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Bclaf1 regulates c-FLIP expression and protects cells from TNF-induced apoptosis and tissue injury

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Abstract

TNF stimulation generates pro-survival signals through activation of NF-κB that restrict the build-in death signaling triggered by TNF. The competition between TNF-induced survival and death signals ultimately determines the fate of a cell. Here, we report the identification of Bclaf1 as a novel component of the anti-apoptotic program of TNF. Bclaf1 depletion in multiple cells sensitizes cells to TNF-induced apoptosis but not to necroptosis. Bclaf1 exerts its anti-apoptotic function by promoting the transcription of *CFLAR*, a caspase 8 antagonist, downstream of NF-κB activation. Bclaf1 binds to the p50 subunit of NF-κB, which is required for Bclaf1 to stimulate *CFLAR* transcription. Finally, in Bclaf1 siRNA administered mice, TNF-induced small intestine injury is much more severe than in control mice with aggravated signs of apoptosis and pyroptosis. These results suggest Bclaf1 is a key regulator in TNF-induced apoptosis, both *in vitro* and *in vivo*.

Keywords apoptosis; Bclaf1; c-FLIP; TNF
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Introduction

TNF is an important pro-inflammatory cytokine involved in many inflammatory diseases (Kriegler *et al*, 1988; Kontoyiannis *et al*, 1999; Feldmann & Maini, 2003; Taylor & Feldmann, 2009). TNF promotes inflammation through NF-κB activation, or alternatively by inducing cell death, depending on cell types and conditions (Varfolomeev & Ashkenazi, 2004; Lee *et al*, 2019). In most cell types, TNF stimulation does not induce cell death, but activates NF-κB resulting in transcriptional upregulation of many pro-inflammatory and anti-apoptotic genes (Chu *et al*, 1997; Wang *et al*, 1998, 2008; Kreuz *et al*, 2001). In contrast, some other cell types are sensitive to TNF-induced apoptosis, for example intestinal epithelial cells (Tracey *et al*, 1986; Takahashi *et al*, 2014). However, it is still not clear how TNF-induced apoptosis is regulated, resulting in differential cellular responses.

TNF engagement of TNFR1 results in the rapid assembly of a signal molecule complex, known as complex I (Micheau & Tschopp, 2003), at the intracellular region of TNFR1, which contains RIPK1, TRADD, TRAF2, and cIAP1/2 (Chen & Goeddel, 2002; Muppidi *et al*, 2004; Karin, 2006). Within the complex, cIAP1/2 mediates K63 ubiquitination of RIPK1, which serves as a platform to further recruit TAK1 and TAB1/TAB2, resulting in TAK1 activation and subsequent activation of NF-κB and MAPK family (Ea *et al*, 2006; Li *et al*, 2006; Wu *et al*, 2006; Bertrand *et al*, 2008). Once activated, the NF-κB heterodimer p50/p65 translocates to the nucleus, where it induces transcription of multiple pro-inflammatory genes as well as anti-apoptosis genes such as *CFLAR* (Kreuz *et al*, 2001; Micheau *et al*, 2001). The formation of complex I is dynamic and transient with TRADD being quickly released into the cytoplasm forming a cytosolic TRADD/FADD/caspase 8 complex, named complex IIa (Micheau & Tschopp, 2003; Dickens *et al*, 2012). The apoptotic potential of complex IIa is suppressed by c-FLIP as it hetero-dimerizes with caspase 8 and blocks caspase 8 activation (Irmler *et al*, 1997). Thus, in most cell types under unperturbed conditions, TNF does not trigger apoptosis.

Under certain conditions, TNF stimulation can induce apoptosis or necroptosis. It is known that TNF in combination with cycloheximide (CHX) treatment can induce apoptosis, as CHX treatment blocks the synthesis of pro-survival proteins induced by NF-κB (Kreuz *et al*, 2001; Micheau *et al*, 2001; Chang *et al*, 2006). Without the inhibition from c-FLIP, caspase 8 is activated and promotes apoptosis. However, if caspase activity is simultaneously inhibited, cells would turn to the necroptotic machinery by activating RIPK1 and RIPK3, which in turn promotes the phosphorylation of MLKL to execute necroptosis (He *et al*, 2009; Sun *et al*, 2012; Ofengeim & Yuan, 2013; Wang *et al*, 2014; Pasparakis & Vandenabeele, 2015; Newton *et al*, 2019). Recent studies reveal that inhibition of TAK1 or cIAP1/2 also sensitize cells to apoptosis, but in a RIPK1 kinase activity-dependent manner (Podder *et al*, 2019; Zhang *et al*, 2019a). By contrast, the apoptosis induced by TNF/CHX is RIPK1 independent (Dziedzic *et al*, 2018; Zhang *et al*, 2019b).

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c-FLIP is a key protein that determines life and death of cells receiving TNF stimulation (Irmler *et al*, 1997; Kreuz *et al*, 2001). Tissue-specific knockout of *Cflar* has revealed a critical role of c-FLIP in maintaining homeostasis of various tissues, including immune cells, intestine, liver, and skin, many of which are related to loss of protection to the TNF-induced cell death due to *Cflar* deficiency (Piao *et al*, 2012; Panayotova-Dimitrova *et al*, 2013). Because c-FLIP exerts its anti-apoptotic function by blocking caspase 8 activation, its cellular level, particularly relative to that of caspase 8, is the key to determine survival and death of a cell (Chang *et al*, 2002; Muendlein & Jetton, 2020). c-FLIP is a short-lived protein and is transcriptionally upregulated by NF-κB when cells are stimulated by TNF (Kreuz *et al*, 2001; Micheau *et al*, 2001). Thus, whether or not TNF stimulation induces enough c-FLIP to inhibit caspase 8 activation in complex IIa may determine the sensitivity of a cell to TNF. However, little is known about the regulation of c-FLIP induction by TNF stimulation in addition to being a NF-κB target.

Bclaf1 (Bcl-2-associated transcription factor 1) is a multifunctional protein, involved in various biological processes, including apoptosis (Kasof *et al*, 1999; Lamy *et al*, 2013), DNA damage response (Lee *et al*, 2012b; Vohhodina *et al*, 2017), cancer progression (Zhou *et al*, 2014; Shao *et al*, 2016, 2020), and viral defense (Ziegelbauer *et al*, 2009; Lee *et al*, 2012a). It has been shown to positively regulate the interferon signaling (Qin *et al*, 2019). However, the role of Bclaf1 in TNF signaling has not been investigated.

In this study, we report that Bclaf1 binds to p50 and enhances the transcriptional upregulation of *CFLAR* in response to TNF stimulation to prevent caspase 8 activation and TNF-induced apoptosis. Bclaf1-depleted cells are sensitized to apoptosis induced by TNF through the suppression of c-FLIP expression. Thus, Bclaf1 is an important component of the anti-apoptotic program of TNF signaling downstream of NF-κB activation that can stimulate c-FLIP expression and inhibit apoptosis.

Results

Bclaf1 deficiency sensitizes cells to apoptosis in response to TNF/CHX

In our previous study, we found Bclaf1 not only functions as a transcription activator in the interferon signaling pathway, but also can bind to genomic DNA without any treatment (Shao *et al*, 2016; Qin *et al*, 2019). In an effort to examine what other pathways Bclaf1 might be involved, we performed RNA-seq analysis of Bclaf1 knockout (KO) HeLa cells in comparison with that of wild-type (WT) cells and revealed TNF signaling pathway as a potential one (Fig EV1A). The transcriptions of many genes related to TNF signaling pathway were significantly altered in KO cells (Fig EV1B). This prompted us to examine whether Bclaf1 played a role in TNF-mediated functions.

We first determined whether Bclaf1 is involved in TNF-induced apoptosis. Two Bclaf1 KO HeLa cell lines generated by CRISPR/cas9 using distinct guide RNAs, or cells in which Bclaf1 was knocked down using two different siRNAs, as well as control cells were treated with TNF (10 ng/ml) and cycloheximide (CHX, 1 μg/ml) for 12 h to induce conventional apoptosis and then subjected to Annexin V/7AAD staining. Analysis of percent of Annexin V positive cells or Annexin $V^+/7AAD^-$ cells showed that TNF^+CHX-induced apoptosis was much more severe in Bclaf1 knockout or knockdown cells than in their respective control cells, including HeLa, MEFs, and HepG2 (Figs 1A–C and D–F). Of note, the condition we used to induce apoptosis is rather mild (Fig EV1C), and blocking apoptosis by adding z-VAD nearly completely abolished Annexin V positive cells (Fig EV1J). Consistently, Western blotting analysis of caspase 3 and PARP cleavage demonstrated more apoptosis occurred in Bclaf1 KO or knockdown cells (Figs 1A–C and EV1G). To determine the involvement of Bclaf1 in the RIPK1-dependent apoptosis induced by TNF, we treated MEFs and HeLa with TNF and SM-164, a cIAP1/2 inhibitor, and found that Bclaf1 knockdown did not affect the apoptosis induced by TNF plus SM-164 in both cell types (Fig EV1H and I). Taken together, these results suggest that Bclaf1 deficiency sensitizes cells to TNF-induced apoptosis in a RIPK1-independent manner.

