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Regulation of *NFKBIZ* gene promoter activity by STAT3, C/EBP β , and STAT1

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Abbreviations¹

¹ChIP, chromatin-immunoprecipitation; Gluc, *Gaussia* luciferase; GOF, gain-of-function; I κ B, inhibitor of nuclear factor κ B; RT-qPCR, reverse transcription-quantitative PCR; siRNA, small interfering RNA; TYK2, tyrosine kinase-2

Abstract

Interleukin-17A (IL-17A) is a cytokine that affects the functions of non-immune cells, including keratinocytes, and thereby amplifies immune responses. An I κ B family protein I κ B- ζ , encoded by the *NFKBIZ* gene, mediates IL-17A-induced inflammatory cellular responses. Previously we reported that a transcription factor STAT3 mediates the transcriptional induction of *NFKBIZ* through its binding to the specific binding site existing in the *NFKBIZ* promoter. However, it remains unclear how other transcription factors regulate *NFKBIZ* transcription. Here, we investigated the *NFKBIZ* promoter regulation by transcription factors C/EBP β and STAT1 and revealed opposing roles of C/EBP β and STAT1 in *NFKBIZ* transcription. We found that siRNA-mediated knockdown of C/EBP β attenuates IL-17A-induced upregulation of *NFKBIZ* in the HaCaT cell line. A putative C/EBP-binding site is located adjacent to the STAT-binding site in the *NFKBIZ* promoter, the deletion of which abolished C/EBP β -driven promoter activation in transient *NFKBIZ* promoter-luciferase assay. Deleting the STAT-binding site also led to a reduction in C/EBP β -driven promoter activation, suggesting a cooperative action between C/EBP- and STAT-binding sites. Furthermore, Co-overexpression of STAT1 suppressed both C/EBP β - and STAT3-driven *NFKBIZ* promoter activation independently of its tyrosine 701 phosphorylation. siRNA-mediated STAT1 knockdown augmented I κ B- ζ induction in IL-17A-treated HaCaT cells, with enhanced expression of an I κ B- ζ target gene *DEFB4A*. Together, these results indicate that both C/EBP β and STAT3 are transcription factors that coordinately induce *NFKBIZ* promoter activation, indicating that STAT1 has an inhibitory role. Thus, these could be a fine-tuning mechanism of IL-17A-I κ B- ζ -mediated cellular responses.

Keywords: inflammation, cytokine, STAT, C/EBP β , I κ B- ζ , transcriptional regulation

Introduction

The I κ B- ζ protein (also known as INAP or MAIL; encoded by the gene *NFKBIZ* in humans or *Nfkbiz* in mice) is an atypical member of the inhibitor of nuclear factor κ B (I κ B) family proteins [1, 2]. I κ B- ζ protein expression is highly upregulated in the epidermal keratinocytes of psoriatic lesions of the patients' skin. It is essential for the transcriptional induction of a panel of psoriasis-related proteins involved in inflammatory signaling, neutrophil chemotaxis, and leukocyte activation [3, 4]. Furthermore, systemic and keratinocyte-restricted *Nfkbiz* gene ablation in mice leads to suppression of psoriasis and associated systemic inflammation [4]. Also, siRNA-mediated local silencing of I κ B- ζ in mouse skin inhibited psoriasis-like skin inflammation [3, 5]. Thus, I κ B- ζ has received interest as a potential therapeutic target for psoriasis.

I κ B- ζ (*NFKBIZ*) expression in cells is induced by inflammatory cytokines such as IL-17A, IL-17F, IL-1 β , or IL-36 family [6-8]. In addition, transcription factors NF- κ B [8, 9] and STAT3 [8, 10, 11] reportedly mediate I κ B- ζ induction. *NFKBIZ* transcript variant 1 mRNA (NM_031419), which encodes a reported I κ B- ζ L isoform of this protein, has been suggested to be the major transcript in keratinocytes and is shown to be transcribed by NF- κ B and STAT3 [8]. Also, the stability of transcribed *NFKBIZ* mRNA and subsequently translated I κ B- ζ protein is regulated post-transcriptionally [11-13] and post-translationally [14], respectively.

