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

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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 HEK293 cells. Using ChIP sequencing, locus-specific methylation analysis, CRISPR/Cas9-mediated genome editing, and RT-qPCR (quantitative RT-PCR) and immunoblot assays, we found that short-term treatment (a few hours) with the proinflammatory cytokine tumor necrosis factor α (TNFα) activates *IL-32* in a DNA demethylation–independent manner. In contrast, prolonged TNFα treatment (several days) induced DNA demethylation at the promoter and a CpG island in the *IL-32* gene in a TET (ten-eleven translocation) family enzyme– and NF-κB–dependent manner. Notably, the hypomethylation status of transcriptional regulatory elements in *IL-32* was maintained for a long time (several weeks), causing elevated IL-32 expression even in the absence of TNFα. Considering that IL-32 can, in turn, induce TNFα expression, we speculate that such feedforward 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 the inflammatory response, IL-32 expression is induced by TNFα[3] 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 TNFα 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 TNFα expression in mouse Raw macrophage cells (1). Moreover, injection of human IL-32 protein into the knee joints of WT 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 TNFα-dependent manner (2). Functionally, IL-32 promotes the differentiation of monocytes toward macrophage-like cells that display phagocytic activity, further supporting a role of IL-32 in the 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 that 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* infection, 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-me-

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This article contains Figs. S1–S8 and Tables S1–S5.

All high-throughput sequencing data have been deposited in the GEO under accession number GSE121361.

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[3] The abbreviations used are: TNFα, tumor necrosis factor α; 5-aza-dC, 5-aza-2′-deoxycytidine; CGI, CpG island; TKO, triple knockout; CREB, cAMP response element–binding protein; CRE, cAMP response element; RT-qPCR, quantitative RT-PCR; TET, ten-eleven translocation; FPKM, fragments per kilobase per million mapped fragments.

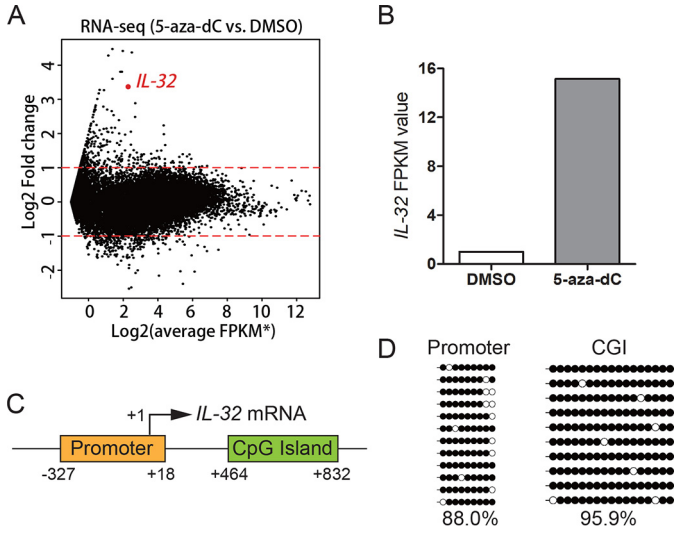

Figure 1. The *IL-32* gene is silenced by DNA methylation in HEK293 cells. *A*, RNA-Seq results showed that 5-aza-dC treatment activates *IL-32* expression in HEK293 cells. The *asterisk* indicates that the FPKM values were added a pseudocount of 0.5 to avoid being divided by zero. *B*, *IL-32* FPKM 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.

diated active demethylation or by passive DNA demethylation caused by interfering with maintenance DNA methylation (21). TET family methylcytosine dioxygenases catalyze active DNA demethylation through the sequential oxidation of 5mC (5-methylcytosine) to 5hmC (5-hydroxymethylcytosine), 5fC (5-formylcytosine), and 5caC (5-carboxylcytosine) (22–24), followed by TDG (thymine DNA glycosylase)-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 TNFα 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. 1, *A* and *B*), 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 the Sequence Manipulation Suite (27) of the *IL-32* gene (Fig. 1*C*) are highly methylated (Fig. 1*D*).

