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FRA1:c-JUN:HDAC1 complex down-regulates filaggrin expression upon TNFα and IFNγ stimulation in keratinocytes

Sung Shin Ahn[a], Hyunjin Yeo[a], Euitaek Jung[a], Yoongho Lim[b], Young Han Lee[a], and Soon Young Shin[a,1]

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Filaggrin (FLG), an essential structural protein for skin barrier function, is down-regulated under chronic inflammatory conditions, leading to disruption of the skin barrier. However, the detailed molecular mechanisms of how FLG changes in the context of chronic inflammation are poorly understood. Here, we identified the molecular mechanisms by which inflammatory cytokines inhibit FLG expression in the skin. We found that the AP1 response element within the –343/+25 of the *FLG* promoter was necessary for TNFα + IFNγ–induced down-regulation of *FLG* promoter activity. Using DNA affinity precipitation assay, we observed that AP1 subunit composition binding to the *FLG* promoter was altered from c-FOS:c-JUN (at the early time) to FRA1:c-JUN (at the late time) in response to TNFα + IFNγ stimulation. Knockdown of FRA1 or c-JUN abrogated TNFα + IFNγ–induced FLG suppression. Histone deacetylase (HDAC) 1 interacted with FRA1:c-JUN under TNFα + IFNγ stimulation. Knockdown of HDAC1 abrogated the inhibitory effect of TNFα + IFNγ on FLG expression. The altered expression of FLG, FRA1, c-JUN, and HDAC1 was confirmed in mouse models of 2,4-dinitrochlorobenzene–induced atopic dermatitis and imiquimod-induced psoriasis. Thus, the current study demonstrates that TNFα + IFNγ stimulation suppresses FLG expression by promoting the FRA1:c-JUN:HDAC1 complex. This study provides insight into future therapeutic strategies targeting the FRA1:c-JUN:HDAC1 complex to restore impaired FLG expression in chronic skin inflammation.

filaggrin | c-JUN | FOS-related antigen 1 | histone deacetylase 1 | keratinocytes

Significance

Filaggrin (FLG) is one of the structural proteins for skin barrier function. Reduced FLG expression is implicated in the pathogenesis of inflammatory skin diseases, such as atopic dermatitis and psoriasis. However, it remains unclear how FLG is down-regulated at the transcriptional level. In this study, we found that the FRA1 and c-JUN components of AP1 interact with histone deacetylase 1 to form a repressor complex that down-regulates FLG promoter activity under tumor necrosis factor α (TNFα) + interferon (IFNγ) stimulation in keratinocytes. Blocking this complex may have therapeutic benefits in patients with inflammatory skin diseases.

The fundamental function of the skin is to protect the host from factors in the external environment, such as irritants, pathogens, and allergens (1). Sturdy construction of epidermal layers is necessary for a healthy skin barrier. When epidermal keratinocytes differentiate and move toward the skin surface, they form epidermal layers and sequentially express various barrier proteins, such as filaggrin (FLG) and LOR (2, 3).

FLG is one of the essential structural proteins that make up the skin barrier (2). FLG is synthesized from proFLG (>400 kDa in humans), a large phosphorylated precursor protein composed of multiple FLG repeats (1, 2). During the transition from the granular to cornified layer, proFLG is dephosphorylated and cleaved to generate functional FLG monomers (1–3). Under healthy conditions, the interaction between FLG monomers and keratin filaments contributes to the cellular compaction and integrity of the stratum corneum, the outermost layer of the skin (2, 4). Therefore, proper regulation of FLG expression is essential for a healthy skin barrier. Indeed, FLG deficiency leads to disorganization of the architecture of keratin filaments and the lamellar bilayer (5–7). Loss-of-function mutations in FLG can produce a truncated proFLG protein, which may be responsible for ichthyosis vulgaris and eczema (4, 7, 8). Furthermore, down-regulated FLG expression has been detected in atopic dermatitis (AD) and psoriasis skin lesions, as well as in keratinocytes treated with proinflammatory cytokines (7–10). For example, TNFα disrupts the skin barrier by reducing the expression of stratum corneum lipids and barrier proteins, such as FLG and LOR (9, 11), and serum levels of TNFα and IFNγ are highly detected in patients with psoriasis (12). FLG is also down-regulated in mouse models of 2,4-dinitrochlorobenzene (DNCB)–induced AD and imiquimod (IMQ)-induced psoriasis (13–16). Thus, FLG down-regulation in chronic skin inflammatory conditions is related to skin barrier disruption, a hallmark characteristic of psoriasis and AD. Previous studies demonstrated that toluene-induced STAT3 suppresses *FLG* expression (17) and that AP1 is involved in *FLG* transcription (18–20). These findings suggest that specific transcription factors are involved in the regulation of FLG expression. However, the detailed mechanisms underlying FLG down-regulation by inflammatory cytokines are still unclear.

This study aimed to investigate the molecular mechanisms of reducing FLG expression by TNFα + IFNγ in primary normal human epidermal keratinocyte (NHEK) and HaCaT keratinocyte cell lines. We found that the AP1 binding motif in the *FLG*

Author affiliations: [a]Department of Biological Sciences, Sang-Huh College of Lifescience, Konkuk University, Seoul 05029, Republic of Korea; and [b]Division of Bioscience and Biotechnology, Bio/Molecular Informatics Center, Konkuk University, Seoul 05029, Republic of Korea

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The authors declare no competing interest.

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[1]To whom correspondence may be addressed. Email: shinsy@konkuk.ac.kr.

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promoter is crucial for FLG down-regulation and that TNFα + IFNγ treatment induces the formation of a repressor complex between FRA1:c-JUN components of AP1 and histone deacetylase (HDAC) 1. We confirmed the altered expression of FLG and the FRA1:c-JUN:HDAC1 complex in vivo using mouse models of DNCB-induced AD (21) and IMQ-induced psoriasis (15).

Results

Effects of TNFα and IFNγ on the Suppression of FLG Expression In Vitro and In Vivo. *FLG* mutations are well known as a strong genetic risk factor for AD and allergic diseases (4, 5, 7, 8, 22). In patients with AD and psoriasis, the level of FLG expression decreased, while the level of serum TNFα and IFNγ significantly increased (9, 12, 23). It has been reported that TNFα alone or in combination with IFNγ inhibits the expression of proFLG (9, 10, 24, 25). We observed that combined treatment with TNFα and IFNγ (TNFα + IFNγ) synergistically reduced proFLG expression compared with treatment with either cytokine alone in HaCaT cells (*SI Appendix*, Fig. S1*A*). We also confirmed that TNFα + IFNγ reduced the proFLG protein and *FLG* messenger RNA (mRNA) abundance in a time-dependent manner in both HaCaT and primary NHEK cells (*SI Appendix*, Fig. S1 *B–F*).

To investigate whether TNFα and IFNγ were involved in mediating the suppression of the expression of *FLG* in vivo, we used a mouse model developed using topical application of DNCB to induce AD-like skin inflammation in BALB/c mice (21) (*SI Appendix*, Fig. S2*A*). Topical application of DNCB led to the development of typical AD-like skin lesions, with symptoms such as superficial erosion and redness (Fig. 1*A*). Under these experimental conditions, we tested the effect of intradermal injection containing neutralizing antibodies against TNFα and IFNγ on DNCB-induced skin lesions (*SI Appendix*, Fig. S2*B*). We observed that DNCB-induced epidermal and dermal thickness (Fig. 1 *B–D*) was reduced by the injection of anti-TNFα and