Tyrosine kinase-2 (TYK2; encoded by a gene *TYK2* in humans or *Tyk2* in mice) is a JAK family kinase member that leads downstream STAT3 activation and acts in response to various cytokines [15]. *Tyk2* gene disruption in mice attenuates imiquimod-, IL-23-, and IL-22-induced skin inflammation with epidermal hyperplasia by suppressing immune cell activation, including Th1 and Th17 cell responses [16, 17]. Besides, we have shown that TYK2 is involved in *NFKBIZ* transcription via the TYK2-STAT3 pathway in keratinocytes [11]. We identified a functional STAT3-binding site at the upstream (position -133 to -125)

of the transcription start site of *NFKBIZ* transcript variant 1 [11]. However, it remains unclear how additional transcription factors modulate the *NFKBIZ* transcription.

IL-17A reportedly induces DNA binding of the C/EBP β transcription factor in various cell lines [18, 19]. A putative C/EBP-binding element exists in the *NFKBIZ* promoter, and the site is located near the above-mentioned STAT3-binding site [11]. As the existence of a set of STAT3 and C/EBP-binding sites nearby of one another on the promoters can support the cooperative action of STAT3- and C/EBP-mediated transcription [20, 21], we speculated that the C/EBP-binding site on the *NFKBIZ* promoter might have a role in *NFKBIZ* (I κ B- ζ) induction and investigated in the present study. Also, we investigated the role of STAT1, a member of the STAT family of transcription factors, in the regulation of *NFKBIZ* transcription, as STAT1 has been known to suppress the transcriptional activity of STAT3 in various cell types [22]. Herein, we extend insight into the *NFKBIZ* promoter regulation mechanism by showing C/EBP β as a positive regulator and STAT1 as a negative regulator.

Materials and Methods

Plasmids

Expression vectors for C/EBP β , FLAG-tagged STAT1, and FLAG-tagged STAT3C, a constitutively active form of STAT3, have been described previously [23, 24]. Human *NFKBIZ* promoter-luciferase reporter (*NFKBIZ* promoter-Luc) and a mutated promoter clone for a STAT-binding site (Δ STAT) were described [11]. These promoter clones simultaneously express naturally-secreted *Gaussia* luciferase (Gluc) under the control of the promoter sequence (–1148 to +98) of *NFKBIZ* transcript variant 1 (NM_031419) and secrete alkaline phosphatase under the control of the CMV promoter, which serves as the internal control. We generated by PCR the *NFKBIZ* promoter clones of partial truncations or C/EBP-binding site deletion, as well as STAT1 gain-of-function mutants [25], A267V and R274W. Primers used for the plasmid constructions are listed in Table S1.

Cell culture and transfection of siRNA

The human keratinocyte cell line HaCaT and human embryonic kidney 293T cells were maintained in DMEM-containing 10% fetal bovine serum. Recombinant human IL-17A was purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human IFN- γ was purchased from Wako Pure Chemical Industries (Osaka, Japan). For siRNA transfection, the Lipofectamine 2000 reagent (Life Technologies, Carlsbad, CA) was used. The siRNAs used are listed in Table S1.

RNA isolation and reverse transcription-quantitative real-time PCR (RT-qPCR)

Total RNA was prepared from cells using the TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). First-strand cDNA was synthesized from 1 μ g of total RNA with ReverTra Ace (TOYOBO, Osaka, Japan). Quantitative real-time PCR analysis was carried

out using KAPA SYBR FAST qPCR master mix (KAPA Biosystems, Woburn, MA, USA) with an Mx3005P real-time PCR system (Stratagene, Santa Clara, CA, USA). Data were normalized to the amount of beta-actin (ACTB) mRNA. The primer pairs used are listed in Table S1.

Immunoblotting

Immunoblotting was performed as described previously [26]. Briefly, cell lysates were resolved on SDS-PAGE and transferred to PVDF transfer membranes (PerkinElmer; Boston, MA, USA). The membranes were incubated with each primary antibody and then secondary antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore; Bedford, MA, USA). The antibodies used are listed in Table S1.