Short-term TNFα treatment induces IL-32 expression in a DNA demethylation–independent manner

Given that the *IL-32* gene is induced by TNFα treatment (7–9) and repressed by DNA methylation in HEK293 cells, we

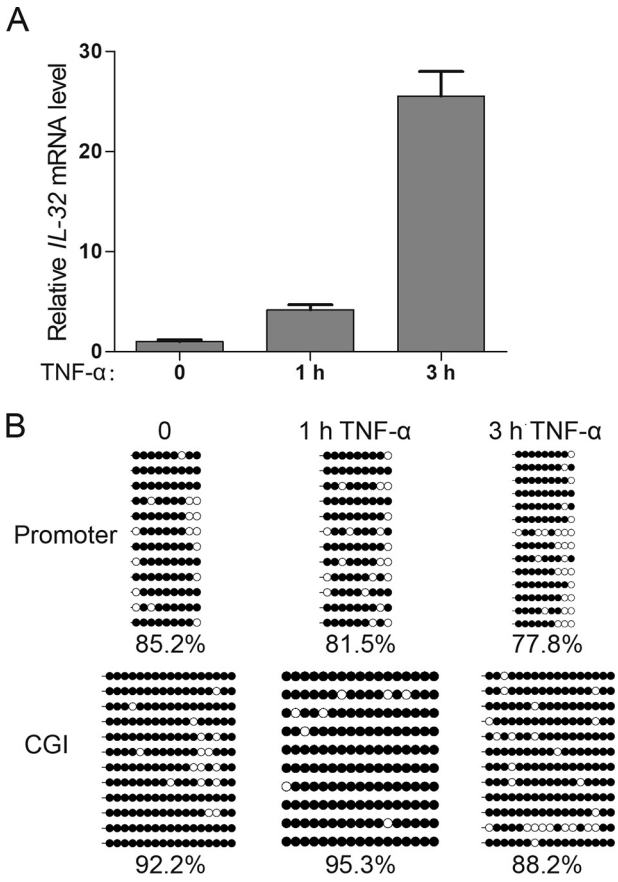

Figure 2. TNFα treatment overcomes DNA methylation–mediated silencing and activates *IL-32* expression. *A*, RT-qPCR results showed that *IL-32* expression is quickly activated upon TNFα treatment. Averages from three independent experiments are shown, and *error bars* represent standard deviation. *B*, locus-specific bisulfite sequencing data showed that the *IL-32* transcriptional regulatory regions remain largely methylated upon 1-h TNFα treatment and that the *IL-32* promoter is slightly demethylated after 3-h TNFα treatment.

wondered whether TNFα treatment is sufficient to overcome DNA methylation–mediated silencing. Thus, we treated HEK293 cells with 50 ng/ml TNFα and analyzed *IL-32* expression at various time points. *IL-32* expression began to be induced as early as 1 h post-TNFα treatment and was potently activated after 3 h of TNFα treatment (Fig. 2*A*).

We next wanted to find out 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 TNFα treatment (Fig. 2, *A* and *B*). These results indicate that TNFα 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 also observed DNA demethylation–independent activation of *IL-32* expression upon short-term TNFα treatment in these cells (Fig. S1).

Long-term TNFα treatment induces significant DNA demethylation of the IL-32 transcriptional regulatory region

We noticed a slight decrease in DNA methylation at the *IL-32* promoter after 3 h of TNFα treatment (Fig. 2*B*). This finding prompted us to perform longer TNFα treatments with

