The proinflammatory cytokine TNF-α induces DNA demethylation–dependent and –independent activation of interleukin-32 expression

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Running title:
TNF-α induced long-term IL-32 activation

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ABSTRACT

Interleukin-32 (IL-32) is a cytokine involved in proinflammatory immune responses to bacterial and viral infections. However, the role of epigenetic events in the regulation of IL-32 gene expression is understudied. Here, we show that IL-32 is repressed by DNA methylation in human embryonic kidney 293 (HEK293) cells. Using ChIP-Seq, locus-specific methylation analysis, CRISPR/Cas9-mediated genome editing, and RT-qPCR and immunoblotting assays, we found that short-term treatment (a few hours) with the proinflammatory cytokine tumor necrosis factor-α (TNF-α) activates IL-32 in a DNA demethylation–independent manner. In contrast, prolonged TNF-α treatment (several days) induced DNA demethylation at the promoter and a CpG island in the IL-32 gene in a TET family enzyme– and NF-κB–dependent manner. Notably, the hypomethylation status of transcriptional regulatory elements in IL-32 was maintained for a long time period (several weeks), causing elevated IL-32 expression even in the absence of TNF-α. Considering that IL-32 can, in turn, induce TNF-α expression, we speculate that such feed-forward events may contribute to the transition from an acute inflammatory response to chronic inflammation.

IL-32 is a proinflammatory cytokine (1-4). The IL-32 gene emerges quite late during evolution and exists only in certain mammals such as humans, chimpanzees, cattle and horses; however, it does not exist in rodents (5,6). Moreover, IL-32 shares little sequence identity with other interleukins (1,5).

Consistent with a role of IL-32 in inflammatory response, IL-32 expression is induced by TNF-α in various human cell types, including synovial fibroblasts, intestinal epithelial cell lines and pancreatic cancer cell lines (7-9). Reciprocally, IL-32 can also induce the expression of TNF-α and other cytokines in human THP-1 monocytic cells (1). Interestingly, although mice do not contain the IL-32 gene, ectopic treatment with human IL-32 can induce TNF-α expression in mouse Raw macrophage cells (1). Moreover, the injection of human IL-32 protein into the knee joints of wild-type mice, but not into the knee joints of Tnf gene knockout mice, provokes severe inflammation, suggesting that IL-32 exerts direct effects on joint inflammation in a TNF-α-dependent manner (2). Functionally, IL-32 promotes the differentiation of monocytes towards macrophage-like cells that display phagocytic activity, further supporting a role of IL-32 in immune response (10).

IL-32 plays important roles in inflammatory autoimmune diseases (11,12). IL-32 is highly expressed in rheumatoid arthritis synovial tissue biopsies (2), inflamed mucosa of inflammatory bowel disease (9) and chronic pancreatitis duct cells (8). These reports suggest that IL-32 is likely a cytokine involved in chronic inflammation, and it may serve as a potential therapeutic target.

As a proinflammatory cytokine, the expression of IL-32 is induced during bacterial and viral infections, and its expression improves host immunity in controlling these infections (13). For example, in patients with active Mycobacterium tuberculosis infections, IL-32 expression is induced, and it protects human macrophages and peripheral blood mononuclear cells against M. tuberculosis (14-16). Likewise, the expression of IL-32 is induced during HIV infection and influenza virus infection, as it contributes to the antiviral response (4,17,18).

DNA methylation is an important gene silencing mechanism that functions by recruiting corepressor proteins to impede the binding of DNA methylation-sensitive transcription factors (19,20). DNA demethylation can be achieved by enzyme-mediated active demethylation or by passive DNA methylation caused by interfering maintenance DNA methylation (21). TET family methylcytosine dioxygenases catalyze active DNA demethylation through the sequential oxidation of 5mC to 5hmC, 5fC and 5caC (22-24), followed by TDG-mediated base excision repair (24).