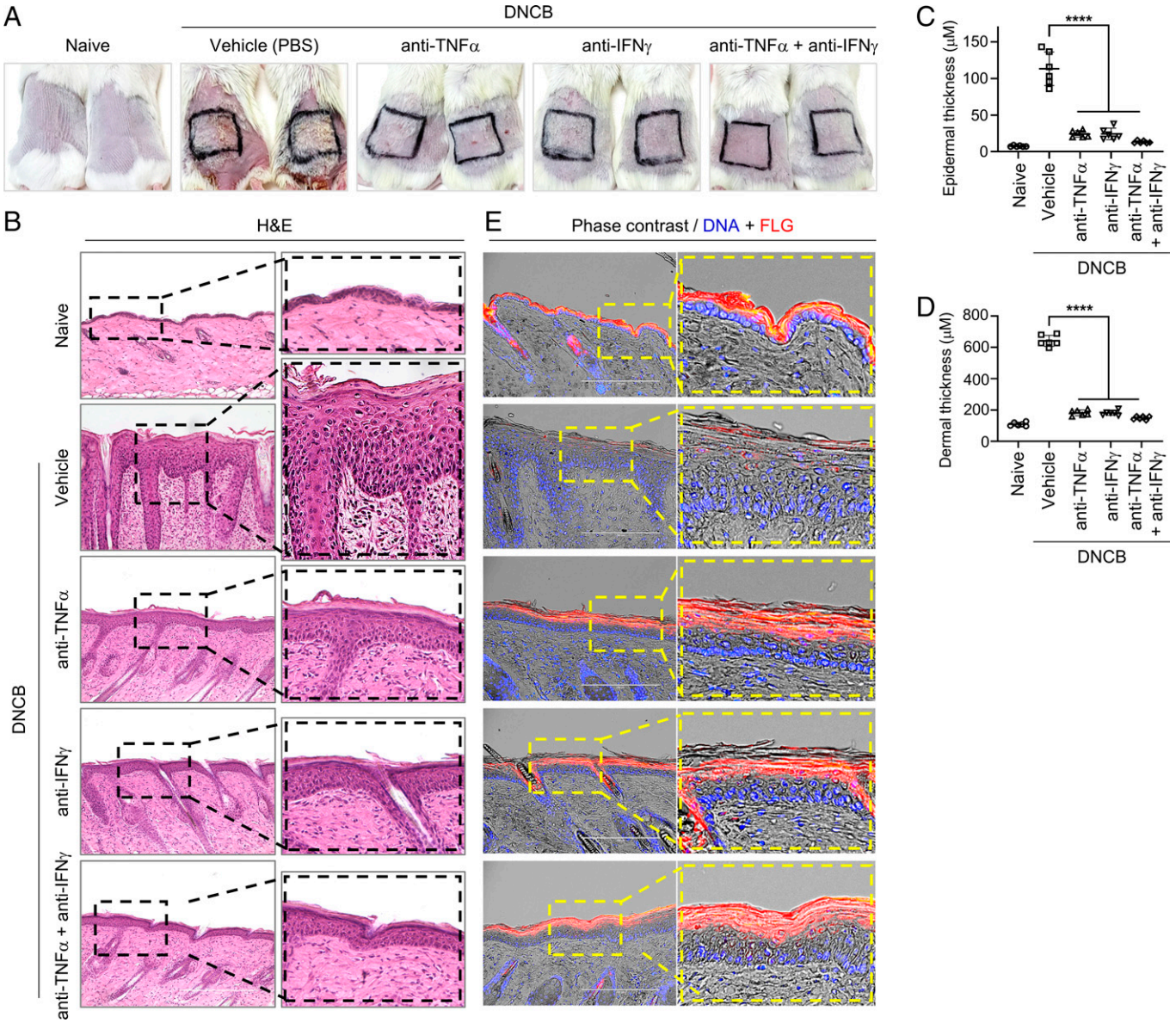

Fig. 1. The effect of antibody blockade of TNFα and IFNγ on a DNCB-induced AD-like mouse model. (*A*) Visual observation of the back skin of BALB/C mice on day 21. (*B*) Paraffin-embedded sections were stained with H&E. (Scale bar, 200 μm.) (*C* and *D*) The thicknesses of the epidermis (*C*) and dermis (*D*) were measured by ImageJ software. Data are presented as mean ± SD ($n = 6$). ****$P < 0.0001$ by Sidak's multiple comparisons test. (*E*) Immunofluorescence staining of tissue sections with anti-FLG antibody using rhodamine red-X–conjugated secondary antibody (red). Nuclei were counterstained with Hoechst 33258 (blue). The areas in the dashed boxes are magnified in *Right*. (Scale bars, 200 μm.)

anti-IFNγ antibodies. Notably, DNCB-induced suppression of FLG staining intensity was restored by intradermal injection of neutralizing antibodies against TNFα and IFNγ (Fig. 1*E*). These findings suggest that TNFα and IFNγ contribute to DNCB-induced AD-like skin lesions and suppression of *FLG* expression in the BALB/c mouse model.

Sustained Expression of FRA1 and c-JUN Is Necessary for the Down-Regulation of *FLG* Promoter Activity by TNFα + IFNγ. Next, we tried to identify the regulatory region contributing to the repression of *FLG* expression induced by TNFα + IFNγ. Serial deletion constructs of the *FLG* promoter were generated, and the promoter–reporters were transiently transfected into HaCaT cells. We found that TNFα + IFNγ stimulation reduced *FLG* promoter–reporter activity, even in the cells transfected with the shortest construct (–343/+25) (Fig. 2*A*). As the known regulatory STAT3 binding sequence within the *FLG* promoter spans from –453 to –428 (17), these data suggest that an unidentified responsive element is located between –343 and +25. We, therefore, analyzed the functional cis-acting elements between –343 and +25 and found a putative AP1 binding site located from –118 to –106 (Fig. 2*B*). To evaluate whether this putative AP1 binding site is involved in the decrease in *FLG* transcription induced by TNFα + IFNγ, we carried out site-directed mutagenesis of the AP1 binding site in the –343/+25 promoter construct. Mutation of the AP1 binding site (mtAP1) abrogated the decrease in *FLG* promoter activity induced by TNFα + IFNγ (Fig. 2*B*), suggesting that the putative AP1 binding site plays a crucial role in the reduction in *FLG* transcription induced by TNFα + IFNγ treatment.

Because AP1 subunits are differentially expressed and play different roles in keratinocytes in response to various stimuli (18–20, 26, 27), we examined the expression of different AP1 protein subunits (c-FOS, c-JUN, FRA1, and FRA2) induced in HaCaT keratinocytes in response to TNFα + IFNγ treatment for 0 to 36 h. Immunoblotting data showed that c-FOS expression was detected at 0.5 h, peaked at 1 h, and then, gradually decreased to basal levels after 12 h of TNFα + IFNγ treatment (Fig. 2*C*). c-JUN phosphorylation was detected only for 0.5 to 1 h, but its expression level persisted for up to 36 h. FRA1 and FRA2 expression also continuously increased over the 36 h of treatment. Consistent expression of c-JUN, FRA1, and FRA2 was also obtained with primary NHEK cells (*SI Appendix*, Fig. S3*A*). Therefore, c-FOS, c-JUN, FRA1, and FRA2 exhibit differential expression patterns depending on the TNFα + IFNγ exposure time.

Next, we performed an electrophoretic mobility shift assay (EMSA) to determine whether TNFα + IFNγ–induced AP1 subunits bind the *FLG* promoter using a biotinylated deoxyoligonucleotide probe corresponding to the AP1 binding sequence. TNFα + IFNγ treatment increased DNA–protein complex levels after stimulation for both 1.5 and 24 h, and the addition of unlabeled oligonucleotides (competitor) effectively competed with the DNA for protein binding (Fig. 2*D*). Primary NHEK cells also showed similar enrichment of DNA–protein complexes at 24 h, with the loss of DNA–protein complexes seen after the addition of the competitor (*SI Appendix*, Fig. S3*B*). These data suggest that the AP1 complex binds to the putative AP1 binding motif within the –343/+25 of the *FLG* promoter for an extended period of at least 24 h upon TNFα + IFNγ stimulation.

