

Loss of sirtuin 1 (SIRT1) disrupts skin barrier integrity and sensitizes mice to epicutaneous allergen challenge

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Background: Skin barrier integrity requires a highly coordinated molecular system involving the structural protein filaggrin (FLG). Mutational loss of the skin barrier protein FLG predisposes subjects to the development of atopic dermatitis (AD).

Objective: We sought to determine the role of sirtuin 1 (SIRT1) in skin barrier function, FLG expression, and development of AD.

Methods: Skin histology of mice with skin-specific SIRT1 deletion and wild-type control animals was examined by using hematoxylin and eosin staining. Protein and mRNA abundance was analyzed by means of immunoblotting, immunohistochemistry, immunofluorescence, and RT-PCR. Serum antibody levels were assessed by means of ELISA.

Results: Here we show that FLG is regulated by the protein deacetylase SIRT1 and that SIRT1 is critical for skin barrier integrity. Epidermis-specific SIRT1 ablation causes AD-like skin lesions in mice, and mice with epidermal SIRT1 deletion are sensitive to percutaneous challenge by the protein allergen ovalbumin. In normal human keratinocytes and mouse skin SIRT1 knockdown or genetic deletion downregulates FLG, and regulation of FLG expression by SIRT1 requires the deacetylase activity of SIRT1. SIRT1 also promotes activation of the aryl hydrocarbon receptor, and the aryl hydrocarbon receptor ligand restores FLG expression in SIRT1-inhibited cells. Compared with normal human skin, SIRT1 is downregulated in both AD and non-AD lesions.

Conclusion: Our findings demonstrate a critical role of SIRT1 in skin barrier maintenance, open up new opportunities to use SIRT1 as a pharmacologic target, and might facilitate the development of mechanism-based agents for AD prevention and therapy. (*J Allergy Clin Immunol* 2014;■■■:■■■-■■■.)

Key words: Sirtuin 1 (SIRT1), skin barrier, filaggrin

Abbreviations used

AD:	Atopic dermatitis
AhR:	Aryl hydrocarbon receptor
ARNT:	Aryl hydrocarbon receptor nuclear translocator
BaP:	Benzo(a)pyrene
cHet:	Epidermis-specific heterozygous SIRT1 deletion
cKO:	Epidermis-specific homozygous SIRT1 deletion
CYP1B1:	Cytochrome P450 1B1, an aryl hydrocarbon receptor (AhR) target gene
EGFR:	Epidermal growth factor receptor
FLG:	Filaggrin
K10:	Keratin 10
KO:	Knockout
MEF:	Mouse embryonic fibroblast
NC:	Negative control
NF-κB:	Nuclear factor κB
NHEK:	Normal human epidermal keratinocyte
OVA:	Ovalbumin
siRNA:	Small interfering RNA
SIRT1:	Sirtuin 1
siSIRT1:	Small interfering RNA targeting SIRT1
TEWL:	Transepidermal water loss
WT:	Wild-type
XRE:	Xenobiotic response element

The skin is the essential barrier protecting against environmental insults, including infectious pathogens, chemicals, and UV radiation, and minimizing water loss from the body. As the most abundant cells forming the epidermis, keratinocytes proliferate and differentiate to form an impermeable barrier. Defects in the skin barrier have an active role in the pathogenesis of several chronic inflammatory skin diseases, including atopic dermatitis (AD).¹⁻⁴

AD is an increasingly common pruritic inflammatory disease affecting 10% to 20% of children and 3% of adults in the United States and other developed countries.^{5,6} The clinical picture evolves in stages and is frequently associated with an increased serum IgE concentration and a number of cutaneous, ocular, and other atopic disorders, such as allergic rhinitis and asthma. There is no cure for AD, and its molecular pathogenesis is still poorly understood. Although for many years AD was considered to be primarily an immunologically driven disease with a secondary barrier defect (the so-called inside-outside hypothesis), investigators hypothesized that the primary defect was in the skin barrier (the outside-inside hypothesis).^{4,6} Abnormal barrier function of the skin has long been noted in patients with ichthyosis vulgaris, a common skin condition characterized by postnatal appearance of dry flaking skin, even in the absence of AD. Recent studies have disclosed a strong association between a defect in the skin barrier and the pathogenesis of AD.⁶ This defect is due to a genetic loss-of-function mutation of the gene filaggrin (*FLG*),