Luciferase assay for NFKBIZ promoter activity

293T cells were transfected with *NFKBIZ* promoter-Luc, together with each expression vector for untagged C/EBP β , FLAG-tagged STAT3C, or FLAG-tagged STAT1. At 48 h after transfection, the culture supernatants of the transfected cells were collected, and *Gaussia* luciferase (Gluc) and alkaline phosphatase activities were measured using a Secrete-Pair Dual Luminescence Assay Kit (GeneCopoeia, MD, USA) according to the manufacturer's instructions.

Chromatin-Immunoprecipitation with Sequencing (ChIP-seq) data visualization

ChIP-seq data deposited in Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>) in the National Center for Biotechnology Information servers were searched and aligned to reference genome (Human GRCh37/hg19) by ChIP-Atlas tool [27] (<https://chip-atlas.org/>) and visualized in the Integrative Genomics Viewer (IGV) [28]

(<https://software.broadinstitute.org/software/igv/>). The IDs of data used were as follows: SRX150578 (K-562), SRX150572 (Hep G2), SRX150709 (A549), SRX190345 (MCF-7) and, SRX150632 (HeLa) for CEBPB; SRX150356 (HeLa) for STAT3; SRX150440 (HeLa) for STAT1.

Statistical evaluation

Data are expressed as mean \pm standard error of the mean. For statistical comparison of two groups, Student's t-test was performed. For multiple comparisons, one-way ANOVA and Tukey multiple comparison tests were used. Statistical differences were determined to be significant at $p < 0.05$.

Results

C/EBP β and STAT3 promote I κ B- ζ induction.

We firstly examined the role of C/EBP β in *NFKBIZ* (encoding I κ B- ζ) expression (Figure 1a). *NFKBIZ* transcript variant 1 (NM_031419) was upregulated at 1.5 h after IL-17A addition in control siRNA-transfected HaCaT cells. The induction was significantly reduced in *CEBPB* siRNA-treated cells (Figure. 1a) and in *STAT3* siRNA-treated cells that served as a positive control (Figure 1b), compared to that in control siRNA-treated cells. The levels of IL-17A-induced I κ B- ζ protein were also reduced by siRNA knockdown of *CEBPB* (Figure 1c) or *STAT3* (Figure 1d). *Gaussia* luciferase-expressing reporter plasmids under the control of the various length of promoter region sequence (position -1147, -219, and -27 upstream from transcriptional start site) of the *NFKBIZ* gene (*NFKBIZ* promoter-Luc) were prepared and transiently introduced into 293T cells (Figure 1e, f, g) to understand the nature of the transcriptional regulation of *NFKBIZ* promoter activity. The effects of transient overexpression of untagged C/EBP β or FLAG-STAT3C by plasmids on transcriptional induction of *NFKBIZ* were examined (Figures 1f, g). Truncation up to -219 had no effects, but truncation up to -27 strongly impaired the reporter activity in responses to either STAT3C or C/EBP β overexpression (Figure 1f, g), suggesting that the promoter sequence -219 to -27 may have a role in *NFKBIZ* induction. These results confirmed the previous result of STAT3 involvement [11] and newly identified the role of C/EBP β in the induced expression of I κ B- ζ .

STAT-binding site and its flanking C/EBP-binding site integrate *NFKBIZ* promoter activation.

We previously identified a STAT-binding element required for STAT3-mediated *NFKBIZ* promoter activation by our ChIP and luciferase reporter assays [11]. The STAT-binding element is located within the region -219 to -27 from the transcription start site, more

