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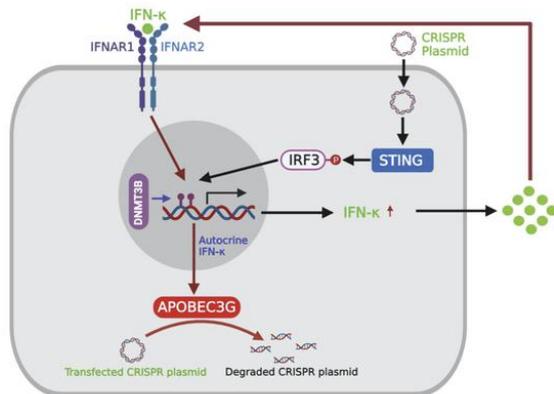
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Keratinocytes sense and eliminate CRISPR DNA through STING/IFN- κ activation and APOBEC3G induction

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One Sentence Summary: IFN- κ resists CRISPR transfection efficiency in keratinocytes

Abstract

CRISPR-Cas9 has been proposed as a treatment for genetically inherited skin disorders. Here we report that CRISPR transfection activates STING-dependent antiviral responses in keratinocytes, resulting in heightened endogenous interferon (IFN) responses through induction of IFN- κ leading to decreased plasmid stability secondary to induction of the cytidine deaminase *APOBEC3G*. Notably, CRISPR-generated KO keratinocytes had permanent suppression of IFN- κ and IFN-stimulated gene (ISG) expression, secondary to hypermethylation of the *IFNK* promoter region by the DNA methyltransferase DNMT3B. JAK inhibition via baricitinib prior to CRISPR transfection increased transfection efficiency, prevented *IFNK* promoter hypermethylation, and restored normal IFN- κ activity and ISG responses. This work shows that CRISPR-mediated gene correction alters antiviral responses in keratinocytes, has implications for future gene therapies of inherited skin diseases using CRISPR technology, and suggests pharmacologic JAK inhibition as a tool for facilitating and attenuating inadvertent selection effects in CRISPR-Cas9 therapeutic approaches.

Introduction

Keratinocytes (KCs) have long been recognized as one of the most difficult cell types to transfect (1, 2), but the mechanisms behind this resistance have remained unknown. KCs are the major cellular constituent of the epidermis, which acts as the primary interface between the body and external agents such as bacteria and viruses. Beyond their critical contribution to the epidermis as a physical barrier, KCs also have a very active immunological role through a range of pattern receptors and the ability to secrete various cytokines (3).

The prokaryote-derived clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 technology has transformed our ability to manipulate specific DNA and RNA sequences in living cells (4). The CRISPR-Cas9 system relies on the guide RNA (gRNA) for targeting specificity and functions by generating targeted DNA breaks that stimulate repair by various endogenous mechanisms. The CRISPR technology can be used both for the insertion or deletion of small DNA segments through the non-homologous end joining pathway (NHEJ) or single-base editing through homology-directed repair (HDR)(4). CRISPR-Cas9 has increasingly been applied to gene therapy for a wide range of human diseases including retinitis pigmentosa (5), Duchenne's Muscular Dystrophy (6), and monogenic dominant diseases such as epidermolysis bullosa (7-10), a devastating, frequently lethal, disease caused by mutations in structural genes involved in epidermal function. The role of KCs' intrinsic resistance to CRISPR-Cas9 transfection has not been addressed, nor has the role of KCs intrinsic antiviral machinery in mediating transfection resistance in KCs. Addressing this in KCs would greatly facilitate future efforts toward efficient gene correction in inherited epidermal diseases.

Results

Keratinocytes have constitutive STING-dependent IFN activity and are highly resistant to CRISPR/Cas9 transfection

To determine the transfection resistance of KCs, we compared transfection efficiency in human embryonic kidney-293T (HEK293T) cells, dermal fibroblasts, and KCs. Transfection efficiency using a liposome-based system was greater than 60% in HEK-293T cells, 7% in fibroblasts, and only 1% in keratinocytes (KCs) (Figure 1A and Supplemental Figure 1A). To further understand these differences in transfection efficacy, we observed that KCs have a constitutive expression of the interferon-stimulated gene (ISG) *MXI*, whereas this was seen in neither fibroblasts nor HEK-293T cells. This corresponded to increased expression of the type I IFN IFN- κ , which was detected only in KCs, and not fibroblasts or HEK-293T cells (Figure 1B, Supplemental Figure 1B, and Supplemental Figure 2A). Notably, we observed a significant increase in both *IFNK* and *MXI* mRNA expression in KCs after CRISPR plasmid transfection (Figure 1C and Supplemental Figure 2B), suggesting that the CRISPR plasmid is recognized by intracellular nucleic acid sensors in KCs. *IFNB1* also showed induction in KCs following CRISPR transfection, although to a much lesser extent (Supplemental Figure 3). The stimulator of interferon genes (STING) is known to control the induction of innate immune genes in response to the recognition of double-stranded DNA (dsDNA)(11).

To address the role of STING in response to CRISPR transfection in KCs, we generated *TMEM173* (STING protein) KO in KCs (Supplemental Figure 4). In contrast to WT KCs, *TMEM173/STING* KO completely abrogated both *IFNK* and *MXI* mRNA expression and the IFN response to CRISPR transfection (Figure 1D and Supplemental Figure 5A). STING activation results in the recruitment of the transcription factor interferon regulatory factor 3 (IRF3) and promotes

phosphorylation of IRF3(12) to activate type I IFNs and ISGs. We assessed phosphorylation of IRF3 (p-IRF3) by western blot in WT and KO KCs including *TMEM173* and *IFNK* KOs. Whereas robust p-IRF3 was seen in WT, control KO, and *IFNK* KO KCs, p-IRF3 was markedly reduced in the *TMEM173* KO KCs upon CRISPR-Cas9 transfection (Figure 1E and Supplemental Figure 6). These data suggest that CRISPR-Cas9 transfection induces IFN- κ and ISGs in KCs through activation of the STING pathway. Notably, this activation of the STING pathway was not dependent upon the constitutive activity of IFN- κ , as IRF-3 was robustly phosphorylated in *IFNK* KO KCs. We next examined whether the upregulation of IFN- κ varied by KC differentiation state. IFN- κ has an established role in host defense against viral pathogens such as human papilloma viruses (HPV)(13, 14), and non-oncogenic HPV infections typically do not involve the basal layer of the epidermis (15) and are instead localized in the upper spinous layers (16). Consistent with a stronger IFN- κ response in the basilar KCs, both *TMEM173*/STING and *IFNK* mRNA expression was highest in undifferentiated KRT5⁺ basal epithelium, in contrast to more differentiated KCs (FLG) and corresponded to open chromatin areas around the *IFNK* promoter as shown by single-cell ATAC-seq (Figure 1F, Supplemental Figure 7). Consistent with this observation, single-cell RNA-sequencing of epidermal cells demonstrated that both IFN- κ and a majority of ISGs are primarily expressed in the basal layer of the epidermis (Figure 1G). These observations would predict that KCs in the basal layer of the epidermis would be more resistant to CRISPR-Cas9 transfection.

STING-dependent induction of the cytidine deaminase APOBEC3G restricts CRISPR-Cas9 transfection efficiency in KCs.