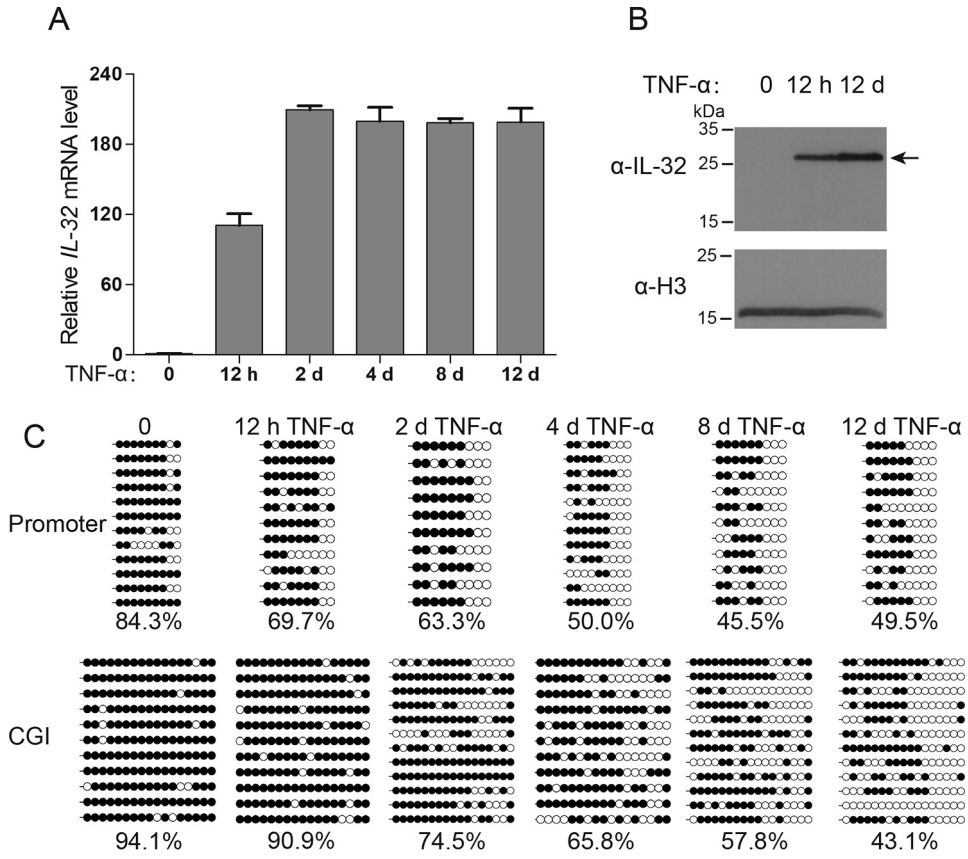

Figure 3. *IL-32* transcriptional regulatory regions undergo DNA demethylation during 12 days of TNFα treatment. *A*, RT-qPCR analysis showed that the *IL-32* mRNA level can be more efficiently activated via long-term TNFα treatment. Averages from three independent experiments are shown, and *error bars* represent standard deviation. *B*, Western blot results showed that the IL-32 protein level can be induced with 12 h and 12 days of TNFα treatment. *C*, locus-specific bisulfite sequencing results revealed that the promoter and CGI of the *IL-32* gene are gradually demethylated during long-term TNFα treatment. *Filled circles* indicate methylated CpG sites, and *open circles* indicate unmethylated CpG sites. CpG site methylation percentages are shown.

measurements of *IL-32* expression and DNA methylation at various time points. As we anticipated, long-term TNFα treatment (12 days) 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. 3, *A–C*).

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

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

Moreover, we noticed that prior exposure to long-term TNFα treatment led to elevated basal *IL-32* expression, even after 10 days of TNFα withdrawal (Fig. 4, *B* and *C*). These results indicated that long-term TNFα treatment not only caused a stable epigenetic change but also led to a sustained change in basal expression of the *IL-32* gene. Similarly, long-term TNFα treatment caused DNA demethylation and elevated basal expression of the *IL-32* gene in HAP1 cells (Fig. S2).

To determine whether the above effect could be maintained for an even longer period, we treated HEK293 cells with TNFα for 12 days and then cultured them in TNFα-free medium for 10, 18, or 30 days. RT-qPCR results showed that the up-regulation of *IL-32* was maintained after 10, 18, and 30 days, although the up-regulated level became more moderate after 30 days (Fig. 4*D*). Consistently, the DNA methylation level of the *IL-32* promoter and CpG island began to increase after 30 days of TNFα withdrawal (Fig. 4*E*). Taken together, these results suggest that long-term TNFα 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 TNFα treatment