specifically, at -133 to -125 [11]. In the present study, we analyzed public C/EBP β ChIP-seq data using ChIP-Atlas (<https://chip-atlas.org/>) [27] and noted that C/EBP β occupies the *NFKBIZ* promoter region in various types of cells (Figure 2a). C/EBP β ChIP-seq peaks are observed approximately 150 bp upstream from transcriptional start site of *NFKBIZ*, and are colocalized with STAT3 at the site in some cell lines as exemplified by HeLa (Figure 2a). A putative C/EBP-binding sequence exists near the above-mentioned STAT3 site (Figure 2b). To clarify whether the putative C/EBP-binding site influences *NFKBIZ* promoter activation, we deleted each of the putative C/EBP-binding site, the STAT-binding site, or both from the *NFKBIZ* promoter-Luc and performed reporter assays (Figure 2b, c, d). The Δ STAT promoter reduced promoter activation while the intact (-1147) promoter saw regular activation via overexpressed C/EBP β (Figure 2c). Δ CEBP and Δ STAT/CEBP promoters showed no significant response to C/EBP β introduction, indicating that the putative C/EBP-binding site is functional to drive the C/EBP β -mediated promoter activation. STAT3C-mediated promoter activation was induced on the intact promoter, whereas this induction was reduced with the Δ STAT promoter and the Δ CEBP promoter. Deletion of both sites (Δ STAT/CEBP) abolished the STAT3C-induced promoter activation (Figure 2d). Collectively, these results suggested that both the C/EBP- and STAT-binding sites are reciprocally required for STAT3- or C/EBP β -induced activation of *NFKBIZ* promoter.

STAT1 counteracts C/EBP β - and STAT3-induced *NFKBIZ* induction.

As the transcription factors STAT1 and STAT3 appear to play opposite roles in various biological contexts [22], we next investigated the role of STAT1 in *NFKBIZ* promoter regulation. Immunoblotting analysis showed that IL-17A-induced increase of I κ B- ζ protein was attenuated by a simultaneous co-treatment with IFN- γ , with a considerable upregulation of endogenous STAT1 protein (Figure 3a). Either IL-17A or IFN- γ did not alter protein levels

of C/EBP β and STAT3 (Figure 3a). The transcriptional activation of STAT1 is mediated through its phosphorylation of STAT1's tyrosine residue Tyr701 (Figure 3b). An experiment in which HaCaT cells pretreated with IFN- γ for short-term (1 h) or long (24 h) resulted in reduced induction of I κ B- ζ protein only upon longer IFN- γ -exposure where the total STAT1 amount in cells increased (Figure 3c). The short-term IFN- γ pretreatment, which induced STAT1 phosphorylation but did not increase total STAT1 levels, did not suppress IL-17A-induced I κ B- ζ protein.

To better understand the function of STAT1 in *NFKBIZ* promoter activation, we conducted STAT1 mutant co-overexpression with STAT3C or C/EBP β . We found significant suppression of promoter activation of either C/EBP β or STAT3C (Figure 3d) by co-transfection of the STAT1 wild-type and by a phosphorylation-defective mutant STAT1 (STAT1 Y701F). Also, the effects of disease-related gain-of-function (GOF) STAT1 mutations, A267V and R274W [25], were investigated. These missense mutations affect the coiled-coil domain of STAT1 which is important to form an antiparallel-type homodimer of non-phosphorylated STAT1 [29]. We found that A267V and R274W mutants suppressed STAT3C-driven *NFKBIZ* promoter activation, with significantly enhanced suppression for R274W mutant compared to wild-type STAT1 (Figure 3d). GOF STAT1 mutants suppressed also C/EBP β -driven *NFKBIZ* promoter activation with a tendency of enhanced suppression for R274W over wild-type STAT1. These results suggest that STAT1 can counteract C/EBP β - or STAT3-induced *NFKBIZ* promoter activity (Figure 3e), presumably by the mechanism regulated by coiled-coil-mediated dimer conformation change. Additionally, IL-17A-induced increase of *NFKBIZ* mRNA (Figure 3f) and I κ B- ζ protein (Figure 3g) levels were significantly augmented in STAT1 siRNA-treated HaCaT cells than in control siRNA-treated cells. Also, the IL-17A-induced increase of *DEFB4A* mRNA level, the induction of which is mediated by I κ B- ζ [3, 6, 30], was significantly enhanced by the siRNA knockdown of STAT1 (Figure 3f).