To determine if the uptake of the CRISPR-Cas9 plasmid is defective in keratinocytes we measured the uptake and stability of CRISPR-Cas9 GFP-tagged plasmids in KCs at different time points

after transfection. While CRISPR-Cas9 GFP⁺ was observed in approximately 6-8% of KCs at early time points, this rapidly decreased to 1-2% over a period of 48 hours (Figure 2A and Supplemental Figure 8A). This uptake followed by rapid disappearance suggests that KCs actively degrade the CRISPR plasmid shortly after transfection, and prior to the interaction of CRISPR-Cas9 with its DNA target. DNases such as DNase I and DNase II, along with the APOBEC3 protein family of cytidine deaminases, have been shown to mediate the clearance of foreign DNA from human cells (17-19). To assess the involvement of DNase I, DNase II, and APOBEC3 family members in the clearance of CRISPR-Cas9 plasmids from KCs after transfection, we used RNA-seq to compare the expression profiles for type I IFN treated versus *IFNK* KO KCs. While the majority of the APOBEC3 family members showed increased mRNA expression, only minor shifts were seen for *DNASE1*, and no changes were observed for *DNASE2* mRNA expression. Correspondingly, *IFNK* KO KCs had decreased mRNA expression of three of the APOBEC3 members; *APOBEC3A*, *APOBEC3F*, and *APOBEC3G*, whereas only *APOBEC3H* was increased (Figure 2B). To determine the potential role of these four APOBEC3 members and *DNASE1* in CRISPR-Cas9 plasmid stability, we used siRNA to knock down each of the four *APOBEC3* genes and *DNASE1* in KCs. Only si*APOBEC3B* and si*APOBEC3G* treatments increased plasmid stability (Figure 2C, Supplemental Figure 8B-D). To determine the relationship of APOBEC3G with epidermal differentiation and *IFNK* mRNA expression, we analyzed RNA-seq data from monolayer KCs and epidermal raft systems. This showed an inverse relationship with the differentiation stage of both *IFNK* and *APOBEC3G*, with more differentiated KCs having lower expression (Figure 2D). Immunostaining corroborated this, with APOBEC3G expression strongest in the basal layer of the epidermis, co-localizing with IFN- κ (Figure 2E). Consistent with the role of TMEM173/STING in regulating IFN responses to CRISPR-Cas9 transfection, we observed

significant suppression of *APOBEC3G* mRNA expression in *TMEM173* KO KCs (Figure 2F and Supplemental Figure 5). These data suggest that STING/IFN- κ -dependent induction of APOBEC3 cytidine deaminases is responsible for CRISPR-Cas9 plasmid degradation in KCs.

CRISPR-Cas9 generated KO KCs have suppressed *IFNK* and ISG mRNA expression

We next examined whether successful transfection of keratinocytes was dependent on repression of *IFNK* expression. Consistent with this notion, we observed that CRISPR/Cas9-generated KOs in KCs have suppressed *IFNK* and ISG mRNA expression, and this was universal across all KC KOs generated, irrespective of the gene target (Figure 3A and Supplemental Figure 9). CpG methylation is a common epigenetic mark for transcriptional regulation (20) and is a known mechanism for controlling *IFNK* expression (21). We, therefore, examined whether CpG methylation could account for *IFNK* and ISG suppression in successfully transfected cells. Treatment of KO KCs with a demethylating agent, 5-dAza-c, led to a significant increase of both *IFNK* (Figure 3B) and *MXI* mRNA expression (Supplemental Figure 10) in all KO KCs treated. Additionally, bisulfite sequencing of the *IFNK* promoter revealed a marked increase in CpG methylation in CRISPR KO compared to WT KCs (Figure 3C and Supplemental Figure 11). DNA methyltransferases (DNMTs) are involved in CpG methylation (22) and are expressed in the skin (23). To determine the role of DNMT methyltransferases in IFN- κ regulation, we generated KCs overexpressing *DNMT1*, *DNMT3A*, or *DNMT3B*. Only *DNMT3B* overexpression led to significant suppression of *IFNK* mRNA expression (Figure 3D), which was accompanied by CpG hypermethylation of the *IFNK* promoter region (Supplemental Figure 12).

We then validated the relevance of this mechanism *in vivo*. Indeed, *DNMT3B* expression positively correlates with epidermal differentiation, exhibiting the highest expression in fully differentiated epidermal rafts (Figure 3E). Consistent with these findings, *DNMT3B* expression was higher in

CRISPR-generated KO compared to WT KCs (Supplemental Figure 13). Confirmatory immunostaining in healthy epidermis showed preferential nuclear expression of the DNMT3B protein in the upper layers of the epidermis, whereas staining was minimal in the lower layers of the epidermis (Figure 3F), where IFN- κ and APOBEC3G were typically co-localized in the basal layer of the epidermis (Figure 2E). These data demonstrate suggest that DNMT3B is a negative regulator of IFN- κ expression via hypermethylation of the *IFNK* promoter IFN- κ and provide novel insights into the mechanisms that regulate IFN- κ activity during epidermal differentiation.

Inhibition of type I IFN autocrine responses through pharmacologic JAK inhibition improves transfection efficiency and prevents generation of *IFNK* low KO KCs.

KC expression of IFN- κ is induced by CRISPR-Cas9 transfection, and IFN- κ directly affects the expression of APOBEC3 cytidine deaminases that in turn promote degradation of intracellular CRISPR-Cas9 plasmids. To determine if inhibiting type I IFN signaling affects CRISPR-Cas9 transfection efficacy we used *IFNK* and *TYK2* KO KCs to interrupt this IFN autocrine loop. Intriguingly, we observed a marked increase in transfection efficiency (indicated by increased GFP positivity) in both *IFNK* and *TYK2* KO KCs. Furthermore, the control KO KCs showed approximately threefold increased transfection efficacy compared to *WT* KCs (Figure 4A), likely due to suppressed IFN- κ autocrine responses (Figure 3A). Consistent with these findings, we also observed increased stability of CRISPR plasmids over time in the *IFNK* KO KCs (Figure 4B). To validate these findings and determine if pharmacologic inhibition of Janus kinase (JAK)/IFN signaling would reproduce these findings, we used the JAK1/JAK2 inhibitor baricitinib. Baricitinib effectively decreased mRNA expression of both *IFNK* and the ISG *MX1* in a dose-dependent manner (Figure 4C) and increased transfection efficiency (Figure 4D) to the same level seen in *IFNK* and *TYK2* KO KCs (Figure 4A). We reproduced these findings in primary

keratinocytes that baricitinib significantly increased the transfection efficiency in primary human keratinocytes (Supplemental Figure 14). To determine whether IFN- κ affects and promotes selection of *IFNK* and ISG low-expressing KC KOs, we performed CRISPR-Cas9 transfection in the presence or absence of baricitinib (Supplemental Figure 15). Indeed, *IFNK* and *MXI* expression remained intact in KO KCs generated in the presence of baricitinib (Figure 4E). The reversal of the *IFNK* expression in the KO lines after baricitinib was illustrated by schematic representation (Supplemental Figure 15). As expected, KO KCs generated in the presence of baricitinib did not have hypermethylation of the *IFNK* promoter region, in stark contrast to KO KCs generated without baricitinib (Figure 4F).