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

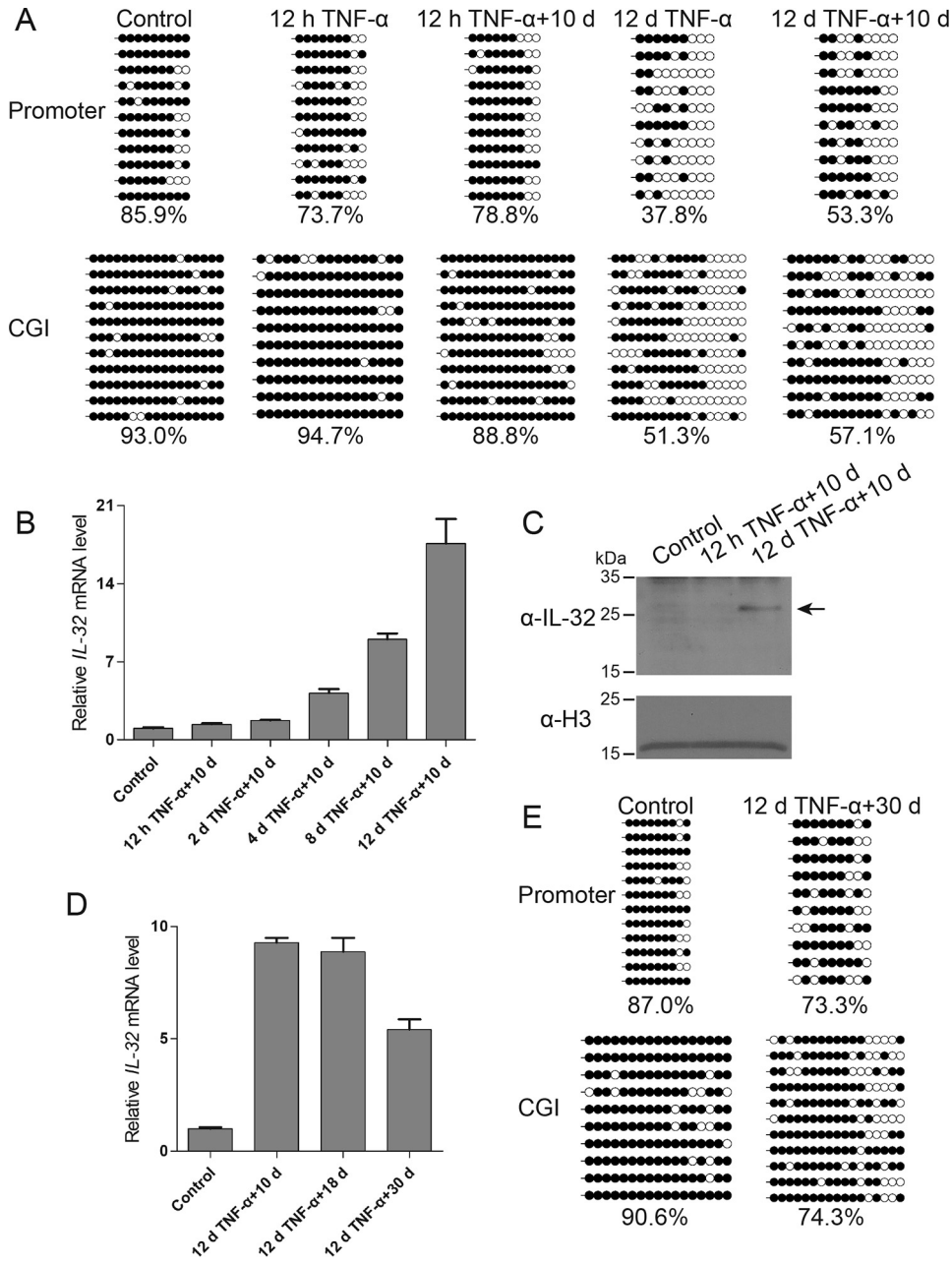

Figure 4. *IL-32* basal expression is up-regulated after long-term TNFα treatment and is accompanied by sustained hypomethylation at the promoter and CGI. *A*, locus-specific bisulfite sequencing data showed that the hypomethylation status of the *IL-32* promoter and CGI can be maintained after 10 days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

To determine whether DNA demethylation at the promoter and CpG island of the *IL-32* gene was mediated by TET enzymes, we generated *TET1* KO, *TET2* KO, *TET3* KO, and *TET*1/2/3 triple knockout (TKO) cells using the CRISPR-Cas9 system. In these cells, frameshift mutations were introduced at the C terminus of the TET family proteins to abrogate their catalytic activity (Figs. S3 and S4, *A* and *B*).

We then performed bisulfite sequencing, and the results revealed that the DNA demethylation induced by TNFα treatment at the *IL-32* gene promoter and CGI was largely abrogated in *TET* TKO cells, with the single knockouts each displaying varied partial defects (Fig. 5*A*). These results suggested that the TET enzymes function together to promote TNFα-induced *IL-32* gene demethylation. We also confirmed that there was no up-regulation of *DNMT* genes in *TET* TKO cells by RNA-Seq experiments (Fig. S4*C*).

