse described with M-MLV reverse transcriptase (Promega, Mannheim, Germany) and oligo-dT primer as described by the manufacturer. A ten-fold serial dilution series of the *HindIII* digested and purified pJet-*Il36a* plasmid, ranging from 6 \times 10⁴ to 6 \times 10⁰ copies/ μ l, was used to construct the standard curve. The concentration of the pJet-*Il36a* plasmid was measured photospectrometrically and the corresponding plasmid copy number was calculated as described previously [47].

qRT-PCR was performed in a Stratagene[™] Mx3005P qPCR instrument (Agilent Technologies, Waldbronn, Germany) using 10 μ l QuantiTect SYBR Green PCR mix (Qiagen), 5 μ l template DNA (either cDNA or plasmid DNA diluted as described above) and the primers *Il36a*_{for} and *Il36a*_{rev} (for details see section plasmid construction) in a total volume of 20 μ l. The PCR conditions have been described previously [3]. Diluted standards were measured in triplicate and a standard curve was generated by plotting the threshold cycles (C_t) against the natural log of the number of molecules. Based on the standard curve the number of *Il36a* cDNA molecules per 500 μ g of total oligo-dT primed cDNA was calculated.

Transient transfection and luciferase assay

Transient transfections were conducted using X-tremeGENE (Roche, Mannheim, Germany), according to the manufacturer's protocol as described [3].

Electrophoretic mobility shift assay

For EMSA equimolar amounts of complementary oligonucleotides were annealed and end labeled with [³²P]dCTP using Klenow Fragment (3' \rightarrow 5' exo-) (New England Biolabs). The following oligonucleotides were used: half-CRE-C/EBP_Il36a (5'-TCAGGTACTTCATCTTACGTCACCTAGT-3', 5'-TCAG ACTAGGTGACGTAAGATGAAGTAC-3'), methylated half-CRE-C/EBP_Il36a (5'-TCAGGTACTTCATCTTAmCGTCACCTAGT-3', 5'-TCAGACTAGGTGAmCGTAAG ATGAAGTAC-3'), consensus C/EBP (5'-TCAGCAGTCAGATTGCCGAAT ATCGGTG-3', 5'-TCAGGACCGATATTGCCGAATCTGACTG-3'). Nuclear extracts were prepared from RAW264.7 cells and primary mouse macrophages as described by Schreiber et al. [48] and band shift assays were performed exactly as described previously [3].

DNA isolation and bisulphite sequencing

Genomic DNA was isolated using innuPREP Blood DNA Mini kit (Analytik Jena, Jena, Germany) according to the manufacturer's instructions. The methylation profile of the proximal *Il36a* promoter was performed by bisulphite sequencing as described previously with minor modifications [49]. In brief, 4 μ g of genomic DNA was digested with *Bg/III*, purified by phenol-chloroform extraction and 500–1000 ng digested DNA was bisulphite-converted as described. Primers were designed using Methy

Primer Express® Software v1.0 (Applied Biosystems/ThermoFisher Scientific) to amplify specific regions of the genome following bisulphite conversion. The *Il36a* promoter region from -439 to -112 relative to the transcriptional start site on the (-)-strand was amplified using the primers *Il36a_BSP_for* (5'-GGAGGGTTTGTAAAGTATTGT-3') and *Il36a_BSP_rev* (5'-AATATCCAATAAATCAACCTAAAA-3'). For bisulphite sequencing, PCR products were gel-purified and cloned into the pCR2.1-Topo Vector System (ThermoFisher Scientific) and sequenced using the M13rev primer (5'-CAGGAACAGCTATGAC-3'). Sequencing results were analyzed using QUMA software [50]. Samples with conversion rate < 90% and sequences identity < 70% were excluded from the analysis. The minimum number of clones for each sequenced condition was ≥10.

SDS-PAGE and Western blotting

Western blotting was performed essentially as described [3] and developed using SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific) or AP-Juice Low Background (PJK GmbH, Kleinblittersdorf, Germany) and a ChemoCam Imager 3.2 (Intas, Göttingen, Germany). Densitometry was performed using LabImage 1D (Kapelan Bio-Imaging, Leipzig, Germany).