Gene expression is often regulated by sequence-specific transcription factors and epigenetic regulators. Given that IL-32 expression is regulated during inflammation, understanding whether epigenetic events occur during the induction of IL-32 expression is interesting. Here, we report that IL-32 is silenced by DNA methylation and that TNF-α induces DNA demethylation-dependent and -independent mechanisms to control IL-32 activation. We also discuss the potential significance of these mechanisms.

RESULTS

IL-32 is silenced by DNA methylation in HEK293 cells

In our previous work, we performed RNA-seq experiments using HEK293 cells treated with the
DNA-demethylating agent 5-aza-2’-deoxycytidine (5-aza-dC) and identified genes silenced by DNA methylation (25,26). IL-32 was one of the genes strongly activated upon 5-aza-dC treatment (Figs. 1A and 1B), suggesting that IL-32 is a gene silenced by DNA methylation in HEK293 cells. Indeed, bisulfite sequencing data revealed that both the promoter and CpG island (CGI) predicted by Sequence Manipulation Suite (27) of the IL-32 gene (Fig. 1C) are highly methylated (Fig. 1D).

**Short-term TNF-α treatment induces IL-32 expression in a DNA demethylation-independent manner**

Given that the IL-32 gene is induced by TNF-α treatment (7-9) and repressed by DNA methylation in HEK293 cells, we wondered whether TNF-α treatment is sufficient to overcome DNA methylation-mediated silencing. We treated HEK293 cells with 50 ng/ml TNF-α and analyzed IL-32 expression at various time points. IL-32 expression began to be induced as early as 1 h post-TNF-α treatment and was potently activated after 3 h of TNF-α treatment (Fig. 2A).

Of course, we next asked whether IL-32 activation was accompanied by DNA demethylation. Interestingly, despite the apparent transcriptional activation, no substantial DNA demethylation at the promoter or CGI of the IL-32 gene was observed after 1 h of TNF-α treatment (Figs. 2A, 2B). These results indicate that TNF-α treatment could activate IL-32 gene expression in a DNA demethylation-independent manner.

We then examined THP-1 cells, a human monocyte-like cell line (28), and HAP1 cells, a human leukemia cell line (29). And we also observed DNA demethylation-independent activation of IL-32 expression upon short-term TNF-α treatment in these cells (Supplemental Fig. S1).

**Long-term TNF-α treatment induces significant DNA demethylation of the IL-32 transcriptional regulatory region**

However, we noticed a slight decrease in DNA methylation at the IL-32 promoter after 3 h of TNF-α treatment (Fig. 2B). This finding prompted us to perform longer TNF-α treatments with measurements of IL-32 expression and DNA methylation at various time points. As we anticipated, a long-term TNF-α treatment (12 d) resulted in clear DNA demethylation of the IL-32 transcriptional regulatory regions; furthermore, the accumulation of DNA demethylation was accompanied by IL-32 induction (Figs. 3A-C).

**Hypomethylation triggered by long-term TNF-α treatment leads to elevated IL-32 expression after the removal of TNF-α**

DNA methylation is a relatively stable epigenetic mark; therefore, we asked whether the methylation status of the IL-32 transcriptional regulatory regions could be stably maintained after TNF-α treatment. We treated HEK293 cells with TNF-α for 12 h or 12 d and then cultured the cells in a TNF-α-free medium for an additional 10-d period. Bisulfite sequencing data revealed that the promoter and CGI of the IL-32 gene remained largely hypomethylated in the cells that underwent 12 d of TNF-α treatment and 10 d of withdrawal (Fig. 4A), indicating that TNF-α induced DNA demethylation could be maintained for a considerable period of time.

Moreover, we noticed that prior exposure to long-term TNF-α treatment led to elevated basal IL-32 expression, even after 10 d of TNF-α withdrawal (Figs. 4B and 4C). These results indicated that long-term TNF-α treatment not only caused a stable epigenetic change but also led to a sustained change in the basal expression of the IL-32 gene.