We next wanted to find out whether *IL-32* gene demethylation mediated by TET enzymes was responsible for the elevated *IL-32* expression levels in cells recovered from long-term TNFα treatment. Although *IL-32* expression was

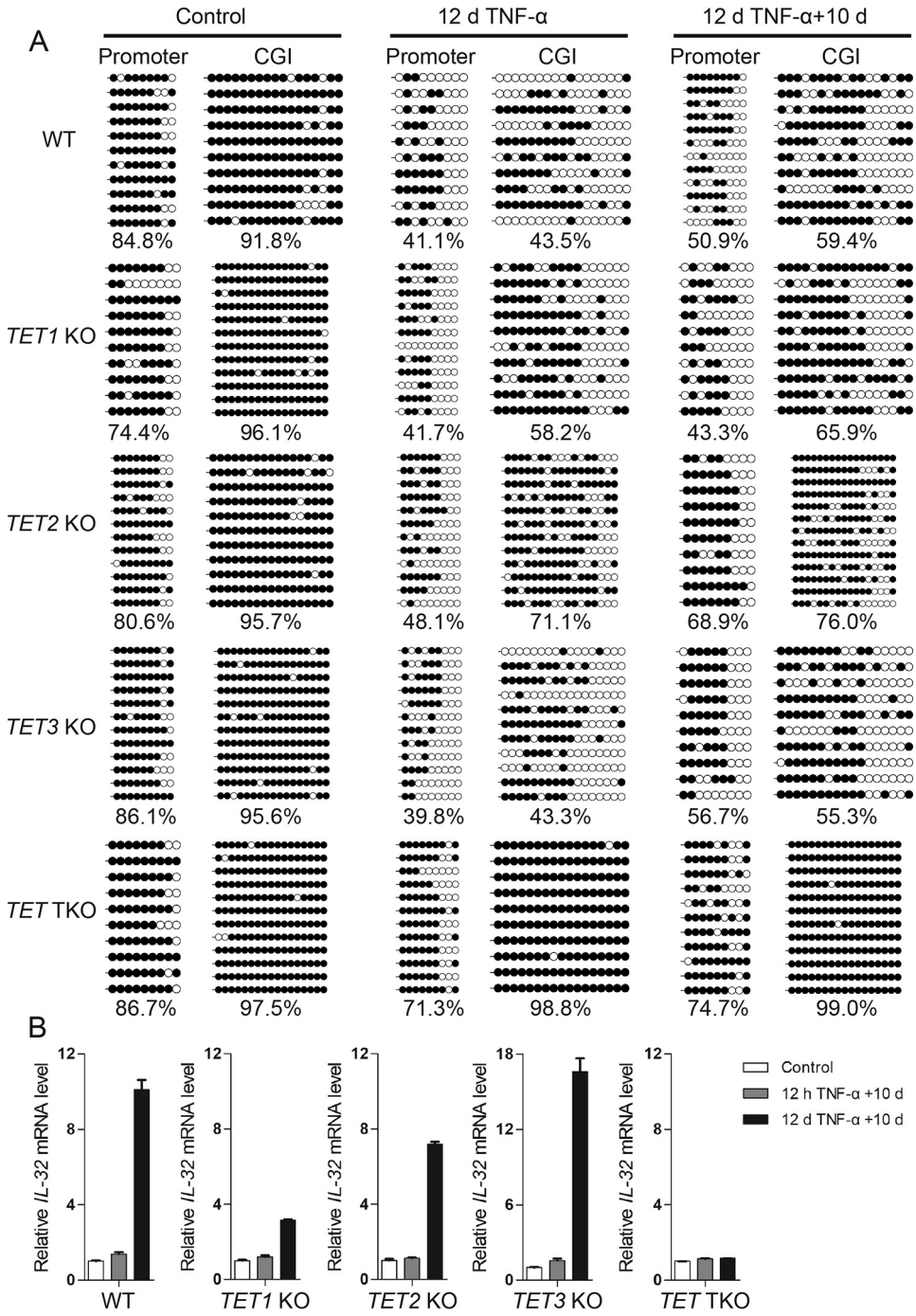

Figure 5. TET enzymes mediate DNA demethylation, leading to up-regulated *IL-32* basal expression upon long-term TNFα treatment. *A*, locus-specific bisulfite sequencing results showed that TET enzymes are responsible for the DNA demethylation events during long-term TNFα treatment. *B*, RT-qPCR results showed that the up-regulated *IL-32* expression that occurred after long-term TNFα treatment depends on TET enzymes. Averages from three independent experiments are shown, and *error bars* represent standard deviation. *d*, day.