These results supported a negative regulatory role of STAT1 in *NFKBIZ* promoter activity regulation.

Discussion

As the role of the I κ B- ζ protein in the pathogenesis of psoriasis and psoriasis-related inflammatory diseases has been revealed in recent years, we investigated its transcriptional regulation mechanism. We found the involvement of C/EBP β in *NFKBIZ* (I κ B- ζ) induction. Analyses using *NFKBIZ* promoter-Luc and its series of truncated constructs showed that C/EBP β and STAT3 increase *NFKBIZ* promoter activity via the corresponding binding sites. Interestingly, the C/EBP β -induced activation of the *NFKBIZ* promoter was reduced by the deletion of the putative C/EBP-binding site itself and the deletion of the flanking STAT3 binding site. Also, the STAT3C-induced *NFKBIZ* promoter activation was reduced by deleting its binding site and the C/EBP-binding site. These data suggest a new reciprocal relationship between STAT3 and C/EBP β for efficient *NFKBIZ* promoter activation. Given the counteracting effect of STAT1 on both STAT3C- and C/EBP β -mediated *NFKBIZ* induction, STAT1 may target the coordinated functioning of STAT3 and C/EBP β on the promoter to suppress *NFKBIZ* transcription.

Our data indicated that the short-term pretreatment with IFN- γ induced tyrosine phosphorylation of STAT1 but did not suppress IL-17A-induced I κ B- ζ protein accumulation at that time point (Figure 3c). Moreover, a non-phosphorylated mutant, STAT1 Y701F, could suppress *NFKBIZ* promoter activity (Figure 3d). Taken together, these data implicated that the tyrosine phosphorylation status of STAT1 does not directly correlate with the suppression of I κ B- ζ expression. However, as observed in Figures 3a and c, the expression level of total STAT1 protein strongly increased upon prolonged IFN- γ treatment, the mechanism of which is presumably that the STAT1 itself is the transcriptional target gene of phosphorylated STAT1 [31]. Given that STAT1 can affect gene transcription without tyrosine phosphorylation in the nucleus [31], it is considered that the IFN- γ stimulation increases the total amount of STAT1 including the unphosphorylated form, resulting in a suppressive impact on the I κ B- ζ

expression in keratinocytes.

As a limitation of the present study, the physiological or clinical significance of the STAT1-mediated *NFKBIZ* promoter regulation has not been elucidated and needs future research. However, STAT3 is one of the major transcription factors in the psoriatic keratinocytes in response to cytokines [8, 32-35]. Furthermore, STAT1 protein levels are increased in lesional psoriatic skin compared to non-lesional skin [36]. Considering this, our findings that STAT1-mediated STAT3-C/EBP β suppression in I κ B- ζ expression may be an additional regulatory layer that affects the pathogenesis of psoriasis in IL-17A-responsive cells like keratinocytes. Moreover, the suppression of I κ B- ζ by STAT1 may partly be responsible for the pathogenesis of chronic fungal skin infections where the GOF human STAT1 mutations impair IL-17A-mediated immunity and STAT3 activity in the patients [25, 37, 38]. For example, in peripheral blood mononuclear cells from patients with the GOF STAT1 mutation, STAT3-dependent gene transcription was reportedly attenuated than in peripheral blood mononuclear cells from healthy control [37], suggesting that the STAT1 coiled-coil mutants may suppress STAT3 function more effectively than wild-type STAT1. This view was supported partly by our results showing an enhanced suppressive function of STAT1 coiled-coil mutants (Figure 3d). Further investigation of these issues would help understand the pathophysiological significance of STAT1-mediated *NFKBIZ* promoter regulation.