Discussion

KCs constitute ~90% of the cells in the epidermis (24). Given the constant onslaught of external agents and microbiota such as bacteria and viruses, KCs are highly active as sentinels and harbor a range of antimicrobial detectors and pattern recognition receptors for a wide range of pathogens (25). IFN- κ is the predominant type I IFN expressed by KCs and is most prominently expressed in the basal layer of the epidermis (26). The role of this axis in anti-viral defenses can be best described in the context of human HPV infections, which are caused by a DNA virus. HPV infections classically involve the mid to upper layers of the epidermis (27), where HPV viral genome amplification occurs (28). Interestingly, HPV viruses antagonize the cGAS-STING-DNA-sensing pathway to facilitate infection (29). Here we demonstrate that CRISPR plasmids activate the same type of anti-viral response through STING and identify cytidine deaminase APOBEC3G as a key regulator in limiting CRISPR transfection in KCs (Figures 2, B, and C).

A surprising observation was that KC KOs generated by CRISPR-Cas9 had permanent suppression of *IFNK* mRNA expression, and ISG responses, secondary to *IFNK* promoter hypermethylation.

Under normal physiologic conditions in the epidermis, IFN- κ expression is sharply localized to the basal layer of the epidermis, and its expression rapidly tapers off in more differentiated layers of the epidermis (26), suggesting that *IFNK* is actively being turned off during the differentiation process. This decrease in *IFNK* coincides with increased expression of the DNA methyltransferase DNMT3B (Figure 3E), which we demonstrate here to be responsible for the *IFNK* promoter hypermethylation in KO KCs, and the subsequent suppression of *IFNK* mRNA expression (Figure 3, C, and D and Supplemental Figure 13). Our data, therefore, suggest that CRISPR transfection is more efficient in cells where IFN- κ has been “turned off” through promoter methylation. This is analogous to HPV infection, which selectively affects KCs that do not express IFN- κ (30), and additionally explains why HPV infections and viral replication predominantly involve mid to upper layers of the epidermis(27). In this context, it is worth noting that DNMT3B expression has been shown to correlate with HPV infection (31, 32), and APOBEC3 members have been shown to restrict HPV infection (33). HPV can also actively suppress IFN- κ expression through the function of the oncogenic proteins E6 and E7 (34, 35)), thereby enabling the virus to gain entry into the lower layers of the epidermis where the epidermal stem cells reside.

Prior studies that have looked at immunological processes that may interfere with CRISPR transfection have focused on the potential immunogenicity of the Cas proteins, particularly regarding pre-existing adaptive immunity to *Streptococcus pyogenes* and *Staphylococcus aureus* (36-38). Furthermore, CRISPR gene editing is more efficient in cells that have lost the function of the tumor suppressor p53, as shown in retinal epithelial cells(39), and in human pluripotent stem cells(40). The interplay between CRISPR-Cas9 and intracellular viral sensing, pathways has not previously been addressed, and it is likely, based on the data presented here, that this IFN κ -APOBEC3G pathway would apply to other transfection systemic dependent on DNA-based

plasmids, but whether the same mechanism extends to RNA-based transfections remains to be determined.

The use of CRISPR to correct various inherited disorders of the skin holds great promise. The data presented here provide several novel insights into the molecular mechanisms behind KC transfection resistance, the consequences including suppression of IFN responses in genetically corrected KCs, and provides a simple way, with the use of JAK inhibition, to circumvent these issues.

Methods

Keratinocyte cell culture and treatment

Immortalized keratinocyte cell line, N/TERT (N/TERT-2G)(41), was used with permission from Dr. James G. Rheinwald for the generation of knock-out (KO) cell lines using non-homologous end joining via CRISPR/Cas9. This cell line has been shown to have normal differentiation characteristics in both monolayer and organotypic skin models(41). N/TERTs were grown in Keratinocyte-SFM medium (ThermoFisher #17005-042) supplemented with 30 µg/ml bovine pituitary extract, 0.2 ng/ml epidermal growth factor, and 0.3 mM calcium chloride(42). Keratinocytes (WT and KO) were treated with a demethylating agent, 5'-Aza-2'-deoxycytidine (5-dAza-C) (10 µM, Millipore-Sigma # A3656-5MG) for the restoration *IFNK* expression following protocol described in this paper(34).

Generation of CRISPR KO lines in N/TERTs KCs

CRISPR KO KCs were generated as previously described in (26). In brief, Single-guide RNA (sgRNA) target sequence was developed (Supplemental Table 1) using a web interface for CRISPR design (<https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design>). Synthetic sgRNA target sequences were inserted into a cloning backbone, pSpCas9 (BB)-2A-GFP (PX458) (Addgene plasmid # 48138), and then cloned into competent *E. coli* (ThermoFisher # C737303). Proper insertion was validated by Sanger sequencing. The plasmid with proper insertion was then transfected into an immortalized KC line (N/TERTs) using the TransfeX transfection kit (ATCC # ACS4005) in the presence or absence of JAK1/JAK2 inhibitor, baricitinib (10 µg/ml). GFP-positive single cells were plated and then expanded. Cells were then genotyped and analyzed by Sanger sequencing.

Generation of overexpressing KCs lines

DNMT1, *DNMT3A*, and *DNMT3B* over expressing KCs were generated by lentiviral transductions of mammalian vector containing Myc-DDK-tagged human *DNMT1* (NM_001130823, Origene), *DNMT3A* (NM_175629, Origene), and *DNMT3B* (NM_006892, Origene) respectively. HEK293T cells were used for viral packaging. Briefly, 10 μ g of expression vector was mixed with an equal concentration of packaging plasmid (TR30037, Origene) in 1ml Opti-MEM medium (31985062, Invitrogen) and 30 μ l turbofectin (TF8100, Origene), incubated at room temperature for 5 minutes. The obtained mixture was added to the HEK293T cells without dislodging the cells. Supernatants from infected HEK293T cells were harvested after 24 hours, filtered using 0.45 μ m syringe filters, aliquoted, and stored at -80c or used immediately. 0.25 x 10⁶ N/TERTs KCs were plated a day before transduction in serum and antibiotics-free medium. The next day, the cells were transduced at a multiplicity of infection of 0.5 along with 8 μ g/ml of polybrene (TR-1003-G, Sigma Aldrich). 24 hours post-transfection, media was replaced with complete growth media. Cells were passaged the following day and the media containing puromycin was used from then on. Puromycin concentration for KCs was determined by performing a drug-kill curve. An empty mammalian expression vector containing Myc-DDK tag only (PS100001, Origene) was used as a negative control for transduction experiments. Un-transduced cells were also treated with puromycin to observe complete cell death. Once all the cells in control wells were killed, limited dilution was performed to obtain single cells which were expanded, and the clones were verified for over-expression by western blotting.

RNA extraction and qRT-PCR

RNA extraction, qRT-PCR, and RNA-Sequencing were performed following the protocol we published earlier(26). RNAs were isolated from cell cultures using Qiagen RNeasy plus kit (Cat

#74136). qRT-PCR was performed on a 7900HT Fast Real-time PCR system (Applied Biosystems) with TaqMan Universal PCR Master Mix (ThermoFisher Scientific) and the TaqMan probe (*IFNK*: Hs00737883_m1; *MXI*: Hs00895608_m1; *TMEM173*: Hs00736955_g1; *APOBEC3A*: Hs02572821_s1; *APOBEC3B*: Hs00358981_m1; *APOBEC3C*: Hs00819353_m1; *APOBEC3D*: Hs00537163_m1; *APOBEC3F*: Hs01665324_m1; *APOBEC3G*: Hs00222415_m1; *APOBEC3H*: Hs00419665_m1; *FLG*: Hs00856927_g1; *CCL5*: Hs00982282_m1; *CXCL10*: Hs00171042_m1; *IFIT2*: Hs01584837_s1; *IFNL1*: Hs00601677_g1; *IRF7*: Hs01014809_g1; *DNMT3B*: Hs00171876_m1; *IFNBI*: Hs01077958_s1; *TREX1*: Hs03989617_s1; *DNASEI*: hs00173736_m1; *DNASEII*: Hs00172391_m1).