We then examined if Bclaf1 has any relationship with TNF-induced necroptosis, as TNF-induced apoptosis and necroptosis are closely related. Bclaf1 was knocked down in MEFs using siRNAs followed by treatment with TNF (10 ng/ml) /CHX (1 μg/ml) /z-VAD (20 μM) (TCZ) or TNF (10 ng/ml) /SM164 (100 nM)/z-VAD (20 μM) (TSZ) to induce necroptosis. In contrast to its involvement in TNF-induced apoptosis, Bclaf1 knockdown did not show any impact on TNF-induced necroptosis, indicated by both percent of cell death and MLKL phosphorylation (Figs 1D and E, and EV1J), a marker for necroptosis.

Collectively, these data indicate that Bclaf1 negatively regulates TNF-induced apoptosis without affecting TNF-induced necroptosis.

Bclaf1 regulates apoptosis through c-FLIP

To explore how Bclaf1 regulates apoptosis, we first examined the role of Bclaf1 in the TNF-induced activation of NF-κB and MAPKs, which are critical events leading to transcriptional induction of pro-inflammatory cytokines and anti-apoptotic proteins following TNFR1 engagement. We found that the kinetics of TNF-induced phosphorylation of p65, P38, JNK, or ERK were not different in HeLa Bclaf1 knockout or knockdown cells compared that of control cells (Fig 2A and B). Further nucleus-cytoplasm separation revealed that the TNF-induced p65 nuclear translocation was not affected by Bclaf1 knockout or knockdown in HeLa cells (Fig 2C and D). These results suggest that Bclaf1 deficiency does not affect the TNF-induced activation of NF-κB and MAPKs.

We then examined the expression of key proteins involved in the apoptotic complex II formation following TNF treatment, and found no difference in the protein levels of TRADD, FADD, and RIPK1 between Bclaf1 knockdown HeLa and control cells. However, the induction of c-FLIP by TNF, which is a prominent and potent caspase 8 antagonist, including both long (c-FLIP-L) and short (c-FLIP-S) isoforms were significantly impaired in Bclaf1 knockdown cells (Fig 3A).

Further analysis of the formation of complex IIa in HeLa and MEFs treated with TNF or TNF/CHX for 4 h by immunoprecipitation using an anti-FADD antibody revealed that less c-FLIP was present in the complex IIa in Bclaf1 knockdown cells compared to that of controls (Fig 3B–D). Consistent with the inhibitory effect of c-FLIP on caspase 8 activation, more processed caspase 8 (p43) were detected in the complex IIa and the cell lysates of Bclaf1

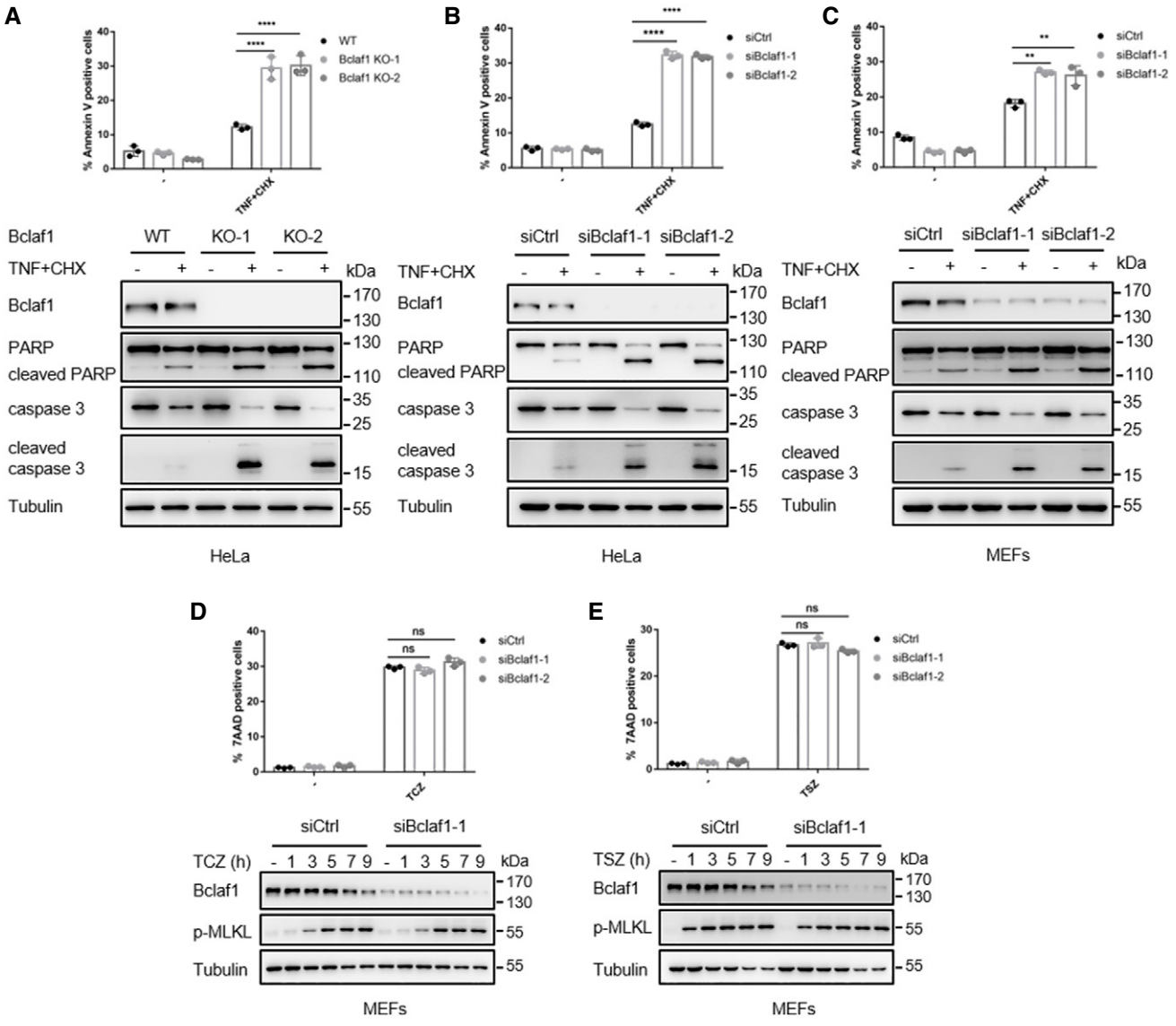

Figure 1. Bclaf1 deficiency sensitizes cells to apoptosis in response to TNF^{+}CHX.

A Bclaf1 wild-type (WT) and two Bclaf1 knockout HeLa cell lines (KO-1 and KO-2) were pretreated with DMSO or cycloheximide (CHX, 1 μg/ml) for 30 min, and then treated with TNF (10 ng/ml) together with CHX for 12 h. Afterward, the cells were subjected to Annexin V/7AAD staining followed by flow cytometry analysis, all Annexin V positive cells were counted for analysis, or protein extractions followed by Western blotting analysis.

B, C HeLa (B) or primary MEFs (C) were transfected with control (siCtrl) or two siRNAs against Bclaf1 (siBclaf1-1 and siBclaf1-2) and then treated and analyzed as described in A.

D, E MEFs transfected with control (siCtrl) or siBclaf1-1 and/or siBclaf1-2 siRNAs were pretreated with CHX (1 μg/ml)/z-VAD (20 μM) (D) or SM-164 (100 nM)/z-VAD (20 μM) (E) for 30 min, and then added TNF (10 ng/ml) for indicated times. The cells were subjected to Annexin V/7AAD staining after 9 h followed by flow cytometry analysis for 7AAD positive and Annexin V negative cells (up), or protein extractions and Western blotting analysis at indicated time periods (down).

Data information: Data are shown as mean ± SD. $n = 3$ biological replicates. ns, not significant; $^{**}P < 0.01$; $^{****}P < 0.0001$. One-way ANOVA test.

knockdown cells treated with TNF or TNF/CHX for 4 and 8 h (Figs 3B–D and EV2A and B), and more mature caspase 8 (p18) in the lysate of 12 h of treatment (Figs 3E and F and EV2C). Because c-FLIP is an unstable protein and known to be further destabilized upon TNF treatment (Chang *et al*, 2006), prolonged treatment with TNF/CHX diminishes c-FLIP. As expected, c-FLIP knockdown also increased TNF-mediated caspase 3 activation and apoptosis in MEFs, and the enhancement is even higher than that of Bclaf1 knockdown cells, which correlates with more profound c-FLIP reduction in c-FLIP knockdown cells (Fig 3G and H). Moreover, Bclaf1 knockdown-mediated elevation of caspase 3 and 8 activation was abolished upon Flag-FLIP overexpression (Fig 3I).

Taken together, these data suggest that Bclaf1 deficiency might impact the downstream events of NF-κB and MAPKs following TNF stimulation, and that the protective role of Bclaf1 might be related to c-FLIP induction.