Some other points also remain unclear. Although the promoter activity was reduced, the -27 construct, which does not have both CEBP and STAT3 sites, still responded to C/EBP β and STAT3 (Figures 1f and g). This discrepancy suggests a possibility that C/EBP β and STAT3 can stimulate *NFKBIZ* induction directly or indirectly through the region between -27 to the first start codon (ATG; +98). Also, Δ CEBP single mutant promoter showed a reduced activity than the intact promoter, whereas Δ STAT/CEBP double mutant promoter showed a

tendency of a higher level of Luc activity than the Δ C/EBP single mutant (Figures 2c and d). It can be speculated that the transcription factor maintaining basal promoter activity can be switched from C/EBP β and STAT3 to other transcription factors when both C/EBP β and STAT3 cannot work sufficiently. The details of this possible redundant regulation by transcription factors remain to be elucidated. Additional factors such as NF- κ B, which has been shown important for *NFKBIZ* transcription [8], or an unidentified transcription factor whose expression depends on C/EBP β or STAT3, should be considered as the candidates.

In summary, the results in the present study suggest that C/EBP β and STAT3 are transcription factors that reciprocally play a promoting role in the *NFKBIZ* promoter activity, and STAT1 is a transcription factor that has a suppressive role. Thus, these could be a novel fine-tuning mechanism controlling IL-17A-I κ B- ζ pathway-induced cellular responses.

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Conflict of interest

The authors have no financial conflicts of interest.

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Figure Legends

Figure 1. C/EBP β and STAT3 promote I κ B- ζ induction. (a, b) HaCaT cells were transfected with siRNAs against *CEBPB* (siCEBPB) (a) or *STAT3* (siSTAT3) (b) for 48 h. Then the cells were stimulated with IL-17A (20 ng/mL) or left untreated for 1.5 h. The mRNA levels of *NFKBIZ* were determined by RT-qPCR. Data are obtained from 3 independent experiments. * $p < 0.05$. (c, d) HaCaT cells were transfected with siRNAs as in (a) and (b). Then the cells were stimulated with IL-17A (20 ng/mL) for the indicated times. The amount of I κ B- ζ protein was determined by western blotting. (e) Schematic representation of luciferase reporter constructs ligated to the *NFKBIZ* promoter region (*NFKBIZ* promoter-Luc). Gluc, *Gaussia* luciferase. TSS, transcription start site. (f, g) 293T cells were transfected with the *NFKBIZ* promoter-Luc constructs with or without the plasmid expressing C/EBP β (f) or STAT3C (g). At 48 h after transfection, the culture supernatants of the cells were collected and assayed for secreted Gluc activity. N = 3. * $p < 0.05$. Total cell lysates from the cells were used to confirm untagged C/EBP β (f) or FLAG-STAT3C (g) by western blotting using anti-C/EBP β and anti-FLAG-tag antibodies.

Figure 2. A STAT-binding site and its flanking C/EBP-binding site integrate *NFKBIZ* promoter activation. (a) ChIP-seq profile of the *NFKBIZ* promoter region. Data showing C/EBP β , STAT3, and STAT1 chromatin binding were obtained and visualized using ChIP-Atlas (<https://chip-atlas.org/>) as described in Materials and Methods. (b) Schematic representation of intact *NFKBIZ* promoter-Luc (-1148) with a putative CEBP-binding site and STAT-binding site and deletion mutants for each site. (c, d) 293T cells were transfected with the *NFKBIZ* promoter-Luc constructs with or without the plasmid for C/EBP β (c) or STAT3C (d). At 36 h after transfection, the culture supernatants of the cells were collected and assayed for Gluc activity. N = 3. * $p < 0.05$. Total cell lysates from the cells were used to

confirm untagged C/EBP β (c) or FLAG-STAT3C (d) by western blotting using anti-C/EBP β and anti-FLAG-tag antibodies.