Protein expression and purification

The DNA binding domain of mouse C/EBPβ was expressed as N-terminal 6×His fusion protein *via* pET45b in *E. coli* BL21 (DE3). Bacteria were cultured in LB medium at 37 °C to an optical density of $A_{600} = 0.5$. Protein production was induced by adding 1 mM isopropyl β-D-thiogalactopyranoside and the cultures were incubated at 30 °C for 5 h. Harvested bacteria were lysed using a French press (three runs at 20,000 psi) in 1× LEW buffer (Macherey-Nagel, Düren, Germany) containing 5% glycerol, 0.5 mM tris (2-carboxyethyl) phosphine (TCEP) and P8849 Protease Inhibitor Cocktail for His-tagged proteins (Sigma). The lysate was cleared at 25,000 ×g for 30 min at 4 °C and the supernatant was loaded on Protino® Ni-TED 2000 packed columns and purified as described by the manufacturer (Macherey-Nagel, Düren, Germany). The eluted protein was dialyzed against storage buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5% (v/v) glycerol, 0.5 mM TCEP) and concentrated to approx. 5 ml using an Amicon® Ultra Centrifugal Filter Ultracel®-3K (UFC900324, Merck/Millipore, Darmstadt, Germany) before loading onto a HiTrap™-Heparin HP column (Cytiva, Marlborough, MA, United States). The Heparin column was then eluted using a step gradient of NaCl (250 mM to 2 M in storage buffer). The eluted fractions containing the purified protein were pooled and dialyzed against the storage buffer and concentrated as described above. The final concentration of the purified C/EBPβ-bZIP protein was estimated by Bradford protein assay (due to low number of aromatic residues).

Fluorescence-based DNA binding assay

Fluorescence polarisation assays were performed using a GENios Pro microplate reader (Tecan, Männedorf, Switzerland) using the following oligonucleotides: *FP_CREBmeth_f* (5'-FAM-CATCTTAmCGTCACCT-3'), *FP_CREBmeth_r* (5'-AGGTGAmCGTAAGATG-3'), *FP_CREB_f* (5'-FAM-CATCT-TACGTCACCT-3'), *FP_CREB_r* (5'-AGGTGACGTAAGATG-3'). The annealed 6-carboxy-fluorescein (FAM)-labeled double-stranded oligonucleotides (5 nM) were incubated with an increasing amount of the protein for 30 min in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5% (v/v) Glycerol, 0.5 mM TCEP, 0.1 mg/ml BSA. Data were normalized and curves were fit individually using the neutcurve Python package (<https://jbloomlab.github.io/neutcurve/>). Averaged K_D values and their standard deviations were reported.

Data processing for visualization of in vitro C/EBPβ binding

We downloaded EpiSELEX-seq data for C/EBPβ (table containing the relative affinity of individual kmers from unmethylated and methylated libraries; GSE98652) [45] and visualized relative affinities of the kmers for the unmethylated library (Lib-U) and methylated library (Lib-M) with emphasis on half-CRE-C/EBP, consensus C/EBP and consensus CRE sequences. Energy logos were prepared by selecting all possible variations of the half-CRE-C/EBP sequence and subsequent visualization using the LogoGenerator of the REDUCE Suite v2.2 (<http://reducesuite.bussemakerlab.org/index.php>).

Statistical analysis

Values are expressed as means ± SD. The exact sample size is given in the figure legends. Analysis using estimation statistics was done with Python

3.8 (Python Software Foundation, <https://www.python.org/>) and the DABEST package v0.3.1 [51]. The generated Gardner-Altman estimation plots display the magnitude and robustness of the effect size and its bootstrapped 95% confidence interval (95% CI).

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ACKNOWLEDGEMENTS

We thank Michael Rehli (University Hospital Regensburg, Germany) for generously providing the pCpGL plasmid and Nanthapon Ruangkiattikul for assistance with the isolation of bone marrow cells

AUTHOR CONTRIBUTIONS

Conceptualization, AN and RG; investigation, AN and NJ; formal analysis, AN and RG; writing—original draft preparation, AN and RG; writing—review and editing, all authors; funding acquisition, RG.

FUNDING

This research was funded by the German Ministry for Science and Education (BMBF; ZooMAP, 01KI0750 and ZooMAPII 01KI1003A, 01KI1003B). RG was supported by grants from the German Research Foundation (DFG, Go983/1, Ge522/6-1). Open Access funding enabled and organized by Projekt DEAL.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41435-021-00153-5>.

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