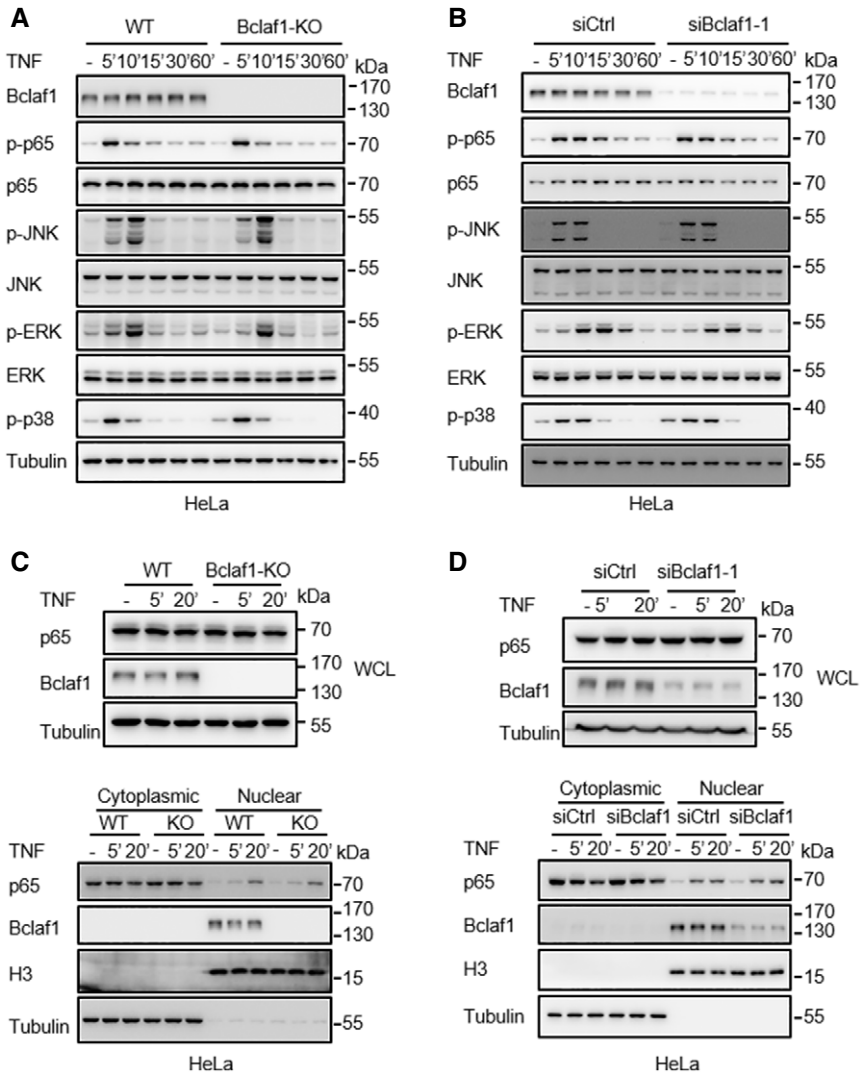

Figure 2. Bclaf1 deficiency does not affect TNF-induced activation of NF-κB and MAPKs.

A, B Cell lysates of HeLa Bclaf1 WT and KO cells (A), and HeLa cells transfected with siCtrl or siBclaf1-1 (B) treated with TNF (10 ng/ml) for the indicated time periods were analyzed by Western blotting with the indicated antibodies.

C, D HeLa Bclaf1 WT and KO cells (C), and HeLa cells transfected with siCtrl or siBclaf1-1 (D) were treated with TNF (10 ng/ml) for the indicated time periods, and then subject to nucleus-cytoplasm fractionation as described in materials and methods followed by Western blotting. Tubulin and histone H3 were used as loading controls for cytoplasmic and nuclear fractions, respectively.

Bclaf1 upregulates the transcription of *CFLAR*

We then explored how Bclaf1 upregulated c-FLIP. We first confirmed in multiple cell lines that Bclaf1 knockout or knockdown indeed impaired the TNF-induced upregulation of c-FLIP including HeLa and MEFs. Western blotting and RT–PCR analysis revealed that Bclaf1 deficiency reduced c-FLIP induction at both protein (Fig 4A–C) and mRNA (Fig 4D–F) levels, suggesting that Bclaf1 might regulate c-FLIP at the transcription level. Indeed, Bclaf1 knockdown did not impact the half-life of c-FLIP excluding the possibility of Bclaf1 stabilizing c-FLIP (Fig EV3A). Because cIAP1/2 are two other pro-survival molecules upregulated by NF-κB upon TNF treatment, we examined if Bclaf1 also regulated these two genes. RT–PCR showed that Bclaf1 knockdown did not cause any change to the mRNA levels of cIAP1/2 (Fig EV3B). Taken together, these results suggest Bclaf1 appears to regulate c-FLIP induction at the transcription level with certain specificity.

We next examined the possibility that Bclaf1 might directly promote *CFLAR* transcription as Bclaf1 has been reported to be a transcriptional regulator. We cloned the *CFLAR* promoter (−1,179~+281) and fused it with a luciferase reporter, referred as FL. Compared with the control, F-Bclaf1 expression significantly increased the luciferase activity of FL reporter (Fig 4G). We further narrowed down the Bclaf1 responsive region to P3 (−288 to +281) in the promoter of *CFLAR*. F-Bclaf1 stimulates both FL and P3 reporter dose-dependently (Fig 4G). To examine whether Bclaf1 can localize in the promoter region of *CFLAR* in cells, we utilized an engineered HeLa cell line, HeLa-Flag-Bclaf1, in which *Flag* was

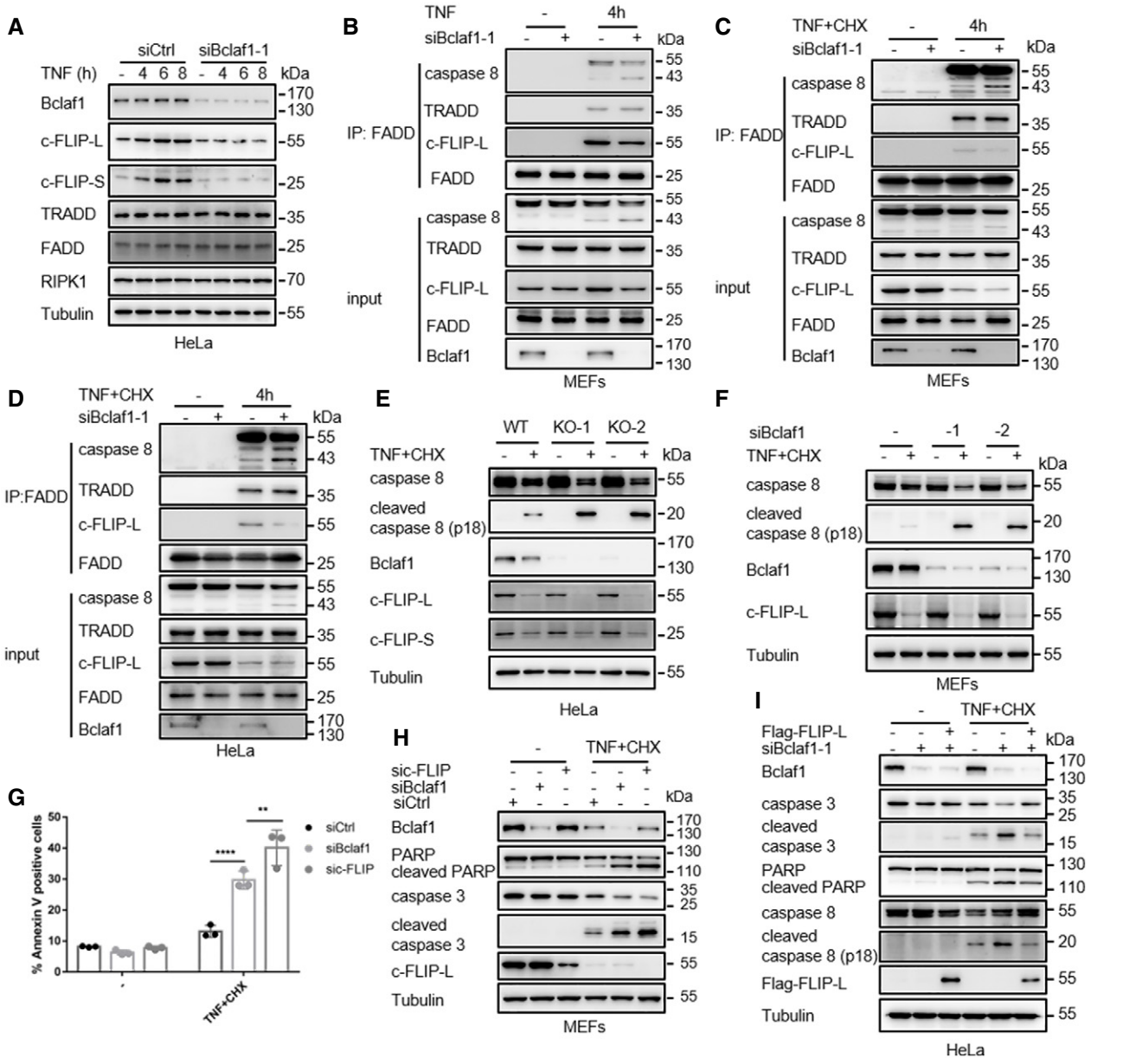

Figure 3. Bclaf1 deficiency inhibits c-FLIP expression and activates caspase 8.

A HeLa cells transfected with siCtrl or siBclaf1-1 were treated with TNF (10 ng/ml) for the indicated time periods followed by Western blotting analysis with the indicated antibodies.

B MEFs treated with TNF (10 ng/ml) for 4 h were subject to FADD immunoprecipitation, and then analyzed by Western blotting.