Similarly, long-term TNF-α treatment caused DNA demethylation and elevated basal expression of IL-32 gene in HAP1 cells (Supplemental Fig. S2).

To determine whether the above effect could be maintained for an even longer period, we treated HEK293 cells with TNF-α for 12 d and then cultured them in a TNF-α-free medium for 10, 18 or 30 d. RT-qPCR results showed that the upregulation of IL-32 was maintained after 10 d, 18 d, and 30 d, although the upregulated level became more moderate after 30 d (Fig. 4D). Consistently, the DNA methylation level of the IL-32 promoter and CpG island began to increase after 30 d of TNF-α withdrawal (Fig. 4E).

Taken together, these results suggest that long-term TNF-α treatment can induce heritable hypomethylation at the promoter and CpG island of the IL-32 gene, causing long-term transcriptional alteration.

**TET enzymes mediate IL-32 demethylation during long-term TNF-α treatment**

DNA demethylation can be achieved by passive demethylation or TET enzyme-mediated active oxidation and demethylation or both (21,30). To answer whether passive demethylation was involved in
TNF-α induced demethylation, we attempted to arrest the cells at S phase and simultaneously treated the cells with TNF-α, unfortunately these cells suffered from severe cell death, and we were unable to draw a clear-cut conclusion about whether there was any involvement of passive demethylation.

To determine whether DNA demethylation at the promoter and CpG island of the IL-32 gene was mediated by TET enzymes, we generated TET1 knockout (KO), TET2 KO, TET3 KO and TET1/2/3 triple knockout (TKO) cells using the CRISPR-Cas9 system. In these cells, frameshift mutations were introduced at the carboxyl terminus of the TET family proteins to abrogate their catalytic activities (Supplemental Figs. S3, S4A, S4B).

We then performed bisulfite sequencing, and the results revealed that the DNA demethylation induced by TNF-α treatment at the IL-32 gene promoter and CGI was largely abrogated in the TET TKO cells, with the single knockouts each displaying varied partial defects (Fig. 5A). These results suggested that the TET enzymes function together to promote TNF-α-induced IL-32 gene demethylation. We also confirmed that there was no upregulation of the DNMT genes in the TET TKO cells by RNA-seq experiments (Supplemental Fig. S4C).

We next asked whether IL-32 gene demethylation mediated by the TET enzymes was responsible for the elevated IL-32 expression levels in cells recovered from long-term TNF-α treatment. Although IL-32 expression was induced by 12 h or 12 d of TNF-α treatment in all of the above cells (Supplemental Fig. S5), elevated IL-32 basal expression was not observed in TET TKO cells withdrawn from long-term TNF-α treatment (Fig. 5B). These results are consistent with the methylation states of the promoter and CGI of the IL-32 gene in these cells and support that long-term TNF-α treatment induces DNA demethylation at the transcriptional regulatory regions of the IL-32 gene, elevating its basal expression level.

**NF-κB-dependent transcriptional activation contributes to IL-32 gene demethylation and long-term elevation of its basal expression**

TNF-α activates the NF-κB signaling pathway and induces nuclear translocation of the canonical p50/p65 heterodimer (31-36). Interestingly, a p65 binding site (κB site) is located in the promoter of the IL-32 gene (Fig. 6A), and its presence was confirmed by our p65 ChIP-seq results (Fig. 6B). Therefore, we knocked out the RELA gene that encodes p65 in HEK293 cells using the CRISPR-Cas9 system (Supplemental Figs. S6A, S6B) and verified the cells using sequencing (Supplemental Fig. S6C) and Western blotting (Fig. 6C). RT-qPCR data revealed that TNF-α-mediated IL-32 activation was significantly impaired in RELA KO cells (Fig. 6D), indicating p65 is the predominant transcription factor mediating IL-32 induction in response to TNF-α. Moreover, in RELA KO cells treated with TNF-α for 12 d, the levels of DNA demethylation at the transcriptional regulatory regions of IL-32 were reduced, especially at the CGI of IL-32 gene (Fig. 6E). The impaired DNA demethylation at the CGI of IL-32 gene was accompanied with a less elevated basal expression of IL-32 in long-term TNF-α treated RELA KO cells (Fig. 6F).