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which encodes the skin barrier protein profilaggrin (proFLG)/FLG.^{7,8} These mutations, which are carried by up to 10% of Europeans, represent a strong genetic predisposing factor for AD, asthma, and allergies.⁹

Although *FLG* mutation carriers have a greatly increased risk of AD, AD develops in only approximately 42% of all mutation carriers.¹⁰ This outcome implies that genetic modifiers and environmental factors are both important. Impaired barrier function increases transepidermal penetration of environmental allergens. This is supported by the finding that *FLG* deficiency in mice facilitates and permits increased percutaneous sensitization with protein allergens, irritants, and haptens.¹¹⁻¹⁴

Sirtuin 1 (SIRT1), a mammalian counterpart of the yeast silent information regulator 2 (Sir2) and a protomember of the sirtuin family, is an nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylase crucial for cell survival, metabolism, senescence, and stress response in several cell types and tissues.¹⁵⁻¹⁹ Both histone and nonhistone targets of SIRT1 have been identified, including forkhead box O (FOXO), p53, peroxisome proliferator-activated receptor (PPAR)-gamma coactivator (PGC-1 α), nuclear factor κ B (NF- κ B), and PPARs.^{16,18,20} SIRT1 also deacetylates xeroderma pigmentosum group A protein (XPA) to regulate UV-induced DNA damage repair.²¹ We have demonstrated previously that SIRT1 positively regulates UV-induced DNA damage repair by promoting xeroderma pigmentosum group C protein (XPC) expression and that it has a critical role in skin tumorigenesis and homeostasis.^{22,23} In addition, SIRT1 has been found to promote differentiation of normal human keratinocytes *in vitro*,²⁴ suggesting a possible role in barrier function and thus the development of AD.

Although genetic *FLG* mutations are strongly associated with the risk of AD, 2 independent studies have shown that both carriers and noncarriers of *FLG* mutations have childhood AD. In one study of patients with AD, 90% of whom were white, only 26.7% of patients with AD carried *FLG* mutations.⁸ In Irish children with AD, approximately 47% of patients carried at least 1 null *FLG* mutation.⁷ These findings indicate that more than half of all patients with AD do not carry *FLG* mutations. One implication is that nongenetic loss of proFLG/FLG, including downregulation of *FLG* expression, might play an important role. *FLG* can be downregulated by disrupting the fine balance of the complex regulation of *FLG* promoter activity during epidermal differentiation.⁹ However, the mechanisms that regulate *FLG* expression remain poorly understood.

Here we show that epidermal SIRT1 deletion downregulates *FLG* and sensitizes mouse skin to the epicutaneous allergen-induced response. SIRT1 regulates *FLG* expression through AhR. Our findings demonstrate a new role of SIRT1 in skin barrier function and shed light on the molecular mechanisms for maintaining the skin barrier and preventing inflammatory skin diseases.

METHODS

Human normal, AD, and non-AD skin

All human specimens were studied after approval by the University of Chicago Institutional Review Board. Formalin-fixed paraffin-embedded tissue blocks were obtained from archives in the tissue bank of the Section of Dermatology, Department of Medicine, University of Chicago. Normal skin (sun protected), AD, and non-AD (spongiotic dermatitis without a history of atopy) samples were used for immunohistochemical analysis of SIRT1 protein levels. The SIRT1 staining intensity was scored blindly by 2 independent investigators as 0 (negative), 1 (low), 2 (medium), or 3 (high), as in our previous studies.^{22,25}

Animal treatments

All animal resources were approved by the University of Chicago Institutional Animal Care and Use Committee. Floxed mice carrying the *Sirt1* allele (Jackson Laboratory, Bar Harbor, Me) were bred with mice expressing Cre recombinase driven by the K14 promoter (Jackson Laboratory) to generate skin keratinocyte-specific heterozygous SIRT1 deletion (cHet) and epidermis-specific homozygous SIRT1 deletion (cKO) mice with a B6 background, as described previously.²³ Mice were backcrossed with SKH1 female mice at least 5 times to generate wild-type (WT) and cKO mice in the SKH1 hairless background.²³ Mice were housed 5 animals per cage, and there was no evidence of dorsal wounds caused by fighting. Female mice (n = 15 for each group) were kept for 24 months for observation of survival and aging-related phenotypes.