Single Cell RNA-sequencing from human skin

Generation of single-cell suspensions for single-cell RNA-sequencing (scRNA-seq) was performed as follows from the normal human epidermis. Samples were incubated overnight in 0.4% dispase (Life Technologies) in Hank's Balanced Saline Solution (Gibco) at 4°C. Epidermis and dermis were separated. The epidermis was digested in 0.25% Trypsin-EDTA (Gibco) with 10U/mL DNase I (Thermo Scientific) for 1 hour at 37°C, quenched with FBS (Atlanta Biologicals), and strained through a 70µM mesh. The dermis was minced, digested in 0.2% Collagenase II (Life Technologies) and 0.2% Collagenase V (Sigma) in a plain medium for 1.5 hours at 37°C, and strained through a 70µM mesh. Epidermal and dermal cells were recombined, and libraries were constructed by the University of Michigan Advanced Genomics Core on the 10X Chromium system. Libraries were then sequenced on the Illumina NovaSeq 6000 sequencer to generate 151-bp paired-end reads. Data processing including quality control, read alignment, and gene quantification was conducted using the 10X Cell Ranger software. Seurat was used for normalization, data integration, and clustering analysis(43). Clustered cells were mapped to

corresponding cell types by matching cell cluster gene signatures with putative cell-type specific markers. The scRNA-seq data can be found at Gene Expression Omnibus (GEO # GSE179162).

Single Cell ATAC-sequencing from human skin

4mm skin biopsies were obtained from the palm/hip of a healthy individual. Biopsies were then incubated in 0.4% dispase overnight to separate the epidermis and dermis. After the separation, the epidermis was transferred to 0.25% Trypsin-EDTA + 10 units/mL DNase mixture and incubated at 37°C for 1hr. The Epidermis mixture was then quenched with FBS and precipitated by centrifugation. Cell pellets were then resuspended in PBS + 0.04% BSA. Cell numbers were counted at this step for future dilution calculation. The nuclei isolation protocol was carried out as described by 10x Genomics. Of note, cells obtained from the epidermis were incubated in lysis buffer on ice for 7 min to achieve the best lysis efficacy. The cell lysis efficacy was determined by Countess II FL Automated Cell Counter. The single-cell ATACseq library was prepared by Advanced Genomics Core at the University of Michigan. 10,000 nuclei/sample and 25,000 reads/nuclei were targeted, and the libraries were sequenced using NovaSeq SP 100 cycle flow cell. The raw data were first processed by the Chromium Single cell ATAC Software Suite (10x Genomics), and then analyzed using the Signac package in R. Briefly, the single-cell ATACseq data will go a serial of analyses including quality control, dimension reduction, clustering, and integration with previously annotated single cell RNAseq data. DNA accessibility profile was then visualized in different cell types and samples. The scATAC-seq data can be found at Gene Expression Omnibus (GEO # GSE226926).

Accell siRNA knockdown

N/TERTs KCs were plated in 96 well plates (30,000 cells/well) and incubated at 37°C with 5% CO₂ overnight. 100 μM Accell siRNA (Dharmacon, *APOBEC3A*: E-017432-00-0005;

APOBEC3B: E-017322-01-0005; *APOBEC3G*: E-013072-00-0005; *APOBEC3H*: E-019144-00-0005; DNASE1: E-016280-00-0005) was prepared in 1x siRNA buffer (Dharmacon# B-002000-UB-100). 1 μ l of 100 μ M siRNA was diluted with 100 μ l Accell delivery medium (Dharmacon # B-005000) for each well of 96 well plates. The growth medium was removed from the cells and 100 μ l of the appropriate delivery mix with siRNA was added to each well and the plate was incubated at 37°C with 5% CO₂. Accell Non-targeting Control siRNA (Dharmacon # D-001910-01-05) was used as a negative control. After 72 Hrs cells were harvested for RNA preparation. RNA isolation and qRT-PCR were as above.

3-D human epidermal tissue cultures

Normal human epidermal KCs were isolated from a pool of neonatal foreskins (n=3) and grown using J2-3T3 mouse fibroblasts as feeder layer as originally described by Rheinwald and Green(44). 3-D human epidermal raft cultures seeded in collagen hydrogels were prepared using three distinct donor pools as described previously(45) and grown at an air-liquid interface for 12 days in E-Medium (DMEM/DMEM-F12 (1:1), 5% Fetal Bovine Serum, adenine (180 μ M), Bovine pancreatic insulin (5 μ g/ml), Human apo- transferrin (5 μ g/ml), triiodothyronine (5 μ g/ml), L- Glutamine (4mM), Cholera toxin (10ng/ml), Gentamicin (10 μ g/ml), Amphotericin B (0.25 μ g/ml)). After 9 days at an air-liquid-interface to allow for epidermal maturation, the RHEs were treated with 0.1% BSA/phosphate-buffered saline (Sigma Aldrich, St Louis, MO) as vehicle control or 10.0 ng/ml TNF-a, IL-17A, IL-22 (R&D Systems, Minneapolis, MN) alone or as a combination for 72 h, harvested, and analyzed for changes in gene expression as described (46). Epidermal tissues were separated from the collagen scaffold and lysed in QIAzol for RNA isolation. RNA-seq and analysis were performed according to the methods mentioned above.

Measurement of CRISPR plasmids stability in KCs

CRISPR plasmid (PX458, Addgene # 48138) was transfected into KCs using a Transfex transfection kit (ATCC # ACS-4005). Cells were then harvested at different time points (0 Hr, 6 Hrs, 12 Hrs, 24 Hrs, and 48 Hrs) and washed with PBS three times to remove extracellular plasmid from the cells. The DNA was then purified using the QIAamp DNA Blood Mini kit (Qiagen # 51106). CRISPR plasmid-specific primers (GFP-F1: GGAGAGGGCAGAGGAAGTCT and GFP-R1: GAACTTCAGGGTCAGCTTGC) were used to perform qPCR with the DNAs isolated from the transfected KCs using SYBR Green PCR Master Mix (ThermoFisher # 4309155) on the 7300 Real-time PCR system (Applied Biosystems).

Transfection efficiency measurement

Different cell types such as keratinocytes, fibroblasts, and HEK293T cells were grown in their respective culture medium and the cells were then transfected with either GFP-linked CRISPR plasmid (PX458 from Addgene) or non-CRISPR plasmid (pCMV-GFP from Addgene) using Transfex kit (ATCC) or FuGene6 (Promega). Cells were then kept in a CO₂ incubator for 24 hours. Cells were then harvested using 0.25% Trypsin-EDTA and then resuspended in PBS. GFP-positive cells were then analyzed at our University of Michigan Flow Cytometry core. We consider the percentage of GFP-positive cells as the transfection efficiency of the respective cell types.