These data collectively support that TNF-α-induced NF-κB signaling pathway activation leads to DNA-demethylation-independent short-term activation and DNA-demethylation-dependent elevation of IL-32 basal transcription in the absence of initial TNF-α treatment.

Transcription factor induced DNA demethylation has been widely reported (37-50). In certain cases, these transcription factors can associate with the TET enzymes (42-47,49). In some other cases, no direct evidence supporting the association between transcription factors and TET enzymes was provided (41,50). We expressed Flag-TET1, Flag-TET2, or Flag-TET3 in HEK293 cells and stimulated cells with 12 h TNF-α. Then we performed immunoprecipitation experiments between p65 and the TET enzymes, but we did not observe any robust interaction. On the other hand, increased chromatin accessibility has been reported to facilitate DNA demethylation mediated by the TET enzymes (51-54). We measured chromatin accessibility at the IL-32 promoter by the FAIRE (formaldehyde-assisted isolation of regulatory elements) assay (55) and observed an increased chromatin accessibility in response to 12 h TNF-α treatment (Supplemental Fig. S6D). Thus, we speculate that p65 induced chromatin opening contributes to DNA demethylation mediated by the TET enzymes.

**CREB and the cAMP-response element (CRE) at the IL-32 promoter are not required for elevated IL-32 basal expression upon long-term TNF-α treatment**

The CpG site within a CRE of the IL-32 promoter was reported to be demethylated during influenza A virus infection, which increased transcription factor
CREB binding (4). We wondered whether this CpG site within CRE was also a target for TNF-α-induced demethylation, playing a role in the long-term activation of IL-32 gene. Therefore, we examined the CRE in the IL-32 promoter (Supplemental Fig. S7A) and confirmed its demethylation by TNF-α treatment (Figs. 2B, 3C, 4A and 5A). We next asked whether this CRE mediates the upregulation of IL-32 transcription through long-term TNF-α stimulation. Frameshift mutations were introduced in both alleles of the CREB1 gene to disrupt CREB binding to CRE (Supplemental Fig. S7B). However, the RT-qPCR results revealed normal IL-32 activation by TNF-α in CREB1 KO cells (Supplemental Figs. S7C, S7D).

In addition, we also mutated this CRE within the IL-32 promoter from TGACGTCA to TTTCGTCA (Supplemental Fig. S7E). Again, RT-qPCR revealed a largely normal elevation of IL-32 basal expression after long-term TNF-α treatment (Supplemental Fig. S7F).

Collectively, these data suggested that the long-term effect of TNF-α treatment is not solely dependent on the DNA demethylation of the CpG site within the CRE of the IL-32 promoter.

**DISCUSSION**

Signaling events triggered by environmental cues are well known for their roles in transcriptional regulation. In most cases, the majority of transcriptional changes triggered by signals are reset, and target gene expression returns to its initial basal level upon withdrawal of the environmental cues that initiated the signaling events (56, 57). However, sometimes, signaling events can also trigger lasting epigenetic changes that facilitate a long-term effect (56-60), which is an interesting field termed “signal to chromatin” (61-63).

DNA methylation is certainly one of the most stable epigenetic marks that can mediate a lasting effect. In recent years, increasing evidence has supported the role of transcription factor binding in facilitating DNA demethylation in neighboring regions (37-50) as well as the role of signaling events in stimulating DNA demethylation (64). However, cases reporting a full axis from signal to TF to DNA demethylation to a lasting transcriptional change in the absence of the initiating signal are still limited (58). Here, we report one such case — an axis involving a TNF-α signal, NF-κB pathway activation and association of p65 at the IL-32 promoter, TET enzyme-mediated IL-32 gene demethylation and the long-term activation of IL-32 expression (Fig. 7).