C, D MEFs (C) and HeLa (D) cells pretreated with CHX (1 μg/ml) for 30 min and then stimulated with TNF (10 ng/ml) in the presence of CHX for indicated times were subject to FADD immunoprecipitation, and then analyzed by Western blotting.

E, F Bclaf1 WT, KO-1, and KO-2 HeLa cells (E) or MEFs transfected with siCtrl or siBclaf1-1 and siBclaf1-2 (F) were pretreated with CHX (1 μg/ml) for 30 min and then stimulated with TNF (10 ng/ml) in the presence of CHX for 12 h followed by Western blotting analysis.

G, H HeLa cells transfected with siRNAs against Bclaf1 or c-FLIP were pretreated with CHX (1 μg/ml) for 30 min and then stimulated with TNF (10 ng/ml) in the presence of CHX for 12 h followed by Annexin V/7AAD staining and flow cytometry analysis (G), or protein extractions followed by Western blotting analysis (H).

I After transfection with siBclaf1-1 for 12 h, the HeLa cells were transfected with Flag-FLIP for 24 h, and then treated as described in H followed by Western blotting analysis.

Data information: Data are shown as mean ± SD. $n = 3$ biological replicates. $^{**}P < 0.01$; $^{****}P < 0.0001$. One-way ANOVA test.

inserted into the *Bclaf1* gene, expressing a N-terminal tagged F-Bclaf1 endogenously, and performed chromatin immunoprecipitation (CHIP) assay using an anti-Flag antibody. Bclaf1 was found to bind to the region of −200~−40 within the promoter of *CFLAR*, and TNF treatment recruited more Bclaf1 to the region (Fig 4H and I).

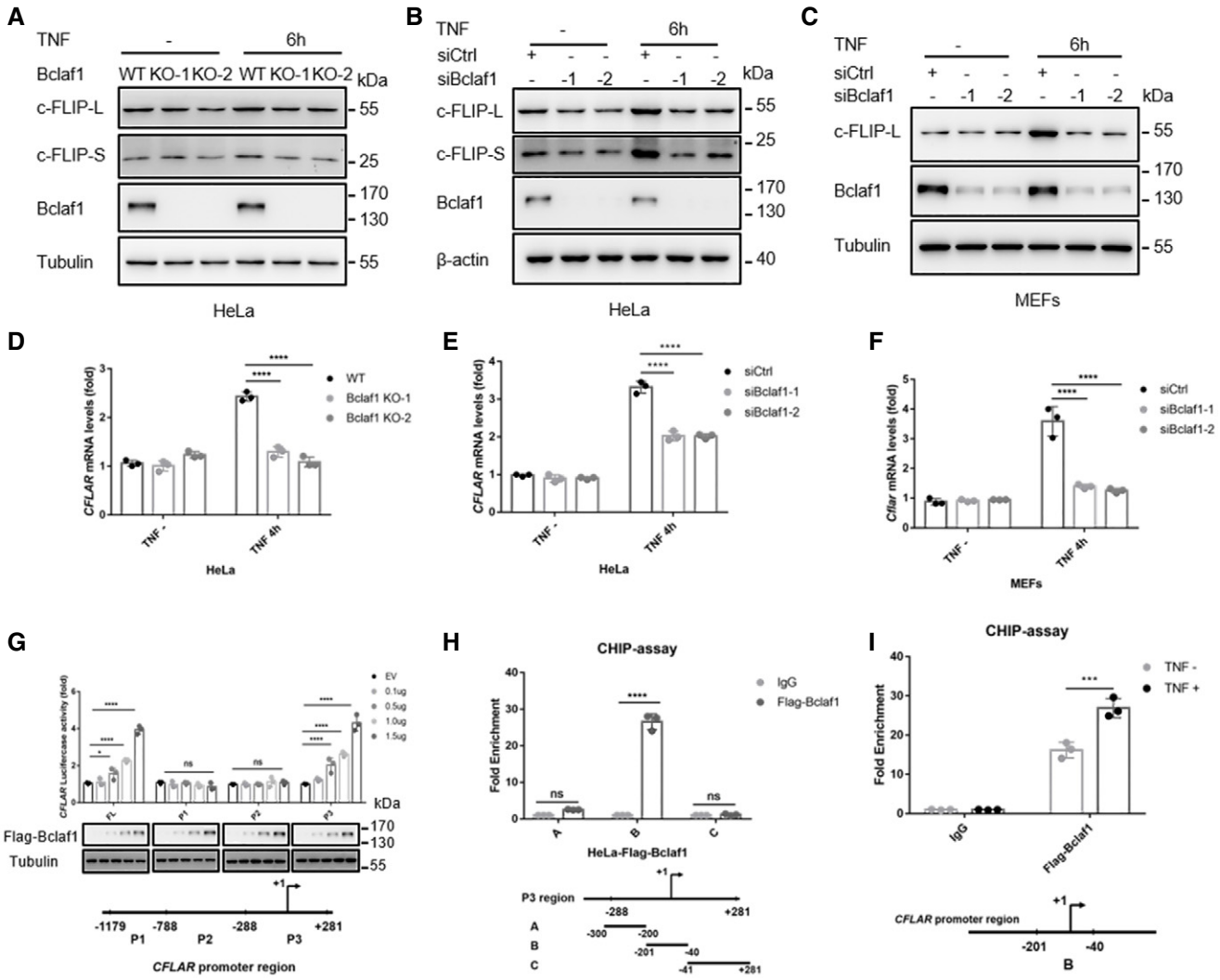

Figure 4. Bclaf1 upregulates the transcription of *CFLAR*.

A–F Bclaf1 WT, KO-1, and KO-2 HeLa cells (A and D), as well as HeLa (B and E) and primary MEFs (C and F), which were transfected with siCtrl or siBclaf1-1 and siBclaf1-2 were treated with TNF (10 ng/ml) for 6 h followed by Western blotting analysis (A–C), or for 4 h followed by total RNA extraction and RT–PCR analysis (D–F).

G *CFLAR* full-length (FL) promoter ranging from −1,179 to +281 and three promoter regions P1 (−1,179 to −788), P2 (−788 to −288), and P3 (−288 to +281) were fused with a luciferase reporter and then co-transfected with an increase dose of Bclaf1 in HeLa-Bclaf1-KO cells. At 24 h after transfection, the cells were collected and luciferase activity was measured. The experiments were performed in triplicates with a Renilla reporter in the transfection mixture for normalization and the activity in the control was arbitrarily set to 1.

H, I CHIP analysis of the binding of Bclaf1 to the promoter of *CFLAR* was performed in HeLa-Flag-Bclaf1 cells treated without (H) or with TNF (I). The regions amplified by RT–PCR were delineated.

Data information: Data are shown as mean ± SD. $n = 3$ biological replicates. *$P < 0.05$; ***$P < 0.001$; ****$P < 0.0001$. One-way ANOVA test.

Bclaf1 upregulates *CFLAR* through p50

Because Bclaf1 mainly localizes in the nucleus and has been shown to regulate transcription by interacting with other transcription factors, we considered the possibility that Bclaf1 might interact and cooperate with a transcription factor activated by TNF to enhance *CFLAR* transcription. To this end, we screened a panel of transcription factors that are activated by TNF stimulation or known to interact with Bclaf1, including NF-κB factor p50 and p65, AP-1, C/EBPα as well as C/EBPβ, for the ability to regulate Bclaf1-mediated transcription. siRNAs targeting each transcription factor were transfected into HeLa-Bclaf1-KO cells before co-transfection with F-Bclaf1 and FL *CFLAR* reporter. In this screen, we identified p50 as a potential regulator of F-Bclaf1 response, as knockdown of p50 reduced F-Bclaf1-induced reporter activities, but others did not (Fig 5A). Consistently, co-transfection of p50 and Bclaf1 into cells generated a dramatic synergistic effect on *CFLAR* reporter activity (Fig 5B), whereas co-transfection of p65 and Bclaf1 showed a very mild effect (Fig EV4A). Moreover, knockdown of Bclaf1 in p65 siRNA-transfected cells was still able to inhibit TNF-induced transcription of *CFLAR* (Fig EV4B).