Bisulfite sequencing analysis of the *IFNK* promoter

Based on the vendor's recommendations, bisulfite treatment was performed on DNA isolated from wild-type and CRISPR knock-out KCs using the EZ DNA methylation-Gold kit (Zymo Research # D5005). Bisulfite-converted DNA was amplified with the following primers, IFNK-BS-F9: TGTTGGGATGGATTATTTAGGTATT and IFNK-BS-R9: TTCAACAAAAAATTTTCTCATTC. PCR products were cloned in pCR2.1-TOPO vector

(ThermoFisher # K204040) and those clones were then subjected to Sanger sequencing using M13Rev and T7 primers.

Western Blot

Total protein was isolated from cells using Pierce RIPA buffer (89900, ThermoFisher) with PMSF Protease Inhibitor (36978, Sigma) and run-on pre-cast gel (456-1094S, Bio-Rad). The membrane was blocked with 3% BSA and then probed by primary antibodies including p-IRF3 (ThermoFisher# PA536775), IRF3 (Abcam # ab68481), p-STING (Cell Signaling # 19781S), p-STAT1 (ThermoFisher # 33-3400) DNMT3B (Cell signaling # 67259S) and β -Actin (A5441, Sigma), followed by secondary antibodies (anti-mouse or rabbit IgG, AP-linked Antibody, Cell Signaling), then washed for 3 times, and substrate added (45-000-947, Fisher Scientific). They were then imaged with chemiluminescent kit and imaged on an iBright imager (ThermoFisher).

Immunostaining

Formalin-fixed, paraffin-embedded tissue slides obtained from healthy individuals were heated for 30 min at 60°C, rehydrated, and epitope retrieved with tris-EDTA (pH 6). Slides were blocked and incubated with primary antibodies against IFN- κ (Abnova # H00056832-M01), APOBEC3G (Abcam # Ab223704), and DNMT3B (Cell Signaling # 67259S) overnight at 4°C. Slides were incubated with biotinylated secondary antibodies (biotinylated goat anti-rabbit IgG antibody, BA1000, Vector Laboratories; biotinylated horse anti-mouse IgG antibody, BA2000, Vector Laboratories) and then incubated with fluorochrome-conjugated streptavidin. Slides were prepared in mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (VECTASHIELD Antifade Mounting Medium with DAPI, H-1200, VECTOR). Images were acquired using an inverted Zeiss microscope. Images presented are representative of at least three biologic replicates.

Statistics

Data were analyzed using GraphPad Prism 9.0. Data are represented as the mean \pm SEM. The numerical results between 2 groups were analyzed by a 2-tailed Student's t-test and multiple comparisons were analyzed by 1-way ANOVA with post-hoc Tukey's test.

Study approval

Human skin samples were obtained under University of Michigan IRB-approved protocol (HUM00151834), and all subjects consented to a 6 mm punch biopsy. The study was conducted according to the Declaration of Helsinki Principles.

Author contributions

MKS and JEG conceptualized the study. MKS, RU, AK, XX, SS, OP, SS, EX, and YJ performed experiments and analyzed data. MKS developed CRISPR/Cas9 method in keratinocytes. CZ, ACB, and LCT analyzed single-cell ATAC-seq and RNA-seq data. BEPW provided RNAs from 3D raft keratinocytes. MKS, JEG, LCT, and JMK acquired funding for this research. MKS and JEG wrote the original draft of the manuscript. MKS, RU, CZ, ACB, LCT, AK, XX, BEPW, SS, OP, SS, EX, YJ, KAG, JJV, JMK, and JEG reviewed and edited the manuscript.

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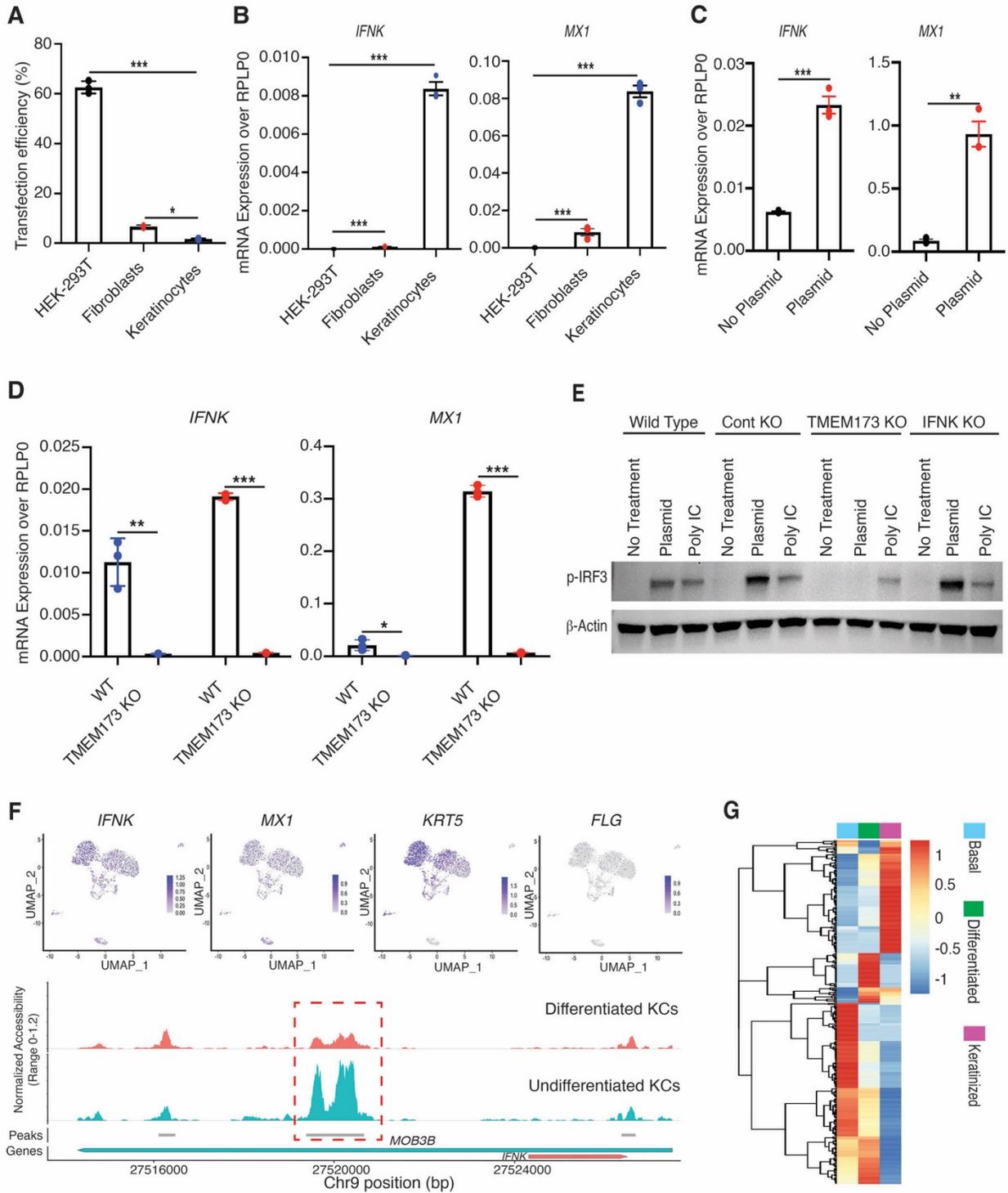