induced by 12 h or 12 days of TNFα treatment in all of the above cells (Fig. S5), elevated *IL-32* basal expression was not observed in *TET* TKO cells withdrawn from long-term TNFα treatment (Fig. 5*B*). 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 TNFα 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

TNFα 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. 6*A*), and its presence was confirmed by our p65 ChIP-Seq results (Fig. 6*B*). Therefore, we

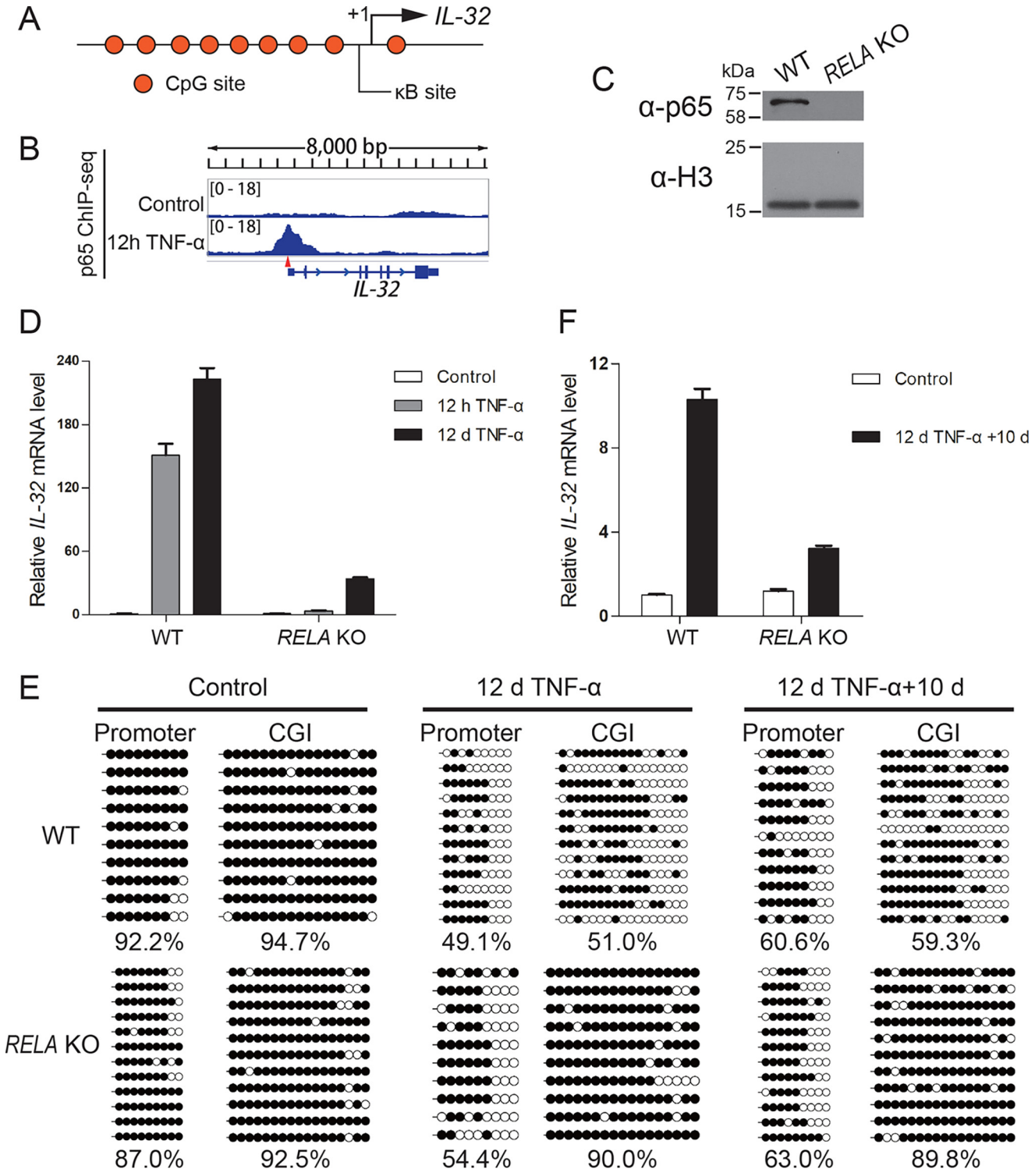

Figure 6. NF-κB–dependent transcriptional activation promotes DNA demethylation and results in *IL-32* up-regulation after long-term TNFα treatment. *A*, schematic representation of a κB site (GGGAGTTTCC) in the *IL-32* promoter. *B*, p65 ChIP-Seq results showed that p65 is enriched at the κB site of the *IL-32* promoter after 12 h of TNFα 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 TNFα stimulation is impaired in *RELA* KO cells. *F*, RT-qPCR results showed that up-regulation of *IL-32* transcription after long-term TNFα treatment is impaired in *RELA* KO cells. Averages from three independent experiments are shown, and *error bars* represent standard deviation. *d*, day.