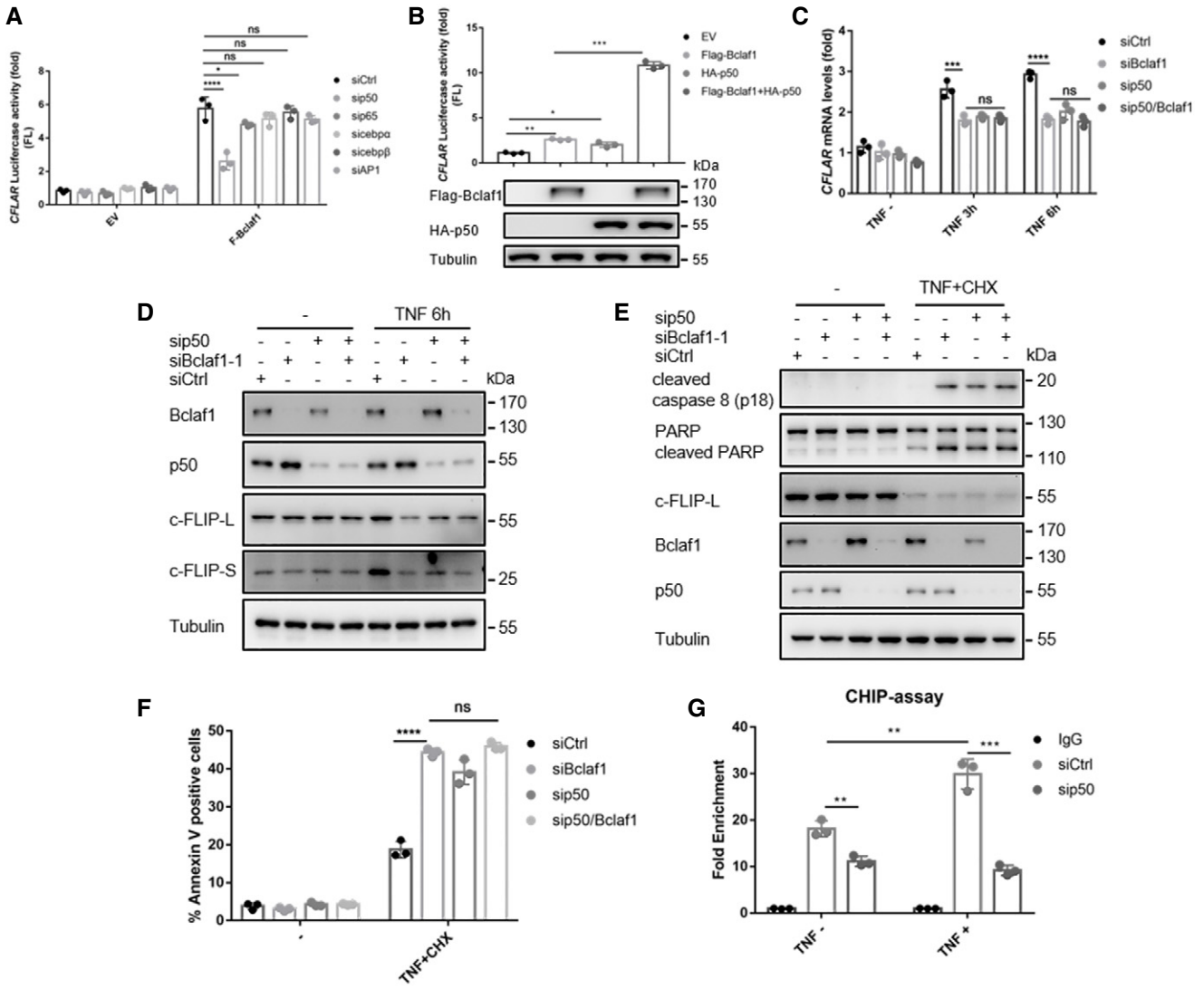

Figure 5. Bclaf1 upregulates *CFLAR* through p50.

A HeLa-Bclaf1-KO cells were transfected with the indicated siRNAs, and then transfected with *CFLAR* promoter and Bclaf1. The luciferase activity was measured as described before.

B *CFLAR* promoter were co-transfected with Bclaf1 or/and p50 into HeLa-Bclaf1-KO cells, and the luciferase activity was measured as described before.

C HeLa cells were transfected with siRNAs against Bclaf1 or p50. 24 h post-transfection, and the cells were treated with TNF (10 ng/ml) for indicated hours. The total RNA was extracted and subjected to RT–PCR analysis.

D HeLa cells were transfected with siCtrl or siRNA against Bclaf1 or p50, treated with TNF (10 ng/ml) for 6 h followed by Western blotting analysis.

E, F HeLa cells were transfected with siCtrl or siRNA against Bclaf1 or p50 were pretreated with DMSO or CHX (1 μg/ml) for 30 min, and then treated with TNF (10 ng/ml) for 12 h. Cells were lysed and then analyzed by Western blotting (E) or collected and subjected to Annexin V/7AAD staining followed by flow cytometry analysis, all Annexin V positive cells were counted for analysis (F).

G CHIP analysis of the *CFLAR* promoter in HeLa-Flag-Bclaf1 cells. Immunoprecipitation was performed using anti-Flag and control IgG antibodies followed by RT–PCR analysis. Data information: Data are shown as mean ± SD. $n = 3$ biological replicates. ns, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. One-way or two-way ANOVA test.

To confirm the involvement of p50 in Bclaf1-mediated *CFLAR* transcription and cell protection against apoptosis upon TNF treatment, we transfected siRNAs for Bclaf1 and p50 individually or in combination into HeLa cells and examined their effects on TNF-induced c-FLIP upregulations and apoptosis. RT–PCR and Western blotting analysis showed that knockdown of either Bclaf1 or p50 reduced both mRNA and protein levels of c-FLIP, but no additive effects were observed when both were knocked down simultaneously (Fig 5C and D). Similarly, co-knockdown of Bclaf1 and p50 did not further enhance the apoptosis induced by knocking down either protein (Fig 5E and F). These results indicated that p50 and Bclaf1 appear to be mutually dependent on each other to regulate the TNF-induced c-FLIP upregulation and apoptosis. In addition, we found the recruitment of Bclaf1 onto the *CFLAR* promoter depended

on p50, at least partially, as the CHIP assay revealed that the binding between Bclaf1 and *CFLAR* promoter was greatly reduced by p50 knockdown in both resting and TNF-stimulated conditions (Fig 5G).

Bclaf1 interacts with p50

We next determined the interaction between Bclaf1 and p50 by co-immunoprecipitation assay. HeLa-Flag-Bclaf1 and WT HeLa cells treated with or without TNF for 2 h were separated into the cytoplasmic and the nuclear fraction. Endogenous F-Bclaf1 complex in each fraction was immunoprecipitated with an anti-Flag antibody and analyzed by Western blotting. As shown in Fig 6A, both p50 and p65 were present in the nuclear Bclaf1 immunocomplex in both untreated and treated cells with that of the latter having greatly more p50. To examine whether p50 binds to Bclaf1 as a p50/p65 heterodimer or independently of p65, we knocked down p65 using siRNAs and examined the interaction of p50 with Bclaf1 in the nucleus. As expected, p65 knockdown reduced nuclear translocation of p50, but p50 was still able to interact with Bclaf1 suggesting that p50 can bind to Bclaf1 without the involvement of p65 (Fig 6B).

To determine the region of Bclaf1 involved in mediating its binding with p50, we overexpressed Flag-tagged full-length and deletion mutants of Bclaf1 with that of HA-tagged p50 in 293T cells and

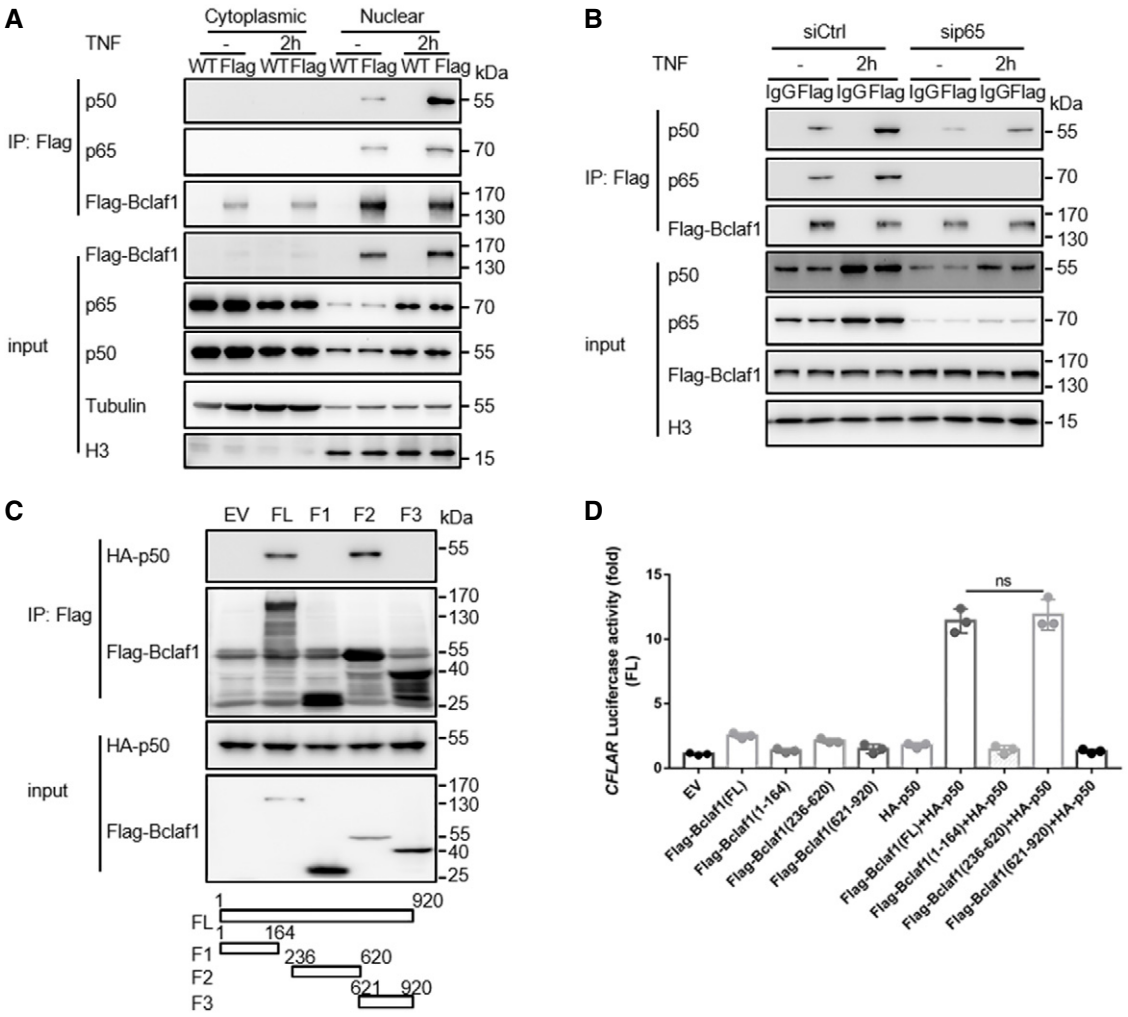

Figure 6. Bclaf1 interacts with p50.