Figure 1. Keratinocytes activate type I IFN responses and sense foreign DNA through the STING pathway and are resistant to CRISPR-cas9 transfection (A) Comparison of transfection efficiency in keratinocytes (KC), fibroblasts (FBs) and human embryonic kidney 293T (HEK-293T) cells (Data are represented as mean \pm SEM; *P < 0.05, ***P < 0.001, 1-way ANOVA with Tukey's test; n=3). (B) *IFNK* and *MXI* mRNA expression in KC, FBs, and HEK-293T cells (Data are represented as mean \pm SEM; ***P < 0.001, 1-way ANOVA with Tukey's test; n=3). (C) Induction of *IFNK* and IFN-stimulated gene *MXI* by CRISPR plasmid transfection (Data are represented as mean \pm SEM; **P < 0.01, ***P < 0.001, 2-tailed Student's t-test; n=3). (D) *IFNK* and *MXI* expression WT and *TMEM173* (STING) KO keratinocytes treated with CRISPR plasmid (Data are represented as mean \pm SEM; *P < 0.05, **P < 0.01, ***P < 0.001, 2-tailed Student's t-test; n=3). Bars with blue dots: no treatment; bars with red dots: CRISPR plasmid treatment (E) Phospho-IRF3 western blot in plasmid-treated KO KCs. (F) Single-cell ATAC-seq from healthy human epidermis shows the overlap between *IFNK*, *MXI*, and *KRT5* open chromatin regions (upper panels). Chromatin accessibility in the *IFNK* promoter region is greater in undifferentiated KCs compared to differentiated KCs (indicated by the dotted red box, lower panel). (G) Heatmap of type I IFN responsive genes from scRNA-seq data of healthy human epidermis shows localization of majority of IFN response genes in the basal epidermal compartment (n=3). (Color code for heat map: red indicates higher expression and blue denotes the lower expression of type I IFN responsive genes)

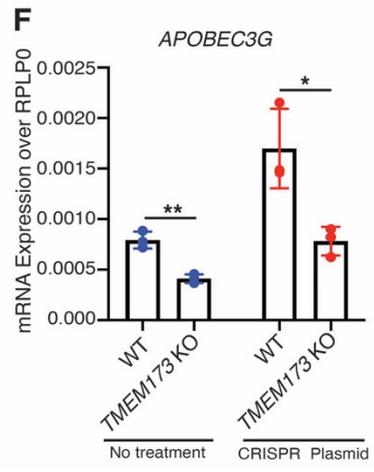
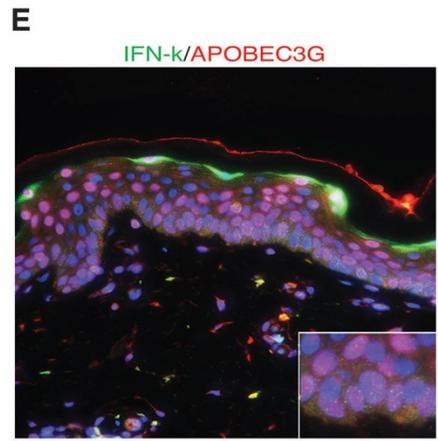
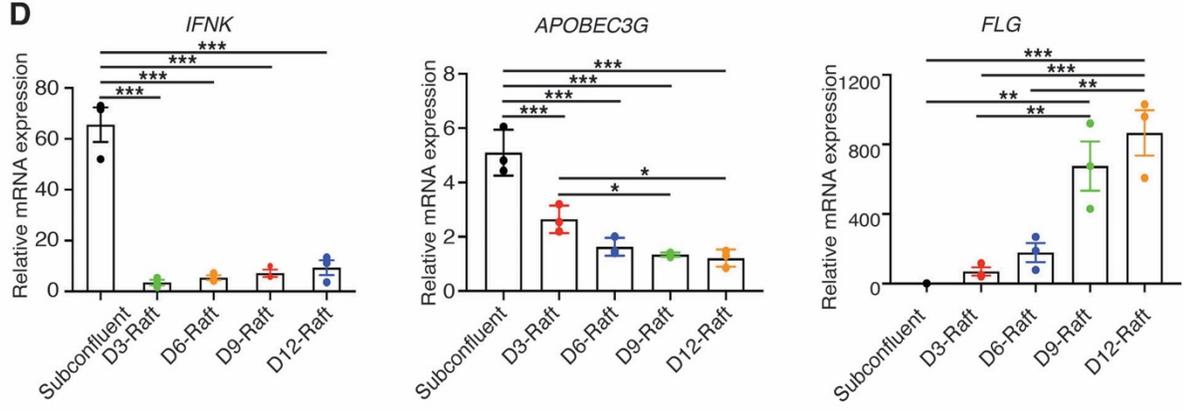
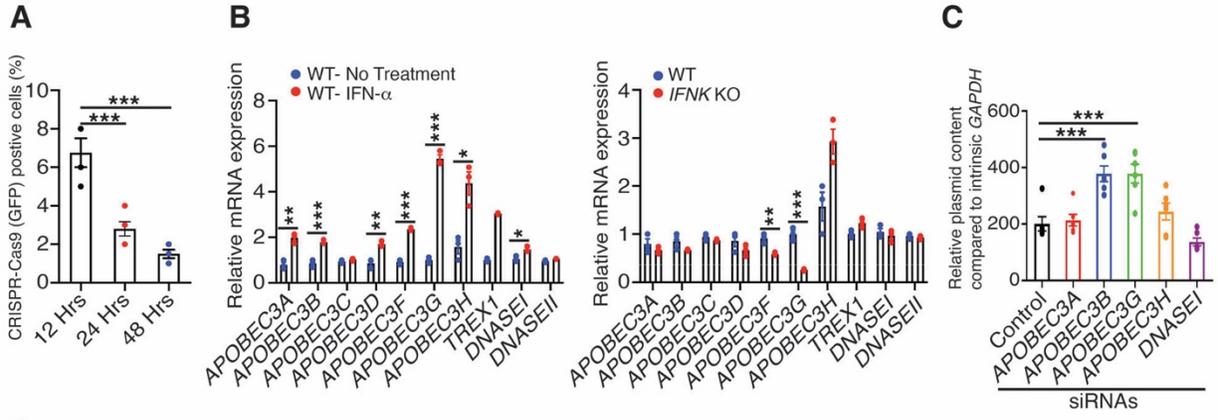


Figure 2. STING-dependent induction of the cytidine deaminase APOBEC3G restricts CRISPR/Cas9 transfection efficiency in keratinocytes. (A) Percentage of GFP positive cells at different time points after CRISPR transfection. (Data are represented as mean \pm SEM; ***P < 0.001, 1-way ANOVA with Tukey's test; n=3). (B) *APOBEC3s* mRNA expression in IFN- α treated KCs and *IFNK* KO KCs (Data are represented as mean \pm SEM; *P < 0.05, **P < 0.01, ***P < 0.001, 2-tailed Student's t-test; n=3) (C) CRISPR plasmid stability in *APOBEC3s* siRNA treated KCs (Data are represented as mean \pm SEM; ***P < 0.001, 1-way ANOVA with Tukey's test; n=5). (D) Expression of *IFNK*, *APOBEC3G*, and *FLG* mRNA in subconfluent monolayer cultures and 3D epithelial raft cultures at different stages of differentiation (day(D)3 through D12) (Data are represented as mean \pm SEM; *P < 0.05, **P < 0.01, ***P < 0.001, 1-way ANOVA with Tukey's test; n=3). (E) APOBEC3G (red) and IFN- κ (green) immunostaining in healthy skin (n=3). (F) *APOBEC3s* mRNA expression in *TMEM173* KO KCs (Data are represented as mean \pm SEM; *P < 0.05, **P < 0.01, 2-tailed Student's t-test; n=3).