knocked out the *RELA* gene, which encodes p65, in HEK293 cells using the CRISPR-Cas9 system (Figs. S6, *A* and *B*, and Table S4) and verified the cells using sequencing (Fig. S6*C*) and Western blotting (Fig. 6*C*). RT-qPCR data revealed that TNFα-mediated *IL-32* activation was significantly impaired in *RELA* KO cells (Fig. 6*D*), indicating that p65 is the predominant transcription factor mediating *IL-32* induction in response to TNFα. Moreover, in *RELA* KO cells treated with TNFα for 12

days, the levels of DNA demethylation at the transcriptional regulatory regions of *IL-32* were reduced, especially at the CGI of the *IL-32* gene (Fig. 6*E*). The impaired DNA demethylation at the CGI of the *IL-32* gene was accompanied by less elevated basal expression of *IL-32* in long-term TNFα-treated *RELA* KO cells (Fig. 6*F*). These data collectively support that TNFα-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 TNFα treatment.

Transcription factor–induced DNA demethylation has been widely reported (37–50). In certain cases, these transcription factors can associate with TET enzymes (42–47, 49). In some other cases, no direct evidence supporting the association between transcription factors and TET enzymes is provided (41, 50). We expressed FLAG-TET1, FLAG-TET2, or FLAG-TET3 in HEK293 cells and stimulated the cells for 12 h with TNFα. Then we performed immunoprecipitation experiments with 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 TET enzymes (51–54). We measured chromatin accessibility at the *IL-32* promoter by formaldehyde-assisted isolation of regulatory elements assay (55) and observed increased chromatin accessibility in response to 12-h TNFα treatment (Fig. S6*D* and Table S5). Thus, we speculate that p65-induced chromatin opening contributes to DNA demethylation mediated by 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 TNFα treatment

The CpG site within the CRE of the *IL-32* promoter has been reported to be demethylated during influenza A virus infection, which increases transcription factor CREB binding (4). We wondered whether this CpG site within the CRE was also a target for TNFα-induced demethylation, playing a role in long-term activation of the *IL-32* gene. Therefore, we examined the CRE in the *IL-32* promoter (Fig. S7*A*) and confirmed its demethylation by TNFα treatment (Figs. 2*B*, 3*C*, 4*A*, and 5*A*). We next asked whether this CRE mediates the up-regulation of *IL-32* transcription through long-term TNFα stimulation. Frameshift mutations were introduced in both alleles of the *CREB1* gene to disrupt CREB binding to the CRE (Fig. S7*B*). However, the RT-qPCR results revealed normal *IL-32* activation by TNFα in *CREB1* KO cells (Fig. S7, *C* and *D*).

In addition, we also mutated this CRE within the *IL-32* promoter from TGACGTCA to TTTCGTCA (Fig. S7*E*). Again, RT-qPCR revealed a largely normal elevation of *IL-32* basal expression after long-term TNFα treatment (Fig. S7*F*). Collectively, these data suggest that the long-term effect of TNFα treatment is not solely dependent on 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, reports of a full axis from signal to transcription factor 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 TNFα signal, NF-κB pathway activation and association of p65 at the *IL-32* promoter, TET enzyme–mediated *IL-32* gene demethylation, and long-term activation of *IL-32* expression (Fig. 7).

In addition to 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 TNFα target, IL-32 has been reported to reciprocally induce the expression of TNFα in certain cell types (1). We suspect that, under certain *in vivo* situations, a strong acute inflammation event or the cumulative effect of several acute inflammation events may lead to demethylation of the *IL-32* gene and a lasting elevation of IL-32 basal expression, which may, in turn, stimulate TNFα 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 because of its relevance to human health. Although this 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.