A HeLa-Flag-Bclaf1 and Bclaf1 WT HeLa cells treated with or without TNF (10 ng/ml) for 2 h were separated into the cytoplasmic and the nuclear fraction. Endogenous Flag-Bclaf1 complex in each fraction was immunoprecipitated with M2 beads and analyzed by Western blotting.

B HeLa-Flag-Bclaf1 cells transfected with siCtrl or sip65 were treated with TNF (10 ng/ml) for 2 h. The nuclear fraction was isolated and subjected to immunoprecipitation with an anti-Flag or control IgG antibody, followed by Western blotting analysis.

C 293T cells transfected with the indicated plasmids were immunoprecipitated with the anti-Flag M2 beads followed by Western blotting analysis. A diagram of full-length Bclaf1 and its deletion mutants are presented.

D *CFLAR* FL promoter was co-transfected with p50 and Bclaf1 FL or its individual deletion mutant into HeLa-Bclaf1-KO cells. The experiments were performed in triplicate with a Renilla reporter in the transfection mixture for normalization.

Data information: Data are shown as mean ± SD. $n = 3$ biological replicates. ns, not significant. One-way ANOVA test.

analyzed their interactions by co-immunoprecipitation. We found that only the middle region (F2) of Bclaf1 could bind to p50 (Fig 6C). Co-transfection of p50 with each Bclaf1 fragment together with *CFLAR* reporter revealed that only the middle region of Bclaf1 showed synergy with p50 in promoting *CFLAR* reporter activity as that of FL Bclaf1 (Fig 6D). Thus, p50 interacts with the middle region of Bclaf1, which is required for transcriptional activation of *CFLAR*.

Bclaf1 protects intestinal epithelia of mice from the TNF-induced damage

To evaluate whether Bclaf1 plays a protective role against TNF-induced tissue injury *in vivo*, we generated Bclaf1 knockdown mice by injecting control siRNAs or siRNAs against Bclaf1 into C57BL/6 mice as described previously (Qin *et al*, 2019). After 4 days, all the mice were treated with mTNF for 2 h before being sacrificed for tissue isolations (Fig 7A). Western blotting and immunohistochemical analysis showed that Bclaf1 was silenced effectively in small intestinal epithelia of the mice treated with Bclaf1 siRNAs (Fig 7B and G). Bclaf1 mainly localized in columnar epithelial cells and lamina propria cells of the small intestine in control mice (Fig 7B).

Hematoxylin-and-eosin staining showed that both control and Bclaf1 siRNAs treatment did not cause any visible morphological changes to the small intestines of the mice. As expected, 2 h after mTNF injection, discernible damage to the villi of the small intestine in the control mice was observed. Remarkably, this damage was drastically exacerbated in Bclaf1-depleted mice (Fig 7C). Measurement of villus heights and crypt depths showed that mTNF-induced much greater reduction in the length of the villi and more increased depth of the crypts in the Bclaf1 knockdown mice compared with that of controls, confirming that TNF-induced much more severe damage to the small intestines in the Bclaf1 knockdown mice (Fig 7D). Both immunohistochemical and Western blotting analysis showed that more cleaved caspase 3 and/or PARP were detected in the Bclaf1 knockdown mice treated with mTNF compared with controls (Fig 7E and G). Phospho-RIPK3 (p-RIPK3) and phospho-MLKL (p-MLKL) staining showed no difference in mTNF-induced necroptotic pathway between control and Bclaf1 knockdown mice (Figs 7F and EV5D and E). The specificity of p-RIPK3 staining was validated by using two different anti-pPIPK3 antibodies, ab222320 (Fig 7F) and ab205421 (Fig EV5D) and by a time course dependency experiment (Fig EV5B and C). Because it was reported that GSDME could be cleaved and activated in small intestinal epithelium by caspase 3, inducing pyroptotic cell death (Wang *et al*, 2017b), we examined whether mTNF treatment induces GSDME cleavage in small intestines and found GSDME was indeed cleaved, and that the cleavage was enhanced in Bclaf1 knockdown mice (Fig 7G).

To further examine the role of Bclaf1 in human intestinal epithelial cells in response to TNF treatment, HIEC-6 cells were transfected with siCtrl or siBclaf1. In contrast to the other cell lines we examined before, which required both TNF and CHX to trigger apoptosis even for Bclaf1 knockdown cells, TNF treatment alone could induce HIEC-6 cells transfected with siBclaf1 to undergo substantial apoptosis despite no apoptosis was observed in control cells, shown by both Annexin V positive cell analysis, and caspase 3 and PARP cleavage (Fig 7H and I). GSDME cleavage was also observed in Bclaf1 knockdown cells. Analysis of c-FLIP induction in HIEC-6 cells demonstrated that TNF-induced c-FLIP induction was inhibited in Bclaf1 knockdown cells at both protein and mRNA levels, with caspase 8 cleavage taken place, consistent with the previous data that Bclaf1 deficiency reduced c-FLIP induction and promoted caspase 8 cleavage (Fig 7J and K).

Together, these data indicate that Bclaf1 deficiency greatly increased the death of intestinal epithelium *in vivo* and HIEC-6 cells *in vitro* caused by TNF, probably involving both apoptosis and pyroptosis, and that Bclaf1 is a critical factor in regulating TNF-induced cell death and tissue injury of intestinal epithelium.

Discussion

It is well established that TNF-induced upregulation of c-FLIP through NF-κB is a main mechanism of cells to inhibit RIPK independent apoptosis (Kreuz *et al*, 2001; Dziedzic *et al*, 2018). However, it is not clear if other factors are involved between NF-κB activation and *CFLAR* transcription. In this study, we demonstrate Bclaf1 participates in the TNF-induced transcription of *CFLAR* by binding to and stimulating the p50 NF-κB subunit. Our results indicate that the cellular level of Bclaf1 is an important factor to determine the sensitivity of a cell to TNF-induced apoptosis, and likely contributes to TNF-mediated pathogenicity.

TNF engagement simultaneously activates survival and death signals. c-FLIP induced by NF-κB is considered as the most prominent anti-apoptotic factor (Kreuz *et al*, 2001), and its relative level to that of caspase 8 may play a very important role in determining the fate of a cell. In this study, we found Bclaf1 positively regulates *CFLAR* transcription downstream of NF-κB activation (Fig 4G–I). In Bclaf1-depleted cells, the TNF-induced upregulation of *CFLAR* is significantly decreased despite that NF-κB p65 nuclear translocation remains normal (Figs 2C and D and 4A–C). The contribution of Bclaf1 to *CFLAR* upregulation relies on NF-κB, particularly p50, because Bclaf1-mediated *CFLAR* upregulation, apoptosis inhibition, and TNF-induced recruitment of Bclaf1 to the *CFLAR* promoter were all abolished upon p50 knockdown (Fig 5A, C and G). Bclaf1 binds to p50, which is required for transcriptional activation of *CFLAR* mediated by Bclaf1 and p50. Because p50 does not possess a transactivation domain, Bclaf1 gives its ability to induce *CFLAR* highly suggests that Bclaf1 can function as a transcriptional activator. Indeed, we have previously shown that Bclaf1 can bind with STAT2 and enhance interferon-stimulated gene transcriptions (Qin *et al*, 2019).