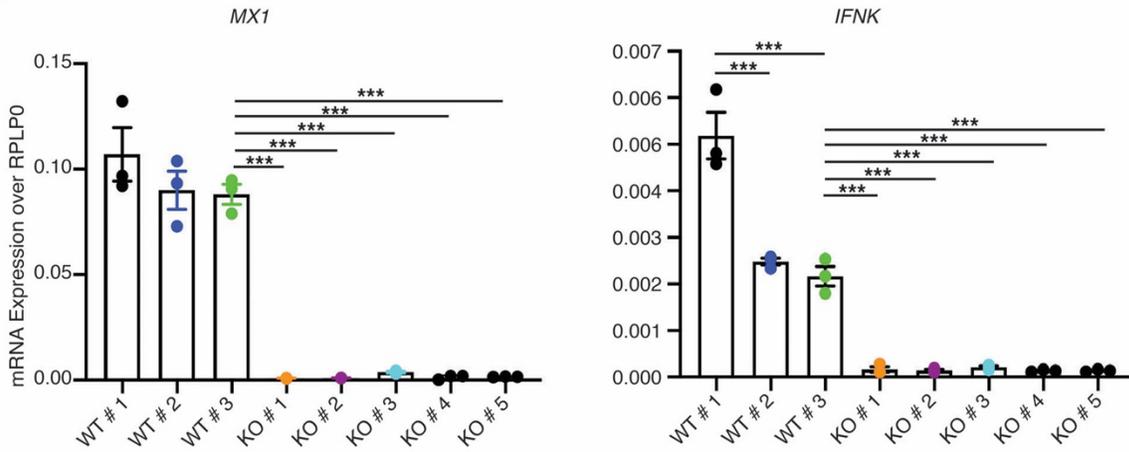
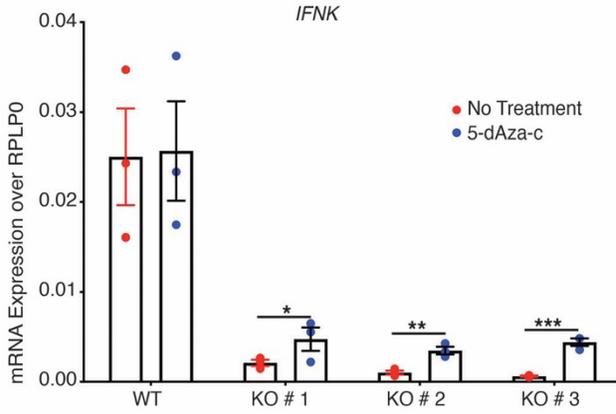
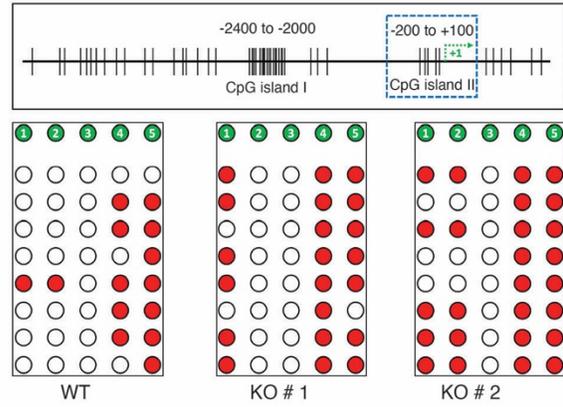
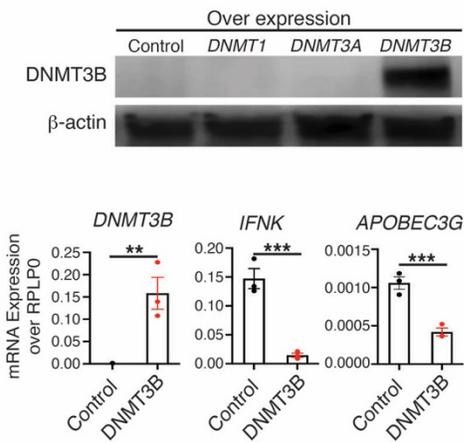
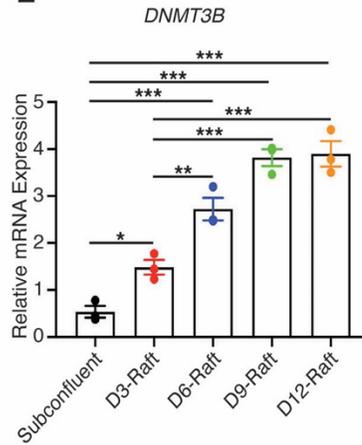
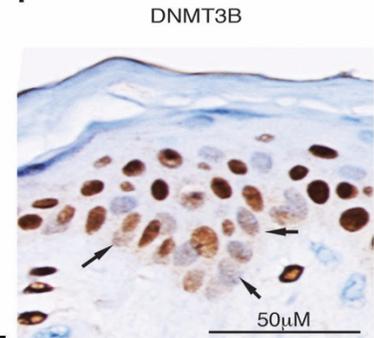
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Figure 3. CRISPR-cas9 generated keratinocytes KOs have suppressed type I IFN responses and *IFNK* expression. (A) Decreased *IFNK* expression and type I interferon response (*MXI* expression) in CRISPR-Cas9 generated KO KCs (Data are represented as mean \pm SEM; ***P < 0.001, 1-way ANOVA with Tukey's test; n=3). (B) Reversal of *IFNK* expression in CRISPR-Cas9 generated KO KCs after treatment with the demethylating agent 5-dAza-c (Data are represented as mean \pm SEM; *P < 0.05, **P < 0.01, ***P < 0.001, 2-tailed Student's t-test; n=3). (C) CpG hypermethylation in the *IFNK* promoter region in KO KCs (KO#1 and KO#2) compared to non-transfected WT control (n=8). (D) Western blot of the DNA methyltransferase, DNMT3B in transgenic overexpressing DNMT1, DNMT3A, and DNMT3B KCs (each lane is representative of n=3 independently transfected KCs (upper panel)). Suppression of *IFNK* and *APOBEC3G* mRNA expression in *DNMT3B* transgenic KCs (lower panel, Data are represented as mean \pm SEM; **P < 0.01, ***P < 0.001, 2-tailed Student's t-test; n=3). (E) *DNMT3B* mRNA expression in 3D epithelial rafts at different stages of differentiation (Data are represented as mean \pm SEM; *P < 0.05, **P < 0.01, ***P < 0.001, 1-way ANOVA with Tukey's test; n=3). (F) DNMT3B protein expression is low in the basal layer (arrows) but increases progressively in the more differentiated layers of the epidermis (n=3).