Figure 3. STAT1 counteracts STAT3- and C/EBP β -induced *NFKBIZ* induction. (a) HaCaT cells were treated with IL-17A (20 ng/mL) and IFN- γ (1 ng/mL) as indicated in the figure for 24 h. Expressions of I κ B- ζ , STAT1, STAT3, and C/EBP β were detected by immunoblot analysis. Representative blots were shown. N = 3. (b) Schematic representation of STAT1 and Y701F mutant. (c) HaCaT cells were pretreated with IFN- γ (1 ng/mL) for 0, 1, or 24 h and then stimulated with IL-17A (20 ng/ml) for 3 h. Immunoblot analysis was performed to detect I κ B- ζ , phosphor-STAT1 (Y701), and total-STAT1. Representative blots were shown. N = 2. ns, nonspecific. (d) 293T cells were transfected with the *NFKBIZ* promoter-Luc construct and C/EBP β or STAT3C plasmid, together with the expression vector for STAT1 WT, Y701F, A267V, R274W, or empty vector. At 48 h after transfection, the culture supernatants of the cells were collected and assayed for Gluc activity. N = 3. * p < 0.05. The expressions of FLAG-STAT1 constructs were checked by western blotting with anti-FLAG-tag antibody. (e) Putative role for STAT1 in the suppression of *NFKBIZ* promoter. (f, g) HaCaT cells were transfected with siSTAT1 for 48 h. Then the cells were stimulated with IL-17A (20 ng/mL) or left untreated for 1.5 h (f) or indicated times (g). mRNA levels were determined by RT-qPCR (f). Immunoblot analysis for I κ B- ζ protein (g). Representative blots were shown. Data are obtained from 3 independent experiments. * p < 0.05.