Bclaf1 was first identified as a transcriptional repressor as it represses a reporter gene when fused to the GAL4-DNA binding domain (Kasof *et al*, 1999). Subsequent studies have indicated that Bclaf1 can function either as an activator or a repressor depending on biological settings. For instance, Bclaf1 promotes transcription of p53 and inflammatory cytokines in response to DNA damage, but represses gene expression in response to RAG-mediated DNA break in early B cells (Liu *et al*, 2007; Soodgupta *et al*, 2019). Bclaf1 has also been identified as a component of the RNA splicing complex and promotes the stability of transcripts by regulating pre-mRNA splicing (Savage *et al*, 2014; Zhou *et al*, 2014; Vohhodina *et al*, 2017). The mechanism by which Bclaf1 enhances gene transcription is not clear. Given that the middle region of Bclaf1 is required to stimulate *CFLAR* transcription, Bclaf1 involved RNA processing

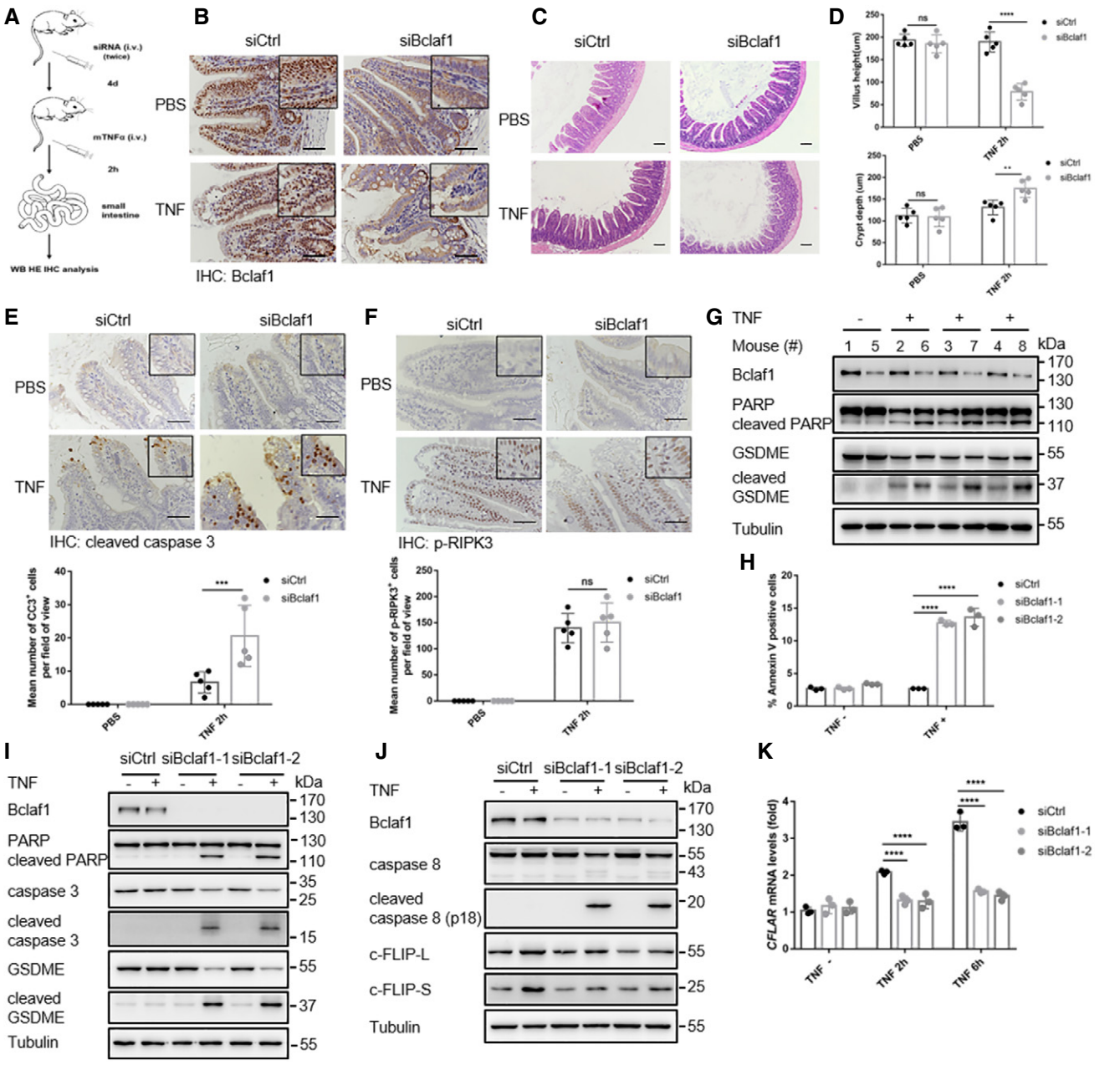

Figure 7. Bclaf1 protects intestinal epithelia of mice from the TNF-induced damage.

A Diagram showing the procedure used for siRNAs mediated knockdown of Bclaf1 in mice and mTNF treatment.

B–G siRNAs against Bclaf1 and control siRNAs (siCtrl) mixed with in vivo-jetPEI (Polyplus) were injected into C57BL/6 mice. The mice were then treated with mTNF for 2 h before sacrifice. The small intestines were excised and processed for immunohistochemical staining for Bclaf1 (B), cleaved caspase 3 (E), phosphorylated RIPK3 (F), and Hematoxylin-and-eosin staining (C) or Western blotting analysis (G). Bclaf1 knockdown efficiency is detected by immunohistochemical analysis (B). Hematoxylin-and-eosin staining showing the morphological change (C). Villus height and crypt depth were measured (D). $CC3^+$ and p-$RIPK3^+$ positive cells in five fields per intestine were quantified. Data are shown as mean ± SD. $n = 5$ mice for each group. ns, not significant; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. One-way ANOVA test. Scale bars: 50 μm in B, E, and F; 100 μm in C.

H–K HIEC-6 cells were transfected siCtrl or siBclaf1-1 and siBclaf1-2. Then, the cells were treated with TNF for 24 h were subjected to Annexin V/7AAD staining followed by flow cytometry analysis (H), or for protein extractions and Western blotting analysis (I and J), or treated for indicated hours followed by total RNA extraction and RT–PCR analysis (K). Data are shown as mean ± SD. $n = 3$ biological replicates. $^{****}P < 0.0001$. One-way ANOVA test.

activity could be excluded as it depends on its N-terminal RS rich region. However, whether Bclaf1 regulates transcription through an epigenetic mechanism, modulation of RNA polymerase activity or other mechanisms needs further investigation.

Although p50 recruits Bclaf1 to the *CFLAR* promoter, it appears that a portion of Bclaf1 binds the promoter constitutively. This binding could be mediated by other DNA binding proteins, or alternatively, Bclaf1 may directly bind a certain DNA sequence. In this

regard, we have shown previously in an *in vitro* binding assay that Bclaf1 can bind the interferon-stimulated response element (Qin *et al*, 2019). Perhaps Bclaf1 could bind certain short DNA sequence in the promoter region of *CFLAR*, and p50 may further recruit Bclaf1 to the region to enhance the interaction or as a part of NF-κB complex. In addition to *CFLAR*, some other NF-κB regulated gene transcription including *IL-8* are also regulated by Bclaf1 (Fig EV5).

The contribution of Bclaf1 to the upregulation of c-FLIP likely determines the sensitivity of certain cell types to TNF. c-FLIP is a very unstable protein with transcriptional control as one of pivotal mechanisms to regulate its cellular level, which in turn influences the sensitivity of a cell to caspase 8 involved apoptotic program. c-Myc has been shown to bind to and repress *CFLAR* promoter, rendering cancer cells less sensitive to TRAIL-mediated apoptosis (Ricci *et al*, 2004). Recently, it has been reported that the E3 ligase PELI1 inhibits TNF-induced apoptosis by modulating *CFLAR* expression levels transcriptionally (Wang *et al*, 2017a). In addition, C/EBPα, NFAT, and many more other transcription factors have been reported to regulate *CFLAR* expression (Zaichuk *et al*, 2004; Ueffing *et al*, 2008; Paz-Priel *et al*, 2009). Depending on the cellular contexts and cell types, c-FLIP upregulation is probably influenced by various factors. Bclaf1 appears to contribute c-FLIP upregulation in multiple cell lines, including HeLa (Fig 4A), MEFs (Fig 4B), HepG2 (Fig EV1F), and HIEC-6 (Fig 7J), and its protective role is particularly evident in intestine epithelial cells, as depleting Bclaf1 shifted HIEC-6 cells from survival to death and caused more severe damage in *vivo* in response to TNF treatment.

We show in this study that Bclaf1 protects cells from the TNF-induced apoptosis. Although initial studies using overexpression system suggest Bclaf1 may promote apoptosis (Kasof *et al*, 1999), cells derived from Bclaf1 knockout mice did not show any defects in apoptosis induced by multiple stimuli (McPherson *et al*, 2009), arguing the direct involvement of Bclaf1 in apoptosis at physiological settings. The stimuli used in this report did not include TNF treatment. Unlike FAS-L, which can directly activate caspase 8, TNF can only activate caspase 8 when the TNF-induced survival pathways are blocked by CHX. Thus, our result that Bclaf1 regulates apoptosis by transcriptionally upregulating *CFLAR* supports the notion that Bclaf1 is not directly acting on the apoptotic machinery, but it also indicates that Bclaf1 can indirectly influence apoptosis by regulating the transcription of c-FLIP, the negative regulator of caspase 8.

Materials and Methods

Plasmids, antibodies, and reagents

Human TNF (300-01A) and Mouse TNF (AF-315-01A) were purchased from PeproTech. Cycloheximide (A8244) and MG132 (133407-82-6) were purchased from APExBIO. Z-VAD was purchased from Beyotime Biotechnology (C1202). SM-164 (S7597) was obtained from Selleckchem. Flag M2 beads (A2220) were purchased from Sigma. Annexin V PE apoptosis detection Kit was purchased from Becton Dickinson Company (559763). In vivo-jetPEI was purchased from Polyplus (PT-201).

Full-length Bclaf1and Bclaf1 truncations were described before (Shao *et al*, 2016; Qin *et al*, 2019). p50 was cloned into the pRK5 vector with an N-terminal HA tag. The *CFLAR* promoter sequence from the position −1,179 to +281 relative to the proposed transcriptional start site of *CFLAR* exon 1 (accession no. AB038965) was cloned into the pGL3-Basic vector (Promega, Madison, WI, USA) (Ricci *et al*, 2004). Three additional reporter plasmids were made by PCR amplification of the sequences corresponding to positions −289 to +281, −788 to −288, and −1,179 to −289.