To further identify the components of the AP1 complex that bound the AP1 probe of the *FLG* promoter at the early and late time points, we performed a DNA affinity precipitation assay (DAPA). As shown in Fig. 2*E*, TNFα + IFNγ treatment for 1.5 h specifically promoted the binding of c-FOS and c-JUN to the AP1 probe. However, after 24 h, FRA1 and c-JUN were bound to the oligonucleotide probe but not to the mutated probe (Fig. 2*F*). These results suggest that the AP1 subunit composition that binds to the *FLG* promoter is functionally altered in keratinocytes in response to TNFα + IFNγ stimulation; c-FOS:c-JUN is at an early stage (0.5 to 3 h), whereas FRA1:c-JUN is at a later time point (over 12 h).

To test the possibility that the late-formed components of the AP1 complex (FRA1:c-JUN) are involved in TNFα + IFNγ–induced *FLG* suppression, we cotransfected with the –343/+25 construct and protein expression plasmids for FRA1 or c-JUN. Ectopic expression of FRA1 (Fig. 2*G*) or c-JUN (Fig. 2*H*) reduced promoter–reporter activity of the –343/+25 construct in a plasmid concentration–dependent fashion. These data suggest that FRA1 and c-JUN are sufficient for TNFα + IFNγ–induced *FLG* suppression.

Knockdown of FRA1 or c-JUN Abrogates TNFα + IFNγ–Induced FLG Suppression. To further validate the role of FRA1 and c-JUN in TNFα + IFNγ–induced FLG suppression, we silenced FRA1 and c-JUN using short hairpin RNA (shRNA) and examined TNFα + IFNγ–induced FLG expression. Knockdown of FRA1 (shFRA1) (Fig. 3*A*) or c-JUN (shJUN) (Fig. 3*B*) abrogated the TNFα + IFNγ–mediated FLG down-regulation, as compared with control expressing scrambled shRNA (shCT). Tanshinone IIA (TanIIA) is an AP1 inhibitor that inhibits AP1–DNA binding activity under conditions involving TNFα + IFNγ stimulation, without affecting the expression of FRA1 and c-JUN (*SI Appendix*, Fig. S4). Pretreatment with TanIIA reduced the ability of TNFα + IFNγ to induce suppression of *FLG* mRNA (Fig. 3*C*) and proFLG expression (Fig. 3*D*). We observed a similar effect of TanIIA in primary NHEK cells (Fig. 3*E*). Immunofluorescence staining confirmed the effect of TanIIA on the restoration of reduced proFLG abundance (Fig. 3*F*). These results confirmed the negative role of the AP1 complex (FRA1:c-JUN) on FLG expression under TNFα + IFNγ stimulation.

HDAC Is Related to FLG Repression Induced by TNFα + IFNγ. Previous studies have reported that the activity of HDACs is crucial in epidermal skin differentiation (28, 29) and that c-JUN and HDAC1 are corecruited to the promoters of target genes (30). Based on these findings, we hypothesized that the FRA1:c-JUN AP1 complex bound to the *FLG* promoter region recruits HDACs as corepressors to inhibit FLG expression. To test this possibility, we pretreated cells with nonselective HDAC inhibitors, including sodium butyrate (NaBT), valproic acid (VPA), trichostatin A (TSA), and suberoylanilide hydroxamic acid (SAHA), before TNFα + IFNγ treatment. We observed that proFLG expression suppressed by TNFα + IFNγ was effectively restored to near control levels by pretreatment with all HDAC inhibitors tested (Fig. 4 *A*–*D*). TSA had a similar effect in primary NHEK cells (Fig. 4*E*). Immunofluorescence staining confirmed the effect of TSA on the restoration of reduced proFLG abundance (Fig. 4*F*). To rule out the pleiotropic effects of HDAC inhibitors, we knocked down *HDAC1*, a representative *HDAC* subunit (31, 32), using shRNA (shHDAC1). TNFα + IFNγ–induced reduction of the proFLG level was abrogated by HDAC1 knockdown (Fig. 4*G*). These data suggest that HDACs play a crucial role in TNFα + IFNγ–induced *FLG* down-regulation.

AP1 and HDAC1 Inhibitors Restored Reduced *FLG* Expression under In Vivo Conditions. Next, we addressed whether AP1 and HDAC inhibitors could restore suppressed FLG expression