Figure 1

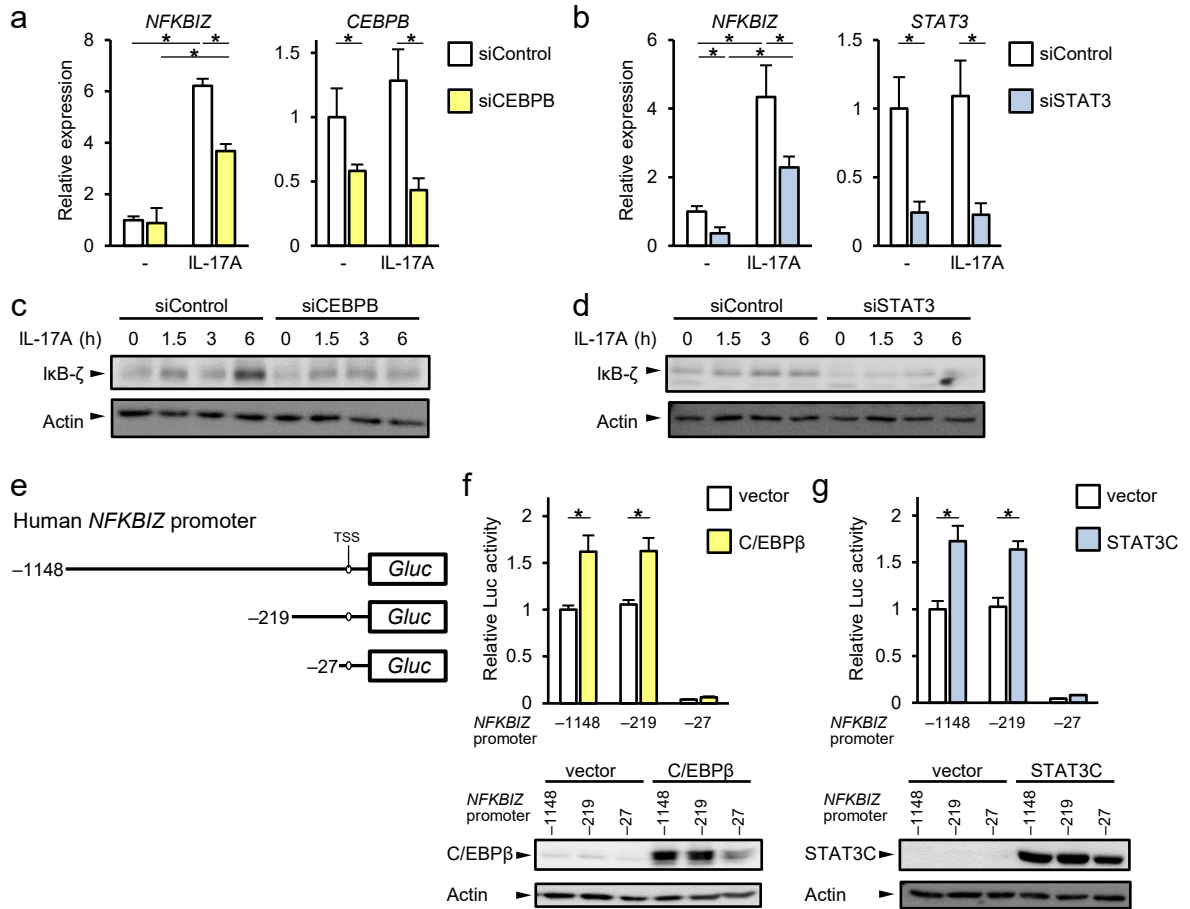


Figure 2

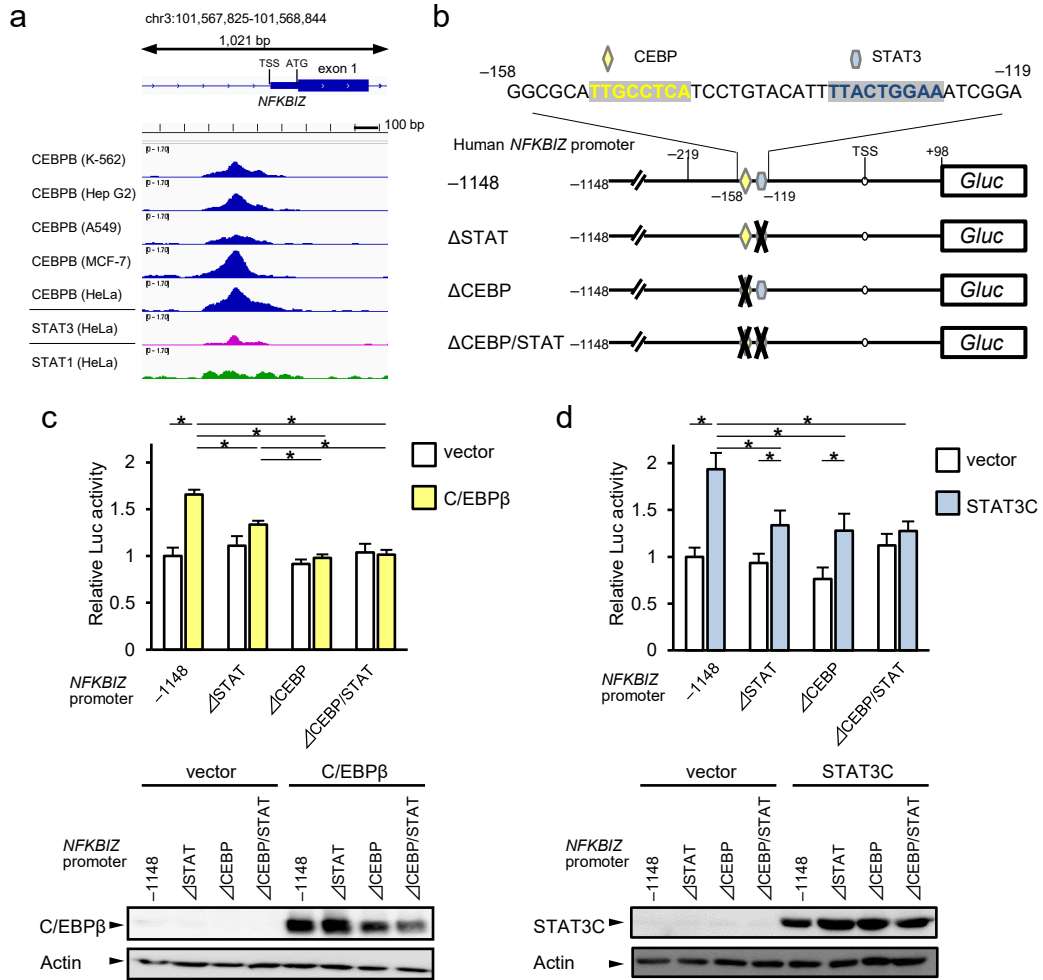


Figure 3