In addition to reporting the abovementioned case, the discovery of DNA demethylation-dependent and -independent mechanisms involved in activating IL-32 expression may have additional significance worthy of further investigation. As a TNF-α target, IL-32 has been reported to reciprocally induce the expression of TNF-α in certain cell types (1). We suspect that under certain in vivo situations, a strong acute inflammation event or the accumulative effect of several acute inflammation events may lead to the demethylation of the IL-32 gene and a lasting elevation of IL-32 basal expression, which may in turn stimulate TNF-α expression in these cells or neighboring cells. Such a self-reinforcing feedforward loop may well contribute to the conversion from acute inflammation to chronic inflammation. Understanding the potential mechanisms governing the conversion from acute inflammation to chronic inflammation is highly important due to its relevance to human health. Although our current study does not offer a clear answer for this important question, it provides an interesting direction for future exploration. One obvious difficulty in following up this study is the lack of a mouse model. The IL-32 gene does not exist in rodents (11), and follow-up studies will likely focus on human diseases. Therefore, one key question is what kind of pathological conditions may be relevant to our observations. We reason that chronic inflammatory diseases and autoimmune diseases are potential candidates on which to focus.

TNF-α antagonists including soluble receptors and antibodies have excellent efficacy in the treatment of chronic inflammatory diseases (e.g., rheumatoid arthritis and inflammatory bowel disease) (65, 66). Establishing a connection between TNF-α-induced demethylation and the long-term activation of proinflammatory genes, including but not limited to IL-32, in any of the above diseases would be highly interesting.

To offer a mechanistic answer for TNF-α-induced long-term gene activation in the absence of TNF-α, the current model is missing one piece. We reason that the long-term effect of TNF-α was due to DNA demethylation that facilitated the association of transcription factor(s) sensitive to DNA methylation. However, in this case, we do not yet know the identity of such transcription factor(s). The CREB binding site in the CRE of the IL-32 promoter and its association with CREB provided an ideal candidate, particularly because this site was found to be demethylated in
A549 cells infected with influenza virus (4) and the association of CREB with CRE is DNA methylation sensitive (67,68). However, in our case, this site does not appear to be the sole answer, because neither mutation of the CREB gene nor mutation of the CRE site in the IL-32 promoter caused sufficient changes (Supplemental Fig. S7). Future studies in this direction are of great interest.

We also performed HPLC-MRM MS/MS experiments at various time points following TNF-α treatment and observed a gradual subtle decline of the global 5mC level (Supplemental Fig. S8). Obviously, TNF-α treatment induced DNA demethylation is not restricted to IL-32 gene. The identification of other potential targets and their biological significance are interesting topics for future investigation.

EXPERIMENTAL PROCEDURES

Cell culture
HEK293 cells were cultured in DMEM/high glucose (HyClone, Cat# SH30022.01) supplemented with 10% fetal bovine serum (Biological Industries, Cat# 04-010-1ACS) and a penicillin-streptomycin solution (BBI Life Sciences, Cat# E607011-0100). Recombinant human TNF-α (Peprotech, Cat# 300-01A) was used at a final concentration of 50 ng/mL. For long-term TNF-α stimulation, TNF-α was added into the culture medium immediately after each passage.

Antibodies
Antibodies against IL-32 (Abcam, Cat# ab172339), p65 (Santa Cruz, Cat# sc-372) and histone H3 (Abcam, Cat# ab1791) were commercially available.

ChiP-seq
ChiP experiments were performed with HEK293 cells using previously described procedures (69). ChiP-seq libraries were constructed with a Kapa hyper prep kit (Kapa Biosystems, Cat# KK8504) and NEBNext multiplex oligos for Illumina (index primers set 1) (NEB, Cat# E7335). Libraries were sequenced via NovaSeq using the 150 bp paired-end mode.