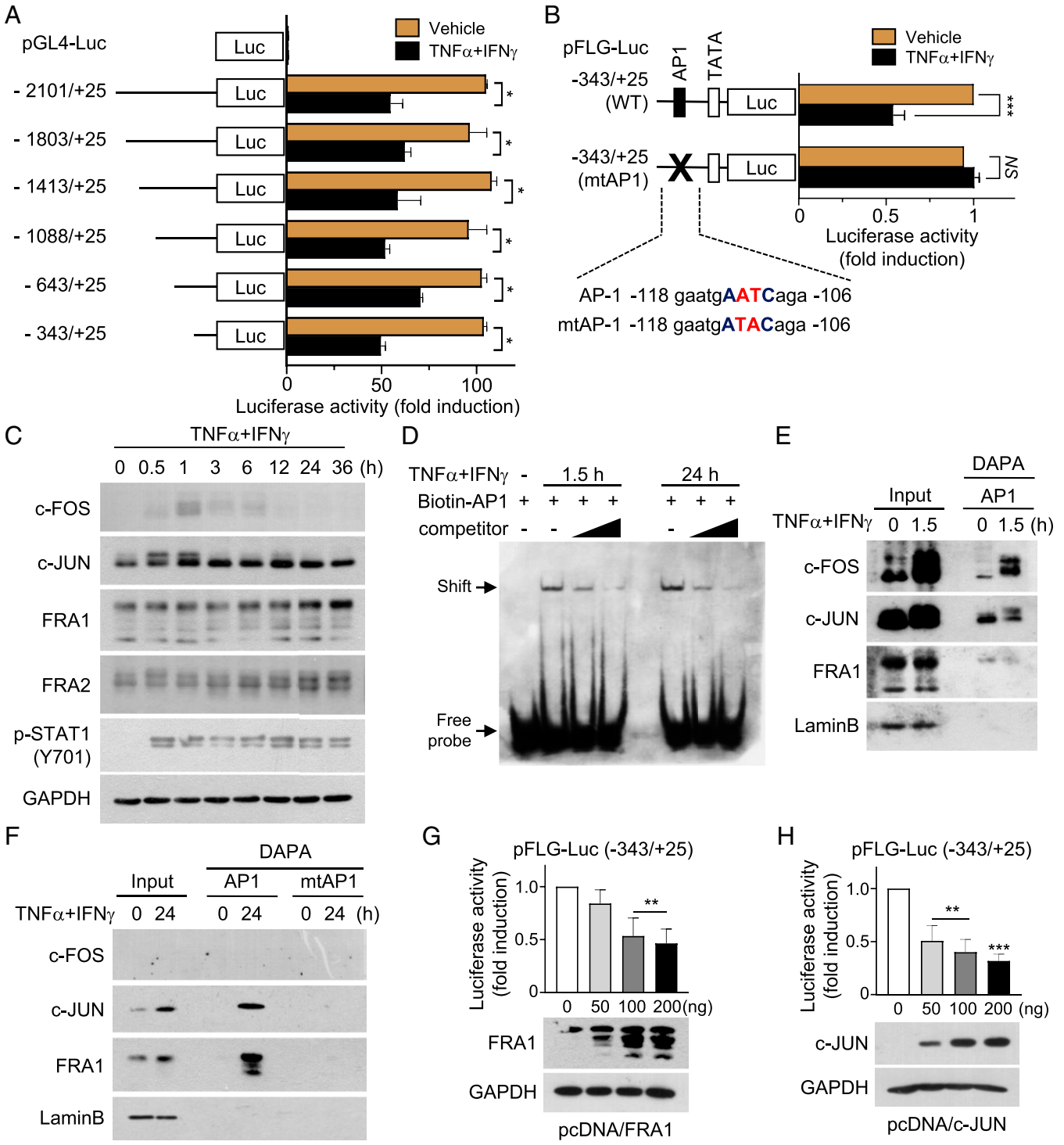

Fig. 2. FRA1 and c-JUN induced by TNFα + IFNγ bind to the AP1 binding motif of the *FLG* promoter and suppress FLG expression. (*A* and *B*) HaCaT cells were transiently transfected with 0.2 μg of a series of 5′-deletion constructs of *FLG* promoter (*A*) or the wild-type (WT) pFLG-Luc (–343/+25) plasmid or a mutated AP1 binding sequence (mtAP1) of this plasmid (*B*). After 24 h, the cells were treated with vehicle (phosphate-buffered saline) or TNFα + IFNγ (10 ng/mL) for an additional 8 h, and luciferase promoter–reporter activities were measured. Bars indicate the mean ± SD ($n = 3$). NS, $P = 0.1412$. *$P < 0.05$; ***$P < 0.001$. (*C*) HaCaT cells were treated with TNFα + IFNγ (10 ng/mL) for the indicated durations. Whole-cell lysates were prepared, and immunoblotting was conducted using antibodies against c-FOS, c-JUN, FRA1, FRA2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (*D*) A biotinylated AP1 binding motif and AP1 complex were examined using an EMSA. Nuclear extracts from HaCaT cells with or without TNFα + IFNγ treatment (1.5 or 24 h) were used as the protein source. Unlabeled AP1 probes (2,500 and 5,000 pmol) were added for the competition assay. (*E* and *F*) A DAPA was performed with nuclear extracts from HaCaT cells treated with TNFα + IFNγ (10 ng/mL) for 1.5 h (*E*) or 24 h (*F*) and biotinylated oligonucleotide probes for AP1 or mtAP1. After precipitation of HaCaT cells with streptavidin-conjugated agarose beads, immunoblotting was carried out using antibodies against c-FOS, c-JUN, FRA1, and LaminB. (*G* and *H*) HaCaT cells were cotransfected with the p*FLG*-Luc (–343/+25) reporter construct and increasing amounts of an FRA1 (*G*) or c-JUN (*H*) expression plasmid. After 24 h, luciferase activities were analyzed (*G*, *Upper* and *H*, *Upper*), and the levels of transfected FRA1 and c-JUN were detected by immunoblotting (*G*, *Lower* and *H*, *Lower*). Bars represent the mean ± SD ($n = 3$). **$P < 0.01$; ***$P < 0.001$.

in DNCB-challenged BALB/c mice. SAHA (HDAC1 inhibitor) or TanIIA (AP1 inhibitor) was topically applied 3 h after DNCB treatment (*SI Appendix*, Fig. S5*A*). Tan IIA and SAHA ameliorated DNCB-induced AD-like skin lesions (*SI Appendix*, Fig. S5*B*). Hematoxylin and eosin (H&E) staining showed that DNCB-induced epidermal and dermal thickening decreased

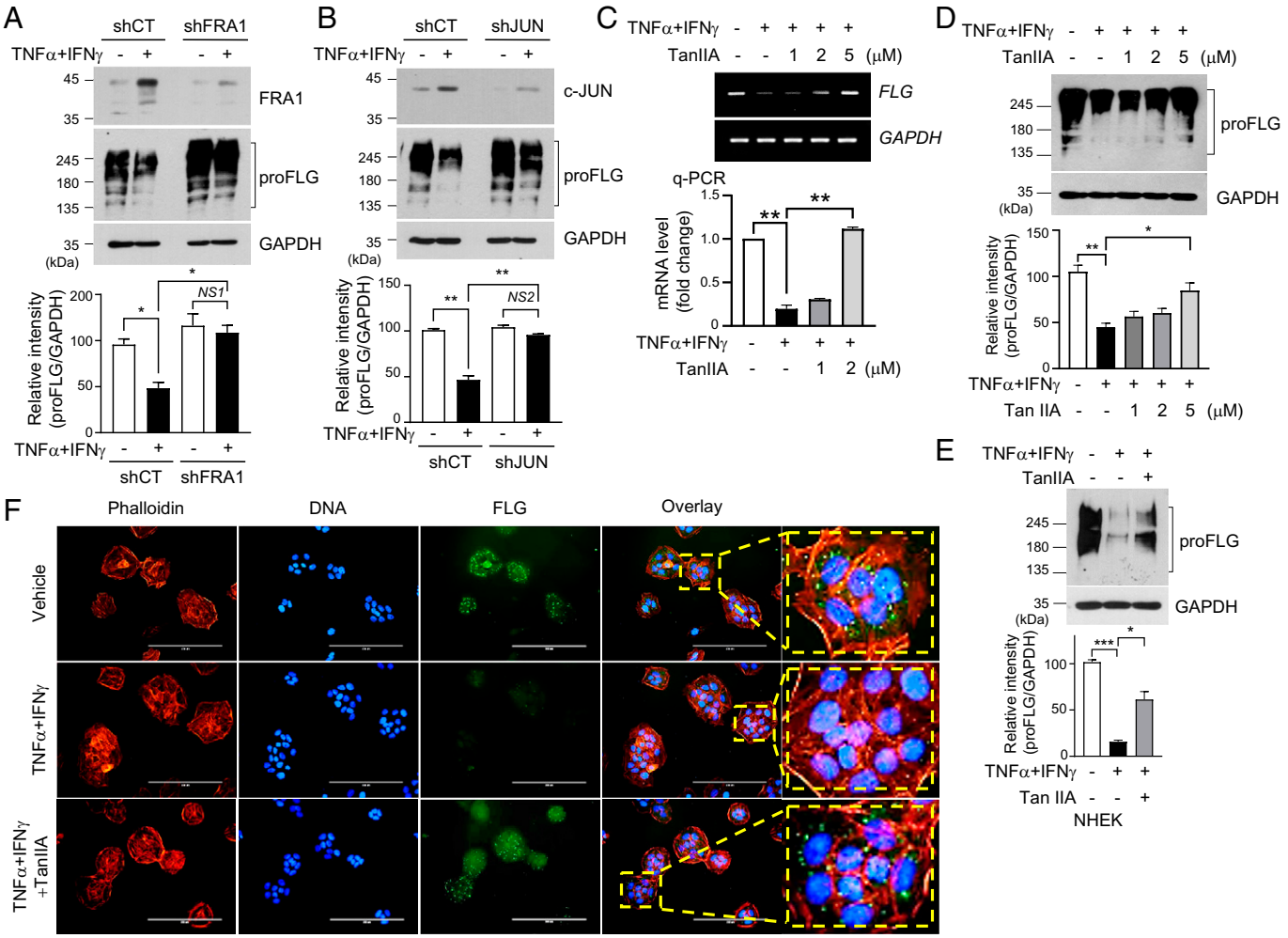

Fig. 3. Inhibition of TNFα + IFNγ–induced FLG suppression via knockdown of FRA1 or c-JUN. (*A* and *B*) Effects of scrambled shRNA (shCT) and FRA1-specific shRNA (shFRA1; *A*) or c-JUN-specific shRNA (shJUN; *B*) on TNFα + IFNγ–induced FLG protein expression by immunoblotting. Relative band intensities for FLG were calculated by comparison with GAPDH and quantified graphically. Bars represent the mean ± SD ($n = 3$). NS1, $P = 0.93$; NS2, $P = 0.34$. $^{*}P < 0.1$; $^{**}P < 0.01$. (*C* and *D*) HaCaT cells were treated with TNFα + IFNγ (10 ng/mL) for 24 h in the absence or presence of different concentrations (0 to 10 μM) of TanIIA, an inhibitor of AP1. *FLG* mRNA levels were examined using RT-PCR and qPCR (*C*) or immunoblotting (*D*). (*E*) NHEK cells were treated with vehicle or TNFα + IFNγ (10 ng/mL) for 24 h in the absence or presence of TanIIA (1 μM), and FLG protein levels were examined by immunoblotting. $^{*}P < 0.1$; $^{**}P < 0.01$; $^{***}P < 0.001$. (*F*) Immunofluorescence staining was carried out using an antibody against proFLG. (Scale bars, 200 μm.)

with the application of these inhibitors (*SI Appendix*, Fig. S5 *C–E*). Notably, the reduction in the staining intensity of FLG was recovered by TanIIA or SAHA (*SI Appendix*, Fig. S5*F*). These results suggest that AP1 and HDAC1 inhibitors restored *FLG* expression in the inflamed skin environment in vivo.