TNFα antagonists, including soluble receptors and antibodies, have excellent efficacy for treatment of chronic inflammatory diseases (*e.g.* rheumatoid arthritis and inflammatory bowel disease) (65, 66). Establishing a connection between TNFα-induced demethylation and 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 TNFα-induced long-term gene activation in the absence of TNFα, our model is missing one piece. We reason that the long-term effect of TNFα 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*

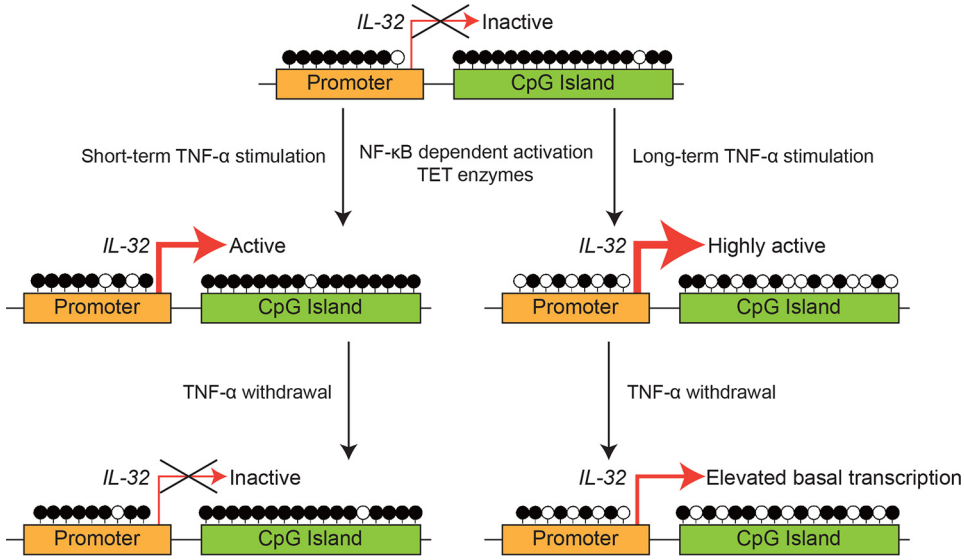

Figure 7. A model for DNA demethylation–dependent and –independent activation of *IL-32* expression upon TNFα treatment.

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 the 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 (Fig. S7). Future studies in this direction are of great interest.

We also performed HPLC-MRM (multiple reaction monitoring) MS/MS experiments at various time points following TNFα treatment and observed a gradual subtle decline of the global 5mC level (Fig. S8). Obviously, TNFα treatment–induced DNA demethylation is not restricted to the *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, catalog no. SH30022.01) supplemented with 10% fetal bovine serum (Biological Industries, catalog no. 04-010-1ACS) and a penicillin–streptomycin solution (BBI Life Sciences, catalog no. E607011-0100). Recombinant human TNFα (Peprotech, catalog no. 300-01A) was used at a final concentration of 50 ng/ml. For long-term TNFα stimulation, TNFα was added to the culture medium immediately after each passage.

Antibodies

Antibodies against IL-32 (Abcam, catalog no. ab172339), p65 (Santa Cruz Biotechnology, catalog no. sc-372), and histone H3 (Abcam, catalog no. ab1791) are commercially available.

ChIP-Seq

ChIP experiments were performed with HEK293 cells using procedures described previously (69). ChIP-Seq libraries were constructed with a Kapa Hyper Prep Kit (Kapa Biosystems, catalog no. KK8504) and NEBNext multiplex oligos for Illumina (index primer set 1, New England Biolabs, catalog no. 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 sequencing experiments (BGI, Shenzhen, China). Sequencing quality was evaluated with FastQC software and aligned to human genome hg38 using STAR Aligner. FPKM values were quantified using Cuffdiff (v2.0.2). FPKM values were added to a pseudovalue 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 extended to the average fragment size. Genome profile files were generated with IGV (integrative genomics viewer) 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 (Table S1) locus-specific methylation analysis, purified genomic DNA was treated with an EpiTect Bisulfite Kit (Qiagen, catalog no. 59104), and the converted DNA was amplified using locus-specific nested PCR primers (Table S2). 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* frameshift mutant, *IL-32* promoter CRE mutant, and *TET* frameshift mutant cell lines, guide RNA sequences (Table S3) 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 included the following: IL-32 forward, TGGCGGCTTATTATGAGGAGC; IL-32 reverse, CTCGGCACCGTAATCCATCTC; GAPDH forward, CTGGGCTACACTGAGCACC; GAPDH reverse, AAGTGGTCGTTGAGGGCAATG.

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