Bioinformatics
50-bp single-end reads were generated by BGISEQ-500 platforms for mRNA-seq experiments (BGI, Shenzhen). Sequencing qualities were evaluated with FastQC software and aligned to human genome hg38 using STAR aligner. RPKM values were quantified using Cuffdiff (v2.0.2). FPKM values were added to a pseudo-value of 0.5 to avoid being divided by zero. ChiP-seq reads were generated by Illumina NovaSeq-6000 platforms (paired-end, 150 bp). Adaptors were removed by Trim_galore software, and then aligned to hg38 genome sequences (< 2 bp mismatches allowed) with Bowtie2. Uniquely mapped reads were kept and then were extended to the average fragment size. Genome profile files were generated with IGV tools and linearly normalized to the same depth of 10 million reads.

IL-32 locus-specific methylation analysis
To perform IL-32 promoter and CpG island (Supplemental Table 1) locus-specific methylation analysis, purified genomic DNA was treated with an EpiTect Bisulfite Kit (Qiagen, Cat# 59104), and the converted DNA was amplified using locus-specific nested PCR primers (Supplemental Table 2). Purified PCR products were cloned, sequenced and then analyzed using a BiQ Analyzer (70).

Genome editing using the CRISPR-Cas9 system
To generate RELA knockout, CREB1 frame-shift mutant, IL-32 promoter CRE mutant and TET frame-shift mutant cell lines, gRNA sequences (Supplemental Table 3) were designed and cloned into lentiCRISPR v2 vectors (Addgene #52961) (71). Individual clones were verified by genotyping PCR and Sanger sequencing.

Primers for RT-qPCR
The sequences of primers used for RT-qPCR include the following: IL-32, forward TGGCGGCTTTATATGAGGAGC and reverse CTGGGCACCGTAATCCATCTC; GAPDH, forward CTGGGCTACACT and reverse AAGTGGTCGTTGAGGGCAATG.

Data availability
All high-throughput sequencing data have been deposited under the GEO accession number GSE121361.
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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

Author contribution
Z.Zhao, Z.Zhang, and B.Z. designed this study. Z.Zhao performed the majority experiments. Z.Zhang. performed the bioinformatics analysis. M.L., J.L., Q.D., and X.L. assisted in performing experiments. B.L. performed the UHPLC-MS/MS experiments under the supervision of H.W. G.L. provided reagents and discussion. Z.Zhao, B.Z., and Z.Zhang. wrote the manuscript, and all authors read and commented on the manuscript.

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**Figure legends:**

Figure 1. The *IL*-32 gene is silenced by DNA methylation in HEK293 cells. *A*, RNA-seq results showed that 5-aza-dC treatment activates *IL*-32 expression in HEK293 cells. * indicates that the FPKM values were added a pseudo count of 0.5 to avoid to be divided by zero. *B*, *IL*-32 FPKM values in 5-aza-dC and DMSO-treated samples. *C*, Schematic representation of the *IL*-32 promoter and CGI. *D*, Locus-specific bisulfite sequencing results revealed that the promoter and CGI of *IL*-32 are highly methylated in HEK293 cells.

Figure 2. TNF-α treatment overcomes DNA methylation-mediated silencing and activates *IL*-32 expression. *A*, RT-qPCR results showed that *IL*-32 expression is quickly activated upon TNF-α treatment. Averages from three independent experiments are shown, and error bars represent standard deviation. *B*, Locus-specific bisulfite sequencing data showed that the *IL*-32 transcriptional regulatory regions remain largely methylated upon 1 h TNF-α treatment and *IL*-32 promoter is slightly demethylated after 3 h TNF-α treatment.
Figure 3. IL-32 transcriptional regulatory regions undergo DNA demethylation during 12 d of TNF-α treatment. A. RT-qPCR analysis showed that the IL-32 mRNA level can be more efficiently activated via long-term TNF-α treatment. Averages from three independent experiments are shown, and error bars represent standard deviation. B. Western blot results showed that the IL-32 protein level can be induced with 12 h and 12 d of TNF-α treatment. C. Locus-specific bisulfite sequencing results revealed that the promoter and CGI of the IL-32 gene are gradually demethylated during long-term TNF-α treatment. Filled circles indicate methylated CpG sites, and open circles indicate unmethylated CpG sites. CpG site methylation percentages are shown.