FRA1:c-JUN:HDAC1 Complex Formation on the AP1 Binding Site of the *FLG* Promoter upon TNFα + IFNγ Stimulation. HDACs, including HDAC1, HDAC2, HDAC3, SIRT1, and SIRT3, were detected in the nuclei of HaCaT cells (*SI Appendix*, Fig. S6*A*). As various HDAC members function differently (28, 31–33), we determined which HDACs are recruited to the FLG promoter by TNFα + IFNγ treatment using DAPA. We found that HDAC1 was pulled down predominantly with the AP1 binding motif in the *FLG* promoter region after 24 h of TNFα + IFNγ treatment, whereas HDAC2, HDAC3, HDAC6, SIRT1, and SIRT3 did not (Fig. 5*A*). A similar result was obtained in primary NHEK cells (Fig. 5*B*).

To address whether HDAC1 physically interacts with the *FLG* promoter, we carried out a chromatin immunoprecipitation (ChIP) assay. HaCaT cells were treated with or without TNFα + IFNγ for 24 h and immunoprecipitated with a rabbit anti-HDAC1 antibody or normal rabbit immunoglobulin G (IgG). Genomic DNA fragments spanning the AP1 binding site were amplified; however, the off-target region was not amplified (Fig. 5 *C* and *D*), suggesting that HDAC1 interacts with the AP1 binding site in the *FLG* promoter at the chromatin level. To further elucidate whether HDAC1 recruited to the AP1 binding site could deacetylate the histone packaged around the AP1 binding site, we performed ChIP analysis with an anti-acetylated lysine 9 of histone H3 (H3K9Ac) antibody. Immunoprecipitated DNA was amplified in control cells but not in TNFα + IFNγ–stimulated cells (Fig. 5*E*), suggesting that HDAC1 recruited to the AP1 binding site could deacetylate histones at the chromatin level. In addition, transient transfection of p300/HAT enhanced *FLG* promoter–reporter activity, which was reduced by cotransfection with FRA1 + c-JUN or HDAC1, suggesting that *FLG* promoter activity was suppressed by HDAC1-mediated histone deacetylation (*SI Appendix*, Fig. S6*B*).

To further investigate whether FRA1:c-JUN:HDAC1 forms a complex with the AP1 binding site in the *FLG* locus at the chromatin level, we deleted the AP1 binding site in the *FLG* locus using the CRISPR-Cas9 system. A control clone (Cont) was generated by transfecting the Cas9 vector only (no guide

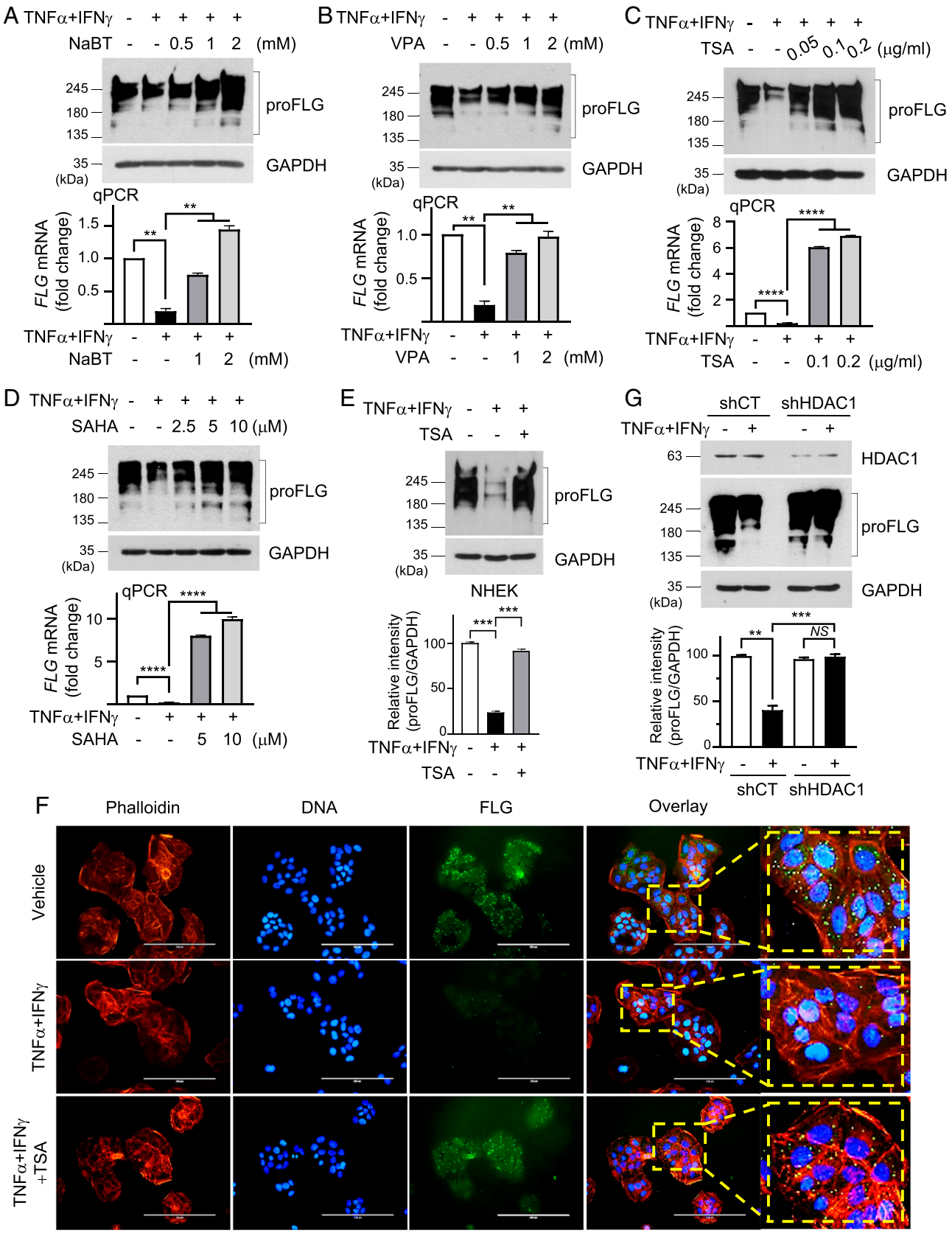

Fig. 4. Inhibition of HDACs abrogates TNFα + IFNγ–induced suppression of FLG expression. (*A–D*) HaCaT cells or NHEK cells were pretreated with various HDAC inhibitors before treatment with TNFα + IFNγ (10 ng/mL). After 24 h, the effects of NaBT (*A*), VPA (*B*), TSA (*C*), and SAHA (*D*) on FLG expression were evaluated by immunoblotting and qPCR analysis in HaCaT cells. (*E*) The effect of TSA in NHEK cells was also measured by immunoblotting. (*F*) Immunofluorescence staining for proFLG was performed in HaCaT cells. (Scale bars, 200 μm.) (*G*) Effects of scrambled shRNA (shCT) or HDAC1-specific shRNA (shHDAC1) on TNFα + IFNγ–induced FLG suppression were examined by immunoblotting. The band intensities corresponding to proFLG levels were normalized to those of GAPDH levels using ImageJ software. Bars represent the mean ± SD ($n = 3$). NS, $P = 0.39$. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