The following primary antibodies were used for Western blotting analysis: anti-Flag (F1804, Sigma), anti-HA (sc-805, Santa Cruz), anti-actin (4970, Cell Signaling Technology), anti-α-Tubulin (PM054, MBL), anti-H3 (17168-1-AP, ProteinTech), anti-Bclaf1 (sc-135845, Santa Cruz), anti-c-FLIP (Dave-2, Alexis Biochemicals; 8510, Cell Signaling Technology), anti-p50 (13586, Cell Signaling Technology), anti-PARP (9542, Cell Signaling Technology), anti-caspase 3 (A19654, abclonal), anti-cleaved-caspase 3 (AC033, Beyotime), anti-caspase 8 (66093, ProteinTech), anti-p65 (8242, Cell Signaling Technology), anti-p-p65 (3033, Cell Signaling Technology), anti-JNK (9252, Cell Signaling Technology), anti-p-JNK (9255, Cell Signaling Technology), anti-ERK (4695, Cell Signaling Technology), anti-p-ERK (4370, Cell Signaling Technology), anti-p-p38 (4511, Cell Signaling Technology), anti-p-RIPK3 (ab222320 and ab205421, Abcam), and anti-p-MLKL (ab196436, Abcam).

Cells and transfections

HEK293T, HeLa, and HepG2 cells were originally obtained from American Type Culture Collection (ATCC) and maintained in our own laboratory. The human normal small intestine cells, HIEC-6, were obtained from Dr. Dong Yulan at China Agricultural University. MEFs were generated from E11.5−E13.5 embryos of mice. All cells were cultured in medium supplemented with 10% (v/v) FBS at 37°C and 5% CO2. HeLa-Bclaf1-KO cell line has been previously reported (Qin *et al*, 2019). The guide RNA sequences for KO-1 and KO-2 were 5′-ATTCTAGAAAGAAGCGATACAGG-3′ and 5′-TTTTGCCCTATCATTTGATTAGG-3′, respectively. HeLa-Flag-Bclaf1 cell line that endogenously expresses Flag-Bclaf1 was generated following the protocol described previously (Qin *et al*, 2019). siRNA was transfected into cells using Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. Control siRNA (siCtrl), human p65, p50, C/EBPα, and mouse Bclaf1 siRNA target sequences were designed and synthesized from Genechem (Shanghai, China) as follows: siCtrl, 5′-UUCUCCGAACGUGUCACGU-3′; sip65, 5′-CGGATTGAGGAGAAACGTAAA-3′; sip50, 5′-CGAATGACAGAGGCGTGTATA-3′; siAP1, 5′-CTACGCAAACCTCAGCAACTT-3′; siC/EBPα, 5′-ACGAGACGTCCATCGACATCA-3′; Mouse siBclaf1-1, 5′-GCTACTTCTGGTGATATTT-3′; Mouse siBclaf1-2, 5′-CGCCGTAAAGAGAGAAGTAAA-3′; sic-FLIP, 5′-CCAAGGAGCAAGATCAAATAT-3′. siRNAs target human Bclaf1 and C/EBPβ were described before (Shao *et al*, 2016). Unstated siRNAs target Bclaf1 in the article are siBclaf1-1.

Cell death and Flow cytometry analysis

Cells were transfected with control (siCtrl) or two siRNAs against Bclaf1 for 24 h. For apoptosis analysis, cells were pretreated with cycloheximide (CHX, 1 μg/ml) or SM-164 (100 nM) for 30 min, and then treated with TNF (10 ng/ml) for 12 h. Cells were harvested, stained with Annexin V/7AAD using apoptosis detection

kit (BD PharmingenTM), and examined by flow cytometry. All Annexin V positive cells were counted as apoptotic cells. For necroptosis analysis, cells were pretreated with z-VAD (20 μM) together with CHX (1 μg/ml), or SM-164 (100 nM) for 30 min, and then treated with TNF (10 ng/ml) for 9 h. Cells were subjected to Annexin V/7AAD staining followed by flow cytometry analysis. 7AAD positive and Annexin V negative cells were counted as necroptotic cells. The data were then analyzed with CellQuest Pro software, version 5.1 (BD).

Real-time PCR

Total RNA was extracted using TRIzol (Invitrogen) following the manufacturer's protocol. A total of 0.8 μg total RNA from different treatments was reversely transcribed using M-MLV reverse transcriptase (Promega) with an oligo (dT) 18 primer. RT–PCR was performed using an UltraSYBR Mixture (Beijing CoWin Biotech, Beijing, China) and a ViiA 7 RT–PCR system (Applied Biosystems). Sample data were normalized to GAPDH expression. The following primers for *CFLAR* were used: *CFLAR* forward, 5′-TCAAGGAGCAGG GACAAGTTA-3′, *CFLAR* reverse, 5′-GACAATGGGCATAGGGTGTT ATC-3′.

Chromatin immunoprecipitation (CHIP)

The CHIP assay was performed using a Magna CHIP™ A/G Chromatin Immunoprecipitation Kit (17-10085, Sigma) following the manufacturer's instruction. Briefly, cells were crosslinked with 1% formaldehyde and neutralized with 0.125 M glycine. Sonication was used to shear purified chromatin to ~500 bp. Anti-Flag and control IgG antibodies were used for immunoprecipitation. After reverse crosslinking, the DNA samples were analyzed by RT–PCR.

Immunoprecipitation and Western blotting

For co-immunoprecipitation experiments, cells were harvested and lysed in lysis buffer (50 mM Tris-Cl at pH 8.0, 150 mM NaCl, 1% Triton X-100, 10 mM DTT, 1× complete protease inhibitor cocktail tablet, and 10% glycerol). For FADD immunoprecipitation, lysates were precleared with 20 μl protein G beads for 1 h at 4°C and then immunoprecipitated overnight at 4°C with 1mg FADD antibodies in the presence of 20 μl protein G beads. The nuclear and cytoplasmic extracts from cells were prepared using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology, Shanghai, China) following the manufacturer's instruction. Equalized extracts were used for the immunoprecipitation and immunoblot analysis, which were described previously (Cui *et al*, 2014). For Western blotting, cells were directly lysed in 2× sodium dodecyl sulfate (SDS) sample buffer. Subsequent procedures were performed as described (Cui *et al*, 2014).

Luciferase assay

Bclaf1-KO HeLa cells or 293T cells were seeded the day before in 24-well plates and then transfected with 100 ng of luciferase reporter plasmids plus 20 ng of pRL-TK plasmids as an internal control. Luciferase activity was measured using a dual-luciferase reporter assay kit (Promega) 24 h after transfection and then normalized.

RNA-seq analysis

RNA-seq analysis was performed on duplicates of Bclaf1-KO and Bclaf1-WT HeLa cells. The RNA sequencing and analysis were performed by the Allwegene Technology Inc., Beijing. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5x). The library preparations were sequenced on an Illumina Hiseq 4000 platform. RNA-seq reads with inferior quality or adapters were filtered. Clean read data were processed using Tophat2 and Cufflinks software to complete the alignment of transcriptomes. Differentially expressed genes and transcripts were then filtered for false discovery rate (FDR)-adjusted $P \leq 0.05$ and expression fold change equal or above 2. KEGG pathway enrichment was analyzed using KOBAS.

Experiments with mice

Animal care and protocols were approved by Animal Welfare Committee of China Agricultural University. siRNAs against Bclaf1 and control siRNAs were delivered into 7-week-old SPF C57BL/6 mice via intravenous injections using in vivo-jetPEI (Polyplus) according to the manufacturer's protocol. Briefly, siRNAs and in vivo-jetPEI complexes were generated following the manufacturer's protocol and injected into the tail veins of mice twice, 24 h apart, using a sterile syringe (1.0 ml) with a 30-gauge needle. After 4 days, the mice were injected into 10 μg of mTNF and sacrificed in 2 h. The small intestines were isolated, and subjected to Western blotting analysis, hematoxylin-eosin solution staining and immunohistochemical staining. For villus length and crypt depth measurement, at least 30 random fields in six sections of each sample were photographed. The five longest villi per field and a total of 180 longest villi (in each sample) were analyzed. The mean values of each mouse's six regions were used for statistical analysis. The villus length (V) and crypt depth (C) from HE-staining were measured by Ipp 6.0 software. Five views from the small intestine of each mouse were analyzed for cleaved caspase 3 positive cells ($CC3^+$) and p-$RIPK3^+$ cells. The mean values of each mouse were used for statistical analysis.

Data availability

The RNA-seq data obtained in this study have been provided in Dataset EV1. Raw sequences were acquired 3 years ago through a company. Since the raw and metadata have not been stored, deposition in a public database was not possible.

Expanded View for this article is available online.

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Author contributions

JT conceived and supervised the study. RZ and JT designed the experiments. RZ performed the majority of the experiments. TX, AS, YL, CQ, MZ, YK, ZY, and YG were involved in specific experiments. CZ was involved in some data analysis. RZ and JT analyzed the data and wrote the manuscript. All authors discussed the results and commented on the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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