Figure 4. IL-32 basal expression is upregulated after long-term TNF-α treatment and is accompanied by sustained hypomethylation at the promoter and CGI. A. Locus-specific bisulfite sequencing data showed that the hypomethylation status of the IL-32 promoter and CGI can be maintained after 10 d of TNF-α withdrawal. B. A time-course experiment revealed that the IL-32 basal expression level is upregulated after long-term TNF-α treatment and TNF-α withdrawal. Averages from three independent experiments are shown, and error bars represent standard deviation in the RT-qPCR results. C. Western blot results showed cells treated long-term with TNF-α display a higher basal protein expression level of IL-32. D. RT-qPCR results revealed that the upregulated IL-32 expression that occurred after long-term TNF-α treatment can be maintained for at least 30 d after TNF-α withdrawal. Averages from three independent experiments are shown, and error bars represent standard deviation. E. Bisulfite sequencing data revealed that cells subjected to 12 d of TNF-α treatment maintained relatively low methylation levels at the promoter and CGI of the IL-32 gene even after 30 d of TNF-α withdrawal.

Figure 5. TET enzymes mediated DNA demethylation, leading to upregulated IL-32 basal expression upon long-term TNF-α treatment. A. Locus-specific bisulfite sequencing results showed that TET enzymes are responsible for the DNA demethylation events during long-term TNF-α treatment. B. RT-qPCR results showed that the upregulated IL-32 expression that occurred after long-term TNF-α treatment is dependent on TET enzymes. Averages from three independent experiments are shown, and error bars represent standard deviation.

Figure 6. NF-κB-dependent transcriptional activation promotes DNA demethylation and results in IL-32 upregulation after long-term TNF-α treatment. A. Schematic representation of a κB site (GGGAGTTTCC) in the IL-32 promoter. B. p65 ChIP-seq results showed that p65 is enriched at the κB site of the IL-32 promoter after 12 h of TNF-α treatment. C. Western blot data validating the RELA KO cell line. D. RT-qPCR results revealed impaired IL-32 induction in RELA KO cells. Averages from three independent experiments are shown, and error bars represent standard deviation. E. Locus-specific bisulfite sequencing results showed that the IL-32 CpG island DNA demethylation reaction that occurs during long-term TNF-α stimulation is impaired in RELA KO cells. F. RT-qPCR results showed that the upregulation of IL-32 transcription after long-term TNF-α treatment is impaired in RELA KO cells. Averages from three independent experiments are shown, and error bars represent standard deviation.

Figure 7. A model for DNA demethylation-dependent and -independent activation of IL-32 expression upon TNF-α treatment.
Figure 1

A. RNA-seq (5-aza-dC vs. DMSO)

B. IL-32 FPKM value

C. IL-32 mRNA

D. Promoter and CGI

- Promoter: 88.0%
- CGI: 95.9%
Figure 2

A

![Bar chart](image)

**Relative IL-32 mRNA level**

| Time (h) | 0 | 1 h | 3 h |
|----------|---|-----|-----|
| **Promoter** | | | |
| 0        | | | |
| 1 h      | | | |
| 3 h      | | | |
| CGI      | | | |
| 0        | | | |
| 1 h      | | | |
| 3 h      | | | |

B

| Time (h) | 0 | 1 h TNF-α | 3 h TNF-α |
|----------|---|-----------|-----------|
| **Promoter** | | | |
| 0        | | | |
| 1 h      | | | |
| 3 h      | | | |
| CGI      | | | |
| 0        | | | |
| 1 h      | | | |
| 3 h      | | | |