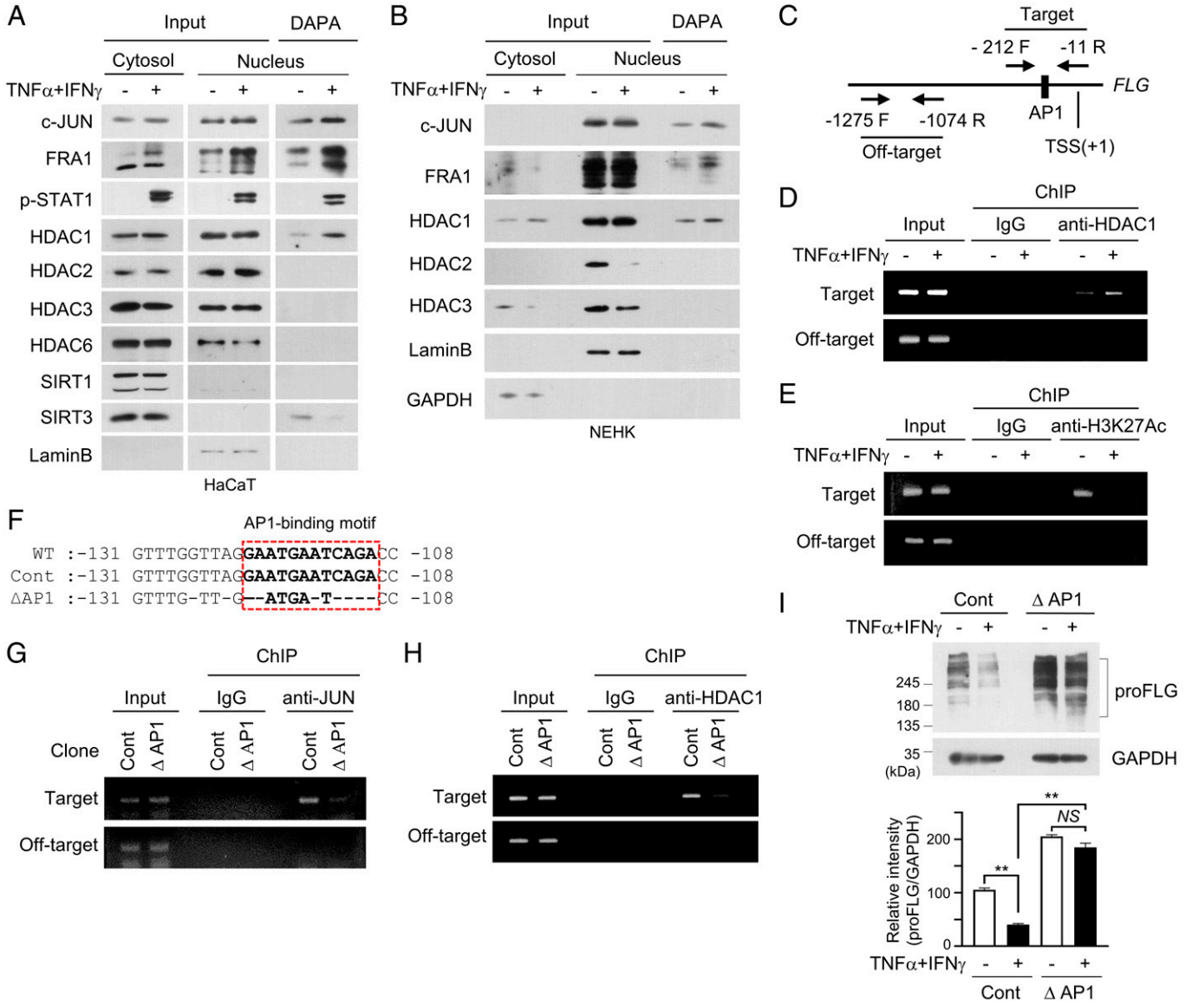

Fig. 5. TNFα + IFNγ induces FRA1:c-JUN:HDAC1 complex formation on the AP1 binding motif in the *FLG* promoter. (*A* and *B*) A DAPA for the biotin–AP1 oligonucleotide probe was performed using nuclear extracts from TNFα + IFNγ–treated HaCaT cells (*A*) or NHEK cells (*B*). Extracted cytoplasmic and nuclear proteins were determined by immunoblotting with specific antibodies. Lamin B was used as a nuclear marker. (*C*) Primer sets used for ChIP analysis. TSS, transcription start site. HaCaT cells were treated with TNFα + IFNγ (10 ng/mL) for 24 h, and chromatin was immunoprecipitated with a rabbit anti-HDAC1 antibody (*D*), a rabbit H3K9Ac antibody (*E*), or normal rabbit IgG. The precipitated DNA was evaluated by PCR using primers for target and off-target. (*F–H*) HaCaT cells were transfected with Cas9 vector only or Cas9 vector ligated with guide RNA (gRNA) targeting the AP1 binding motif in the *FLG* locus. DNA sequences at the AP1 site are presented for the wild type (WT), clones transfected with Cas9 vector only (Cont), and clones with AP1 motif deletions (ΔAP1; *F*). HaCaT/Cont and HaCaT/ΔAP1 cells were treated with TNFα + IFNγ, and ChIP assay was performed using a rabbit anti-JUN antibody (*G*) and a rabbit anti-HDAC1 antibody (*H*). The data were analyzed by PCR using target and off-target primers. ChIP experiments were repeated independently three times with similar results. (*I*) The effect of the deletion of the AP1 motif in the *FLG* locus on the suppression of TNFα + IFNγ–induced *FLG* expression was determined by immunoblotting. The band intensities corresponding to proFLG levels were normalized to those of GAPDH levels using ImageJ software. Bars represent the mean ± SD ($n = 3$). NS, $P = 0.07$. $^{**}P < 0.01$.

RNA (gRNA)), and a clone with the deletion in the AP1 motif (ΔAP1) was produced by transfecting the Cas9 vector containing the gRNA targeting the AP1 binding sequences in the *FLG* locus (Fig. 5*F* and *SI Appendix*, Fig. S7). ChIP experiments performed using the same primers (Fig. 5*C*) showed that DNA immunoprecipitated with anti-JUN (Fig. 5*G*) and anti-HDAC1 (Fig. 5*H*) antibodies was not amplified in the HaCaT/ΔAP1 clone, suggesting that the AP1 binding site in the *FLG* locus was functionally disrupted in the chromatin environment. We then investigated whether deletion of the AP1 binding site in the *FLG* locus affected *FLG* expression. HaCaT/Cont and HaCaT/ΔAP1 cells were treated with TNFα + IFNγ for 24 h. Subsequently, proFLG levels were measured using immunoblotting. We observed that the difference in proFLG levels, relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was significantly less in the HaCaT/ΔAP1 clone than in the HaCaT/Cont clone (Fig. 5*I*). These findings collectively suggest that FRA1:c-JUN inhibited *FLG* expression induced by TNFα + IFNγ stimulation by forming a complex with HDAC1 at the AP1 binding site in the *FLG* promoter in keratinocytes.

Expression of FLG, FRA1, c-JUN, and HDAC1 in Inflammatory Skin Tissues in DNCB-Induced AD and IMQ-Induced Psoriasis Mouse Models. We used mouse models of DNCB-induced AD and IMQ-induced psoriasis to verify the roles of HDAC1 and the FRA1:c-JUN AP1 complex in FLG suppression in vivo. Topical application of DNCB to the dorsal skin of BALB/C mice induced AD-like skin lesions, such as superficial erosion and

redness (Fig. 6*A*). H&E staining shows that DNCB caused epidermal and dermal thickening of dorsal skin tissues (Fig. 6 *B* and *C*). In this DNCB-induced AD-like skin inflammation model, we examined the expression patterns of FLG, FRA1, c-JUN, and HDAC1 by immunofluorescence staining. The intensity of FLG staining was weak in DNCB-treated skin compared with control skin (Fig. 6*D*). In contrast, the staining intensity of FRA1, c-JUN, and HDAC1 was increased in DNCB-treated skin compared with control skin (Fig. 6 *E*–*G*). Furthermore, DAPA analysis revealed that c-JUN, FRA1, and HDAC1 were interacted with the AP1 site in the *FLG* promoter but not with mtAP1 when using tissue proteins from DNCB-treated mice (Fig. 6*H*).