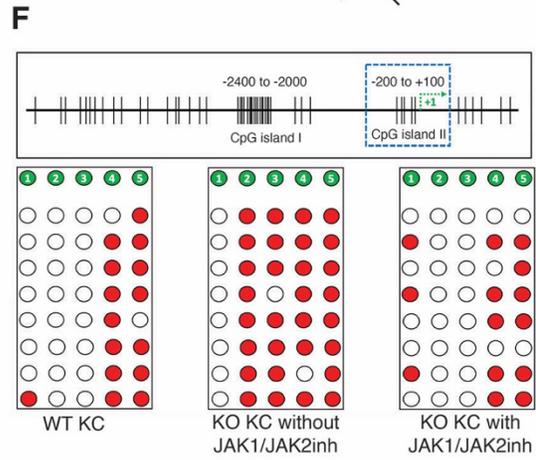
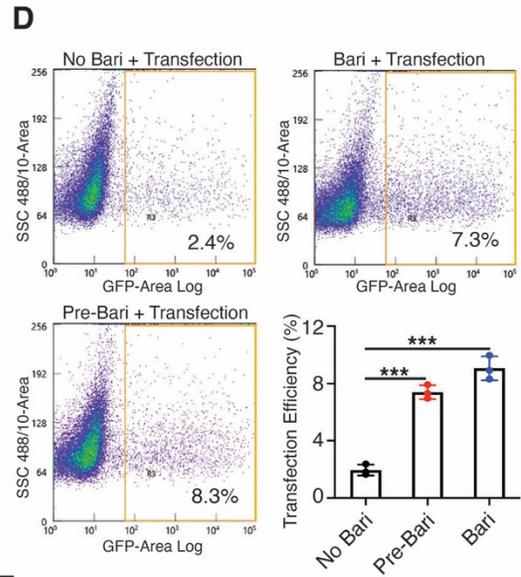
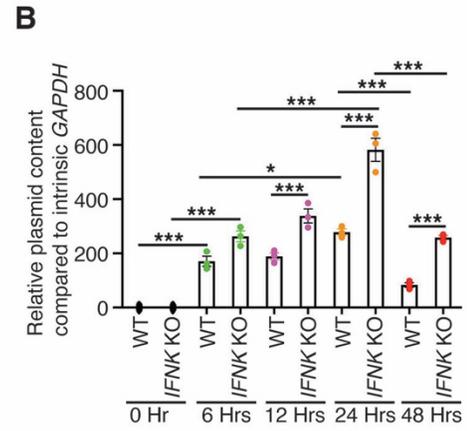
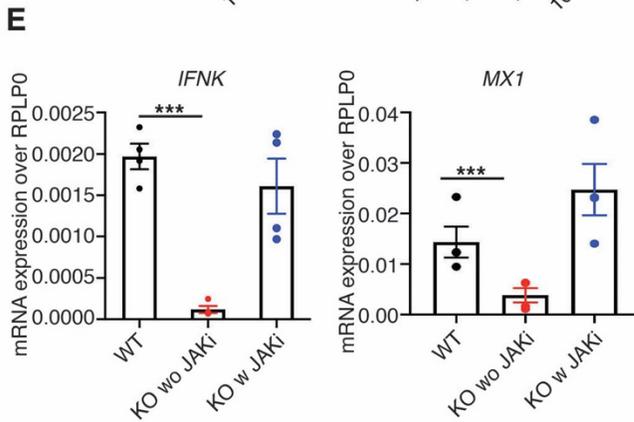
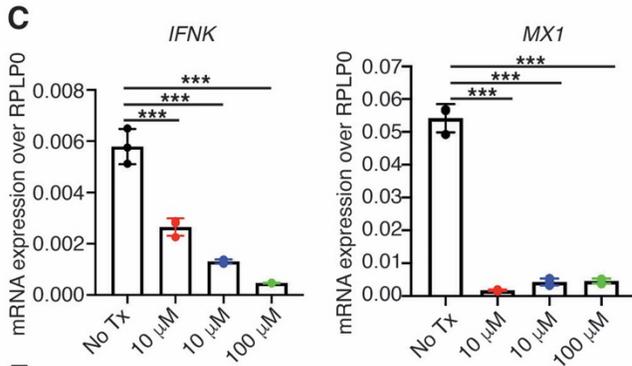
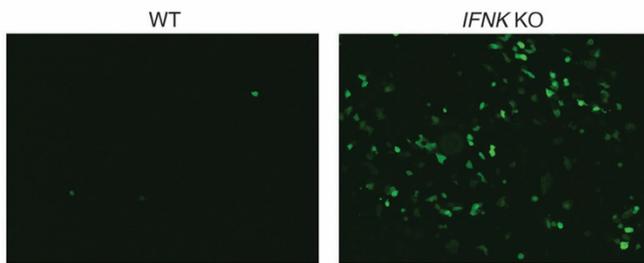
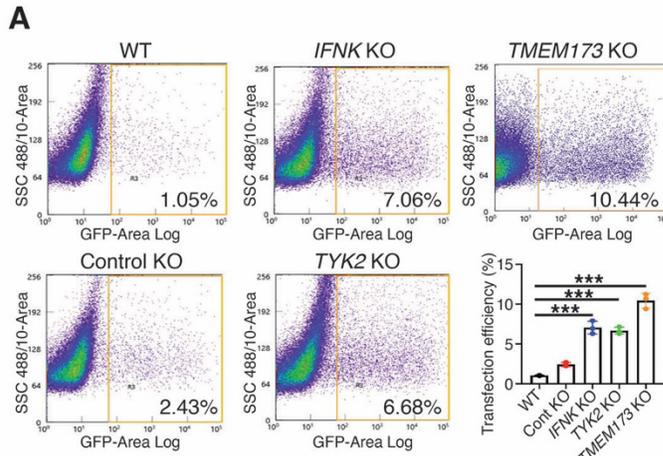


Figure 4. JAK1/JAK2 inhibition prevents suppression of type I IFN response in CRISPR-cas9 generated KO keratinocytes. (A) Increased transfection efficiency in CRISPR-cas9 generated KO KCs (control), and keratinocytes with KO of either *IFNK* or *TYK2*, or *TMEM173*, all of which were KOs generated without baricitinib pretreatment. (Data are represented as mean \pm SEM; ***P < 0.001, 1-way ANOVA with Tukey's test; n=3). (B) CRISPR-cas9 generated *IFNK* KO (*IFNK* KO) KCs, have increased CRISPR-cas9 plasmid stability (Data are represented as mean \pm SEM; *P < 0.05, ***P < 0.001, 1-way ANOVA with Tukey's test; n=3). (C) Suppression of *IFNK* and *MX1* mRNA expression in baricitinib (JAK1/JAK2 inhibitor) treated KCs (Data are represented as mean \pm SEM; ***P < 0.001, 1-way ANOVA with Tukey's test; n=3). (D) CRISPR/Cas9 transfection efficiency in baricitinib-treated KCs (Data are represented as mean \pm SEM; ***P < 0.001, 1-way ANOVA with Tukey's test; n=3). (E) *IFNK* and *MX1* mRNA expression in the CRISPR-cas9 generated KO KCs with (w) or without (wo) JAK1/JAK2 inhibitor. KO KCs with JAKi (right bar of each of the panels) were selected after JAKi treatment. (JAKi, Data are represented as mean \pm SEM; **P < 0.01, ***P < 0.001, 2-tailed Student's t-test; n=3). (F) CpG methylation in the *IFNK* promoter region in JAK1/JAK2 inhibitor treated CRISPR KO keratinocytes. KO KCs with JAKi (right panel) were selected after JAKi treatment (n=8).