Cutaneous treatment with ovalbumin

SIRT1 WT and cKO mice with an SKH-1 background were treated with ovalbumin (OVA; Sigma Chemicals, St Louis, Mo) by means of 3 cycles of daily cutaneous exposure to OVA for 5 consecutive days, as described previously.¹¹ OVA (fraction V, Sigma) was prepared in PBS (Dulbecco PBS, Sigma) at 1 mg/mL. Mice were restrained, and 50 μ L of OVA solution or 50 μ L of PBS was applied to the dorsal skin and allowed to air dry.

Measurement of transepidermal water loss

Transepidermal water loss (TEWL) was measured on the dorsal skin of shaved mice with a DPM9003 device (Nova Technology, East Hanover, NJ), as described previously.²⁶ Measurements were performed at room temperature, and results were recorded. Three readings from the dorsal skin were taken on each mouse and averaged.

Microarray gene expression

RNA was extracted from normal human epidermal keratinocytes (NHEKs) transfected with NC/small interfering RNA targeting SIRT1 (siSIRT1) with the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Total RNA concentration and purity were determined with NanoDrop (Thermo Scientific, Waltham, Mass), and total RNA integrity was confirmed with the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, Calif). The RNA samples were processed with an Affymetrix GeneChip microarray (Affymetrix, Santa Clara, Calif) at the Functional Genomics Core Facility of the University of Chicago.

Cell culture and small interfering RNA/plasmid transfection

SIRT1 WT and knockout (KO) mouse embryonic fibroblast (MEF) cells (a gift from Dr Xiaoling Li, National Institute of Environmental Health Sciences/National Institutes of Health) were maintained in a monolayer culture in 95% air/5% CO₂ at 37°C in Dulbecco modified Eagle medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen, Carlsbad, Calif). MEF cells were cultured for fewer than 20 passages. NHEKs were obtained from Clonetics (Lonza, Allendale, NJ) and cultured in KGM Gold BulletKit medium (Clonetics, Lonza), according to the manufacturer's instructions. NHEKs were cultured for fewer than 4 passages. Cells were transfected with negative control (NC) or small interfering RNA (siRNA; ON-TARGETplus SMARTpool; Dharmacon, Pittsburgh, Pa) targeting SIRT1 by using the Amaxa Nucleofector (Lonza, Basel, Switzerland), according to the manufacturer's instructions, as described previously.^{22,23}

Immunohistochemistry and immunofluorescence

Immunohistochemical staining of SIRT1, keratin 10 (K10), loricrin, and *FLG* was performed in human skin and dermatitis specimens. SIRT1 levels were determined by using the alkaline phosphatase–anti-alkaline phosphatase method in which the substrate staining (red) is easily distinguishable from

melanin (brown). Immunofluorescence analysis of SIRT and cytochrome P450 1B1 (CYP1B1) was performed, as described in our recent studies.^{27,28}

Western blotting

Protein concentrations were determined by using the BCA assay (Pierce, Rockford, Ill). Equal amounts of protein were subjected to electrophoresis. Western blotting was performed, as described previously.²² Antibodies used included SIRT1, CYP1B1, AKT, p53, p21, glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology, Dallas, Tex), phosphorylated AKT at serine 473 (Cell Signaling, Danvers, Mass), FLG, and acetylated p53 at K382 (Abcam, Cambridge, Mass).

Real-time PCR

Quantitative real-time PCR assays were performed with ABI7300 (Applied Biosystems, Foster City, Calif). Real-time RT-PCR fluorescence detection was performed in 96-well plates with the SYBR Green PCR Master Mix (Applied Biosystems). Amplification primers for FLG and CYP1B1 were purchased from OriGene (Rockville, Md). 5'-ACTGGAACGGTGAAGGTGACA-3' (forward) and 5'-ATGGCAAGGACTTCTGTAAAC-3' (reverse) were used for β -actin. The cycle threshold for each sample was determined in triplicate. In all RT-PCR data, mRNA abundance of specific targets was relative expression (fold) to the control group (taken as 1) after normalization to β -actin.²²

Luciferase reporter assays

The plasmid mixtures, containing 1 μ g of xenobiotic response element (XRE) promoter luciferase construct (XRE-luc in pGL3 vector, kindly provided by Dr Michael S. Denison from the University of California) and 0.025 μ g of pRL-TK (used as a transfection efficiency control; Promega, Madison, Wis), were used for transfection with electroporation, according to the manufacturer's protocol. The luciferase activity was measured, as described previously.²²

Toluidine blue staining for mast cells

Toluidine blue staining for mast cells was performed according to the manufacturer's protocol (IW-3013; NovaUltra, Waltham, Mass). Briefly, the sections were deparaffinized and hydrated and then stained in toluidine blue working solution. After washing, the sections were dehydrated and then placed under cover slips with resinous mounting medium. The numbers of mast cells were calculated as the average from 4 or 7 different fields of each sample ($\times 40$ magnification).