In the IMQ-induced psoriasis mouse model (*SI Appendix*, Fig. S8*A*), severe skin lesions developed with skin erythema,

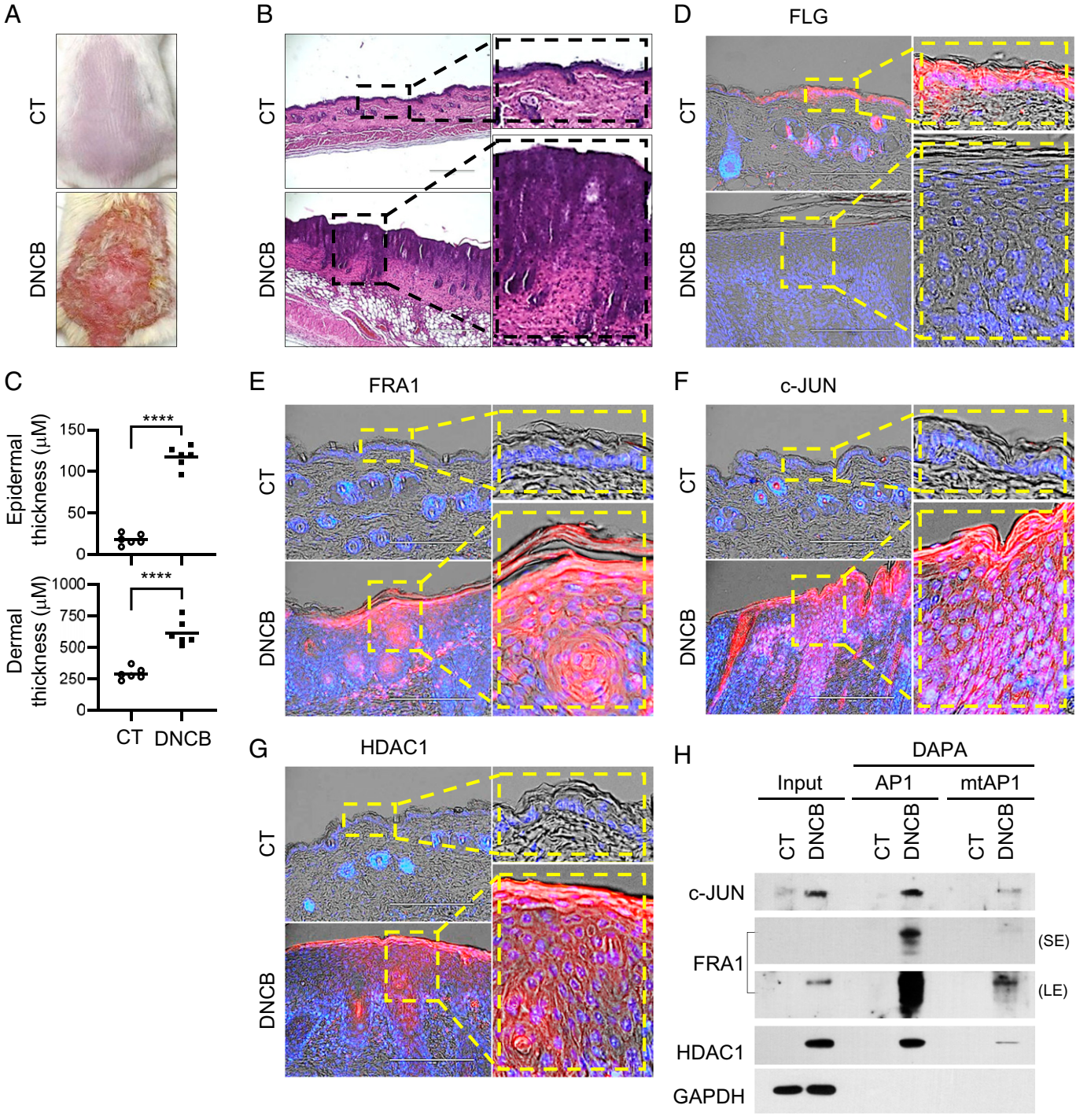

Fig. 6. Expression of FLG, FRA1, c-JUN, and HDAC1 in the skin of DNCB-challenged BALB/C mice. (*A*) Representative photographs of the back skin of BALB/C mice: untreated control (CT) or DNCB on day 21. (*B*) Histological examination of paraffin-embedded sections. (Scale bars, 400 μm.) (*C*) The thickness of the epidermis and dermis was analyzed using ImageJ software. Data are expressed as the mean ± SD ($n = 6$). ****$P < 0.0001$ by Sidak's multiple comparison test. (*D*–*G*) Skin tissue sections were deparaffinized and subjected to immunofluorescence staining with antibodies against FLG (*D*), FRA1 (*E*), c-JUN (*F*), or HDAC1 (*G*) and rhodamine red-X–conjugated secondary antibodies (red fluorescence). DNA was counterstained with 1 mg/mL Hoechst 33258 (blue fluorescence). (Scale bars, 200 μm.) (*H*) A DAPA was performed by incubating protein extracts from dorsal skin tissue with biotinylated oligonucleotide probes for AP1 or mtAP1 and precipitation with streptavidin-conjugated agarose beads. Immunoblotting was performed with antibodies against c-JUN, FRA1, HDAC1, and GAPDH. LE: long exposure, SE: short exposure.

scaling, and thickening (*SI Appendix*, Fig. S8 *B* and *C*). H&E staining and calculation data of tissue thickness show that IMQ induced hyperkeratosis of the back skin tissues (*SI Appendix*, Fig. S8 *D* and *E*). Immunofluorescence staining analysis revealed that IMQ application decreased FLG staining and correspondingly, increased FRA1, c-JUN, and HDAC1 staining (*SI Appendix*, Fig. S8 *F*–*I*). Collectively, these findings suggest that the FRA1:c-JUN AP1 complex and HDAC1 participate in suppressing FLG expression in chronic inflammatory environments, such as AD and psoriasis.

Discussion

In this study, we obtained multiple lines of evidence that TNFα + IFNγ treatment induces FRA1, c-JUN, and HDAC1 recruitment to the AP1 binding motif of the *FLG* promoter to repress *FLG* expression in keratinocytes. We found that AP1 is required for FLG inhibition induced by TNFα + IFNγ. After primary NHEK cells or HaCaT keratinocytes were stimulated with TNFα + IFNγ, FRA1 and c-JUN were up-regulated and formed a repressor complex with HDAC1 at the AP1 binding site of the *FLG* promoter. We also confirmed in vivo that the expression patterns of FLG, FRA1, c-JUN, and HDAC1 in mouse models of DNCB-induced AD and IMQ-induced psoriasis were consistent with the results from keratinocyte cell lines treated with TNFα + IFNγ. Nonetheless, we cannot exclude the possibility that RNA stability and posttranslational regulations may also be involved in suppressing FLG expression in inflammatory skin environments.