Promoter values: 85.2%, 81.5%, 77.8%
CGI values: 92.2%, 95.3%, 88.2%
Figure 3

A

Relative IL-32 mRNA level

TNF-α: 0 12 h 2 d 4 d 8 d 12 d

B

TNF-α: 0 12 h 12 d

α-IL-32

α-H3

C

Promoter

0 12 h TNF-α 2 d TNF-α 4 d TNF-α 8 d TNF-α 12 d TNF-α

84.3% 69.7% 63.3% 50.0% 45.5% 49.5%

CGI

94.1% 90.9% 74.5% 65.8% 57.8% 43.1%
Figure 4

A

|     | Control  | 12 h TNF-α | 12 h TNF-α+10 d | 12 d TNF-α | 12 d TNF-α+10 d |
|-----|----------|------------|-----------------|------------|-----------------|
| Promoter | 85.9% | 73.7% | 78.8% | 37.8% | 53.3% |
| CGI | 93.0% | 94.7% | 88.8% | 51.3% | 57.1% |

B

Relative IL-32 mRNA level

C

α-IL-32

α-H3

D

Relative IL-32 mRNA level

E

Promoter

CGI

87.0% 73.3%

90.6% 74.3%
### Figure 5

#### A

|          | Control    | 12 d TNF-α | 12 d TNF-α+10 d |
|----------|------------|------------|-----------------|
| **WT**   |            |            |                 |
| **Promoter** | 84.8% | 91.8% | 74.4%           |
| **CGI**  |            |            |                 |
| **TET1 KO** |        | 74.4% | 96.1%           |
| **Promoter** | 41.1% | 43.5% | 50.9%           |
| **CGI**  |            |            |                 |
| **TET2 KO** |        | 80.6% | 95.7%           |
| **Promoter** | 48.1% | 71.1% | 68.9%           |
| **CGI**  |            |            |                 |
| **TET3 KO** |        | 86.1% | 95.6%           |
| **Promoter** | 39.8% | 43.3% | 56.7%           |
| **CGI**  |            |            |                 |
| **TET TKO** |        | 86.7% | 97.5%           |
| **Promoter** | 71.3% | 98.8% | 74.7%           |
| **CGI**  |            |            |                 |

#### B

- **WT**
- **TET1 KO**
- **TET2 KO**
- **TET3 KO**
- **TET TKO**

|          | Control | 12 h TNF-α+10 d | 12 d TNF-α+10 d |
|----------|---------|-----------------|-----------------|
| **Relative IL-32 mRNA level** |         |                 |                 |
| **WT**   |         |                 |                 |
| **TET1 KO** |       |                 |                 |
| **TET2 KO** |       |                 |                 |
| **TET3 KO** |       |                 |                 |
| **TET TKO** |       |                 |                 |
Figure 6

A. Schematic representation of the IL-32 promoter region showing CpG sites and κB sites.

B. ChIP-seq analysis of p65 binding in the IL-32 promoter region under Control and 12 h TNF-α conditions. The graph shows the enrichment of p65 at different genomic locations.

B. Bar graph showing the relative IL-32 mRNA level in WT and RELA KO cells under Control, 12 h TNF-α, 12 d TNF-α, and 12 d TNF-α + 10 d conditions.

D. ChIP-seq analysis of α-p65 and α-H3 binding in the IL-32 promoter region under Control and 12 h TNF-α conditions.

E. Table showing the percentage of promoter and CGI sites with p65 binding under different conditions.
Figure 7

IL-32

Promoter
CpG Island

Inactive

Short-term TNF-α stimulation

NF-κB dependent activation
TET enzymes

IL-32

Promoter
CpG Island

Active

IL-32

Promoter
CpG Island

Highly active

Elevated basal transcription

Long-term TNF-α stimulation

TNF-α withdrawal

TNF-α withdrawal
The proinflammatory cytokine TNF-α induces DNA demethylation–dependent and – independent activation of interleukin-32 expression
Zuodong Zhao, Mengying Lan, Jingjing Li, Qiang Dong, Xiang Li, Baodong Liu, Gang Li, Hailin Wang, Zhuqiang Zhang and Bing Zhu

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