Serum IgE levels

IgE concentrations were measured with a mouse IgE ELISA kit (BioLegend, San Diego, Calif), according to the manufacturer's protocol.

Electron microscopy

Transmission electron microscopy was carried out on dorsal skin of 3 female WT or SIRT1 cKO mice at 6 weeks of age. Sections were embedded in Spurr resin (Electron Microscopy Science, Hatfield, Pa) and examined in an FEI Tecnai G2 F30 (FEI, Hillsboro, Ore) by our electron microscopy core facility on campus.

Statistical analyses

Statistical analyses were performed with Prism 5 software (GraphPad Software, San Diego, Calif). Kaplan-Meier survival estimates and log-rank tests were used to evaluate skin lesion onset in mice. Data were expressed as means of 3 independent experiments and analyzed with the Student *t* test or 2-way ANOVA. A *P* value of less than .05 was considered statistically significant.

RESULTS

Development of AD-like skin lesions in mice with epidermis-specific SIRT1 deletion

Recently, we have demonstrated the essential role of epidermal SIRT1 in UV-induced skin carcinogenesis and skin injury by using mice with keratinocyte-specific SIRT1 deletion (see Fig E1 in this article's [Online Repository](http://www.jacionline.org) at www.jacionline.org).²³ Because SIRT1 is critical for preventing age-related pathologies,¹⁵ we asked whether SIRT1 has an active role in skin homeostasis during aging. We monitored WT mice and mice with epidermal SIRT1 heterozygous (cHet) and homozygous deletion (cKO) housed in our clean barrier facility for 24 months. At 51 weeks of age, cKO mice spontaneously had AD-like skin lesions (Fig 1, A and B). In comparison, at 64 weeks of age, cHet mice started to have similar AD-like skin lesions (Fig 1, A and B). The clinical severities of skin lesions included erythema, edema, erosion, and scaling. In contrast, no cutaneous manifestation was observed in SIRT1 WT mice. To further elucidate AD-associated immunologic status of SIRT1 cHet and cKO mice before development of AD-like lesions, we measured serum IgE concentrations, histology, and numbers of cutaneous eosinophils and mast cells in mice without visible AD-like lesions because IgE antibodies, eosinophils, and mast cells have been linked to the pathophysiology of allergic disorders, including AD.²⁹ IgE concentrations were significantly higher in SIRT1 cHet and cKO mice than in age-matched 18-month-old SIRT1 WT mice (Fig 1, C). At 6 months of age, most of the dorsal skin examined in cHet and cKO mice did not show significant acanthosis or alterations to the epidermis (Fig 1, D). However, we observed a significant increase in the number of eosinophils in the SIRT1 cHet and cKO mouse skin (Fig 1, E). In addition, the numbers of mast cells increased significantly in the dermis of SIRT1 cHet and cKO mice compared with those seen in WT mice (Fig 1, F and G). To determine the effect of SIRT1 deletion on the integrity of the skin barrier before formation of AD-like lesions, we measured TEWL. SIRT1 cHet and cKO mice showed increased TEWL compared with WT mice (Fig 1, H). These results indicate that SIRT1 loss causes age- and SIRT1 dose-dependent spontaneous development of AD-like lesions.

Reduced FLG expression in mice with epidermal SIRT1 deletion

To determine the molecular role of SIRT1 in skin barrier function, we carried out a microarray analysis of gene expression in undifferentiated NHEKs transfected with siSIRT1 or NC siRNA. Among the genes that were upregulated or downregulated by SIRT1 knockdown by greater than 2-fold, we found that SIRT1 inhibition decreased FLG expression (see Table E1 in this article's [Online Repository](http://www.jacionline.org) at www.jacionline.org), whereas it had less or little effect on other epidermal differentiation genes, including involucrin, keratin 14, keratin 5, desmogleins, kallikrein-related peptidases (KLKs), matriptase, or serine peptidase inhibitor, Kazal type 5 (SPINK5) (see Fig E2 in this article's [Online Repository](http://www.jacionline.org) at www.jacionline.org). Further analysis showed that *FLG* mRNA expression and proFLG protein abundance both were significantly reduced in undifferentiated NHEKs transfected with siSIRT1 compared with those with NC (Fig 2, A and B). Furthermore, the mRNA level of *FLG* and the protein levels of proFLG and filaggrin monomer (FLG) were decreased in the skin of SIRT1 cKO mice compared with those seen in WT mice (Fig 2, C-E). However, epidermal SIRT1 deletion did not affect the protein