There were several studies about possible transcription factors involved in FLG regulation. For example, it has been reported that STAT3 induces *FLG* suppression by toluene (17) and that AP1 is involved in *FLG* transcription (18). While these previous studies found a relationship between FLG and transcription factors in expression patterns, we uncovered the repressor complex recruited to the *FLG* promoter to inhibit *FLG* expression under TNFα + IFNγ stimulation. First, we found that FRA1 and c-JUN expression were increased by TNFα + IFNγ and recruited to the AP1 binding motif in the *FLG* promoter. Furthermore, we ascertained that stable expression of FRA1 and c-JUN was involved in the repression of FLG expression. However, in this study, we could not elucidate how TNFα + IFNγ stimulation induces stable expression of FRA1 and c-JUN. Next, we set out to identify corepressor proteins. Previous studies have demonstrated that specificity protein 1 (SP1) recruits HDAC1 and c-JUN upon phorbol 12-myristate 13-acetate (PMA) stimulation (33), and c-Jun N-terminal kinase (JNK) signaling induces the association of HDAC1 and c-JUN (30). Based on these findings, we speculated that some repressors, such as HDACs, may be involved in FRA1:c-JUN–mediated *FLG* suppression. To test this possibility, we examined HDAC family members that interacted with FRA1:c-JUN. We observed that HDAC1 interacted predominantly with FRA1:c-JUN on the AP1 binding site of the *FLG* promoter. Thus, we suggested that FRA1:c-JUN recruited members of the HDAC family, mostly HDAC1, to form a repressor complex that can inhibit *FLG* transcription. However, we cannot exclude the possibility that other components of AP1 or HDAC members also participate in TNFα + IFNγ–induced FLG suppression. This study could not elucidate the molecular mechanism underlying how TNFα + IFNγ recruit HDAC1 to FRA1:c-JUN at the AP1 binding site in the *FLG* promoter. Previous studies have demonstrated that STAT3 is associated with FRA1/c-JUN to transactivate the matrix metalloproteinase-9 (MMP9) promoter in MCF7 breast cancer cells (34) and that STAT3 is associated with HDAC1 to repress interleukin 6 (IL6)-induced C-C motif chemokine ligand 2 (CCL2) transcription in HepG2 liver cancer cells (35). In addition, IFNγ induces the association between STAT1 and HDAC1 (36). As STAT1 activation is increased in inflamed skin (37), we hypothesize that IFNγ-activated STAT1 may have a similar role in interacting with FRA1:c-JUN, which can recruit HDAC1 to the FLG promoter in keratinocytes. We observed that TNFα increased c-JUN and FRA1 levels, while IFNγ increased phosphorylation of STAT1 (Y701) after 24 h of stimulation (*SI Appendix*, Fig. S9*A*). In addition, DAPA analysis shows that TNFα has a strong effect on c-JUN and FRA1 binding but that IFNγ was more effective than TNFα in HDAC1 binding to the AP1 binding motif (*SI Appendix*, Fig. S9*B*). As phosphorylated STAT1 (Y701) interacted with the AP1 binding site upon IFNγ stimulation but not by TNFα, these data suggest that TNFα may be sufficient for FRA1:c-JUN complex formation, but additional STAT1 activation signals by IFNγ are necessary to recruit HDAC1 to the FRA1:c-JUN complex at the AP1 binding site (*SI Appendix*, Fig. S10). These findings may explain how TNFα and IFNγ synergistically reduce *FLG* expression. Further research is needed to clarify the role of downstream signaling pathways of TNFα and IFNγ in the formation of the repressor complex on the AP1 binding site in the *FLG* promoter. As HDAC1 is known to interact with other proteins to form multiprotein complexes, such as Swi-independent 3 (SIN3) or nucleosome remodeling and deacetylase (NuRD) complexes (31, 32), more experiments are needed to clarify other proteins that make up the corepressor complex with FRA1:c-JUN:HDAC1 for inhibition of FLG expression. It was also reported that class I HDACs are essential for maintaining skin homeostasis and that HDAC inhibitors have anticancer effects in skin cancer (28, 31). Collectively, it suggests that HDAC1 may be involved in the regulation of other barrier proteins and that disrupting the interaction of HDAC1 and FRA1:c-JUN may restore FLG expression. These speculations warrant further study.

Down-regulation of FLG expression is associated with the pathogenesis of allergic skin diseases, such as AD and psoriasis (5, 6, 38). A mouse model similar to these diseases was used as a chronic inflammation model to validate in vitro results using TNFα or IFNγ treatment. So, we used DNCB solution (21) and IMQ cream (16) to establish these models and confirmed that the expression patterns of FRA1, c-JUN, HDAC1, and FLG in vivo were consistent with the in vitro data. These data suggest that normalizing the elevated expression levels of FRA1, c-JUN, or HDAC1 may be therapies for patients with AD or psoriasis.

In summary, this study demonstrates the molecular mechanism by which chronic inflammation induces down-regulation of FLG expression. We found that the FRA1:c-JUN:HDAC1 complex interacts with the AP1 binding site in the *FLG* promoter to down-regulate FLG expression under TNFα + IFNγ stimulation in human keratinocytes. Our findings suggest that inhibiting overexpression of FRA1:c-JUN or dissociating the FRA1:c-JUN:HDAC1 complex may be a therapeutic intervention to restore FLG expression in patients with chronic inflammatory skin diseases, such as AD and psoriasis.

Materials and Methods

Detailed materials and reagents used in this study are provided in *SI Appendix*.

Cells and Culture Conditions. Detailed culture conditions for human keratinocyte HaCaT cells and primary NHEK cells are provided in *SI Appendix*.

RT-PCR and Real-Time qPCR. Detailed RNA isolation, complementary DNA (cDNA) synthesis, primers, and qPCR probes are provided in *SI Appendix*. RT-PCR and qPCR were performed as described previously (39). The relative expression levels of *FLG* mRNA were normalized to those of GAPDH using the software program provided by the manufacturer (Bio-Rad).

Immunoblot Analysis. Immunoblot analysis was performed as previously described (14). Detailed procedures are provided in *SI Appendix*.

Immunofluorescence Microscopy. Keratinocytes cultured on coverslips were treated with drugs, fixed, permeabilized, and incubated with specific primary antibodies for 24 h at 4 °C and then, secondary antibodies for 1 h at 25 °C, followed by incubation with 1 μg/mL Hoechst 33258 as described previously (40). Fluorescent stained cells were detected using an EVOS FL fluorescence microscope (Advanced Microscopy Group).

Construction and Mutagenesis of Human FLG Promoter–Reporter Constructs. Detailed procedures for the generation of serial deletion constructs of human FLG promoter–reporter and PCR primers (*SI Appendix*, Table S1) are provided in *SI Appendix*. The QuikChange site-directed mutagenesis system (Stratagene) was used for site-specific mutation of the AP1 binding site (mtAP1) (41).

Luciferase Promoter–Reporter Assay. HaCaT keratinocytes were separately transfected with each *FLG* promoter–reporter construct using Lipofectamine 2000 (Invitrogen). Detailed procedures for the *FLG* promoter activity assay are provided in *SI Appendix*.

EMSA. DNA binding proteins were analyzed with the LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific). Detailed procedures for EMSA are provided in *SI Appendix*.

DAPA. DAPA was performed as reported previously (42). Briefly, after HaCaT cells were treated with or without TNFα + IFNγ, the extracted nuclear proteins were incubated overnight with streptavidin-conjugated agarose beads (Invitrogen) and biotinylated oligonucleotides identical to the probes used for EMSA. After washing with phosphate-buffered saline, the beads were collected and performed immunoblot analysis.

ChIP Assay. ChIP assay was carried out as reported previously (41). Briefly, HaCaT cells cultured with or without TNFα + IFNγ were exposed to 1% formaldehyde for DNA cross-linking and then, lysed using the Pierce Magnetic ChIP Kit (Thermo Fisher Scientific). The chromatin was sonicated and immunoprecipitated using a rabbit anti-HDAC1 antibody or normal rabbit IgG The precipitated DNA was amplified by PCR (*SI Appendix*, Table S1).

Induction and Assessment of Inflammation Severity of a DNCB-Induced AD Mouse Model and an IMQ-Induced Psoriasis Mouse Model. AD-like and psoriasis-like skin lesions were induced, as described previously (39, 43). Detailed experimental procedures and assessment of the severity of inflammation are provided in *SI Appendix*. The mouse experiments were carried out under the guidelines for animal experiments and procedures of the Konkuk University Institutional Animal Care and Use Committee and approved by this committee (approval nos. KU19129 [DNCB model], KU19080 [IMQ model], and KU22011 [antibody injection and inhibitors treatment in the DNCB model]).

Fluorescent Immunohistochemical Staining. Paraffin-embedded dorsal skin sections were prepared and fluorescent immunohistochemical staining was performed, as reported previously (39). Detailed experimental procedures are provided in *SI Appendix*.

Statistical Analyses. All data were presented as the mean ± SD. Statistical analyses were conducted using one-way ANOVA followed by Sidak's multiple comparisons test in GraphPad Prism version 8.4.2 (GraphPad Software, Inc.). For all analyses, a P value of less than 0.05 indicated a statistically significant difference.

Data Availability. All study data are included in the article and/or *SI Appendix*. Data will be shared and made available by request from a qualified academic investigator.

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