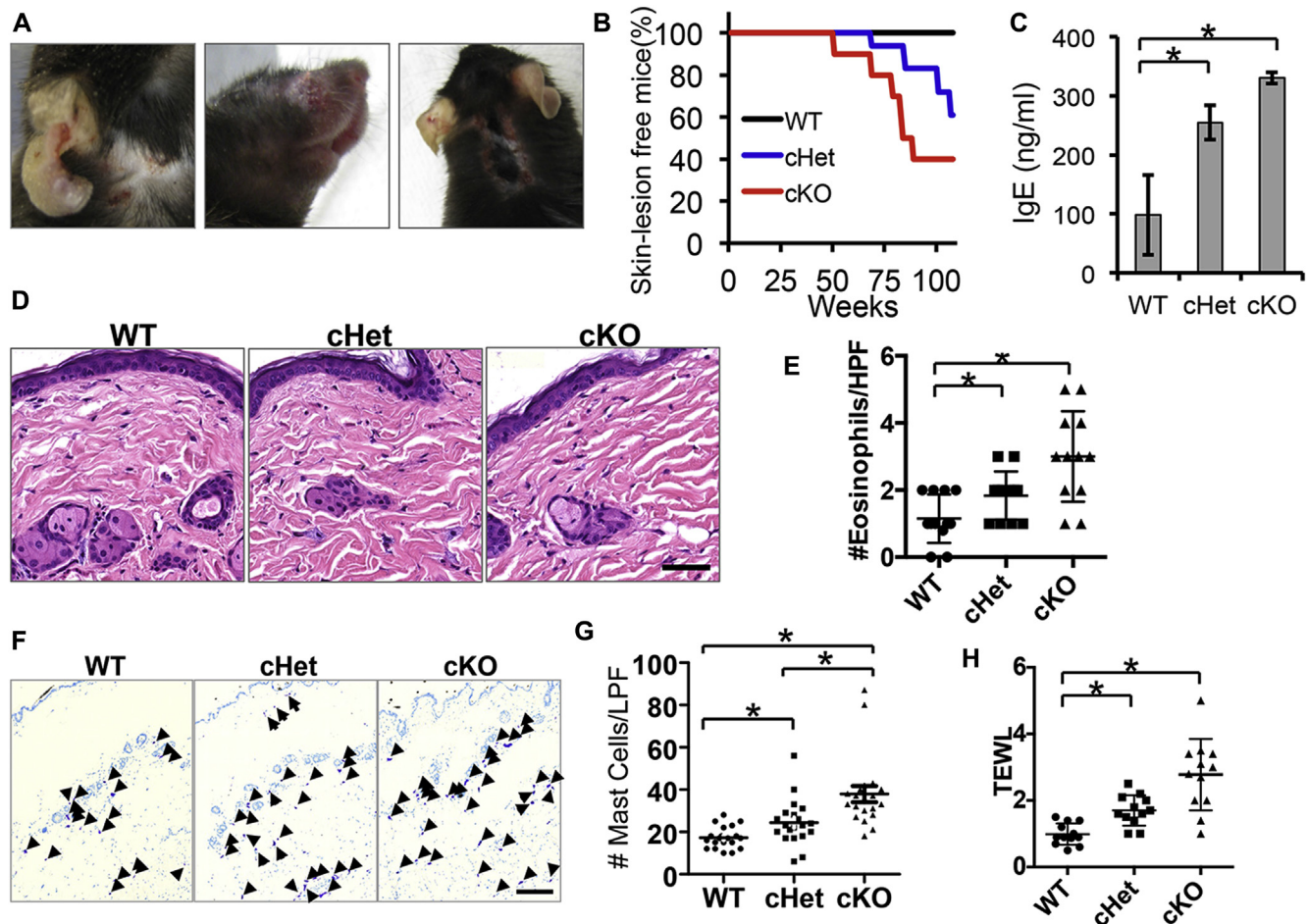


FIG 1. Development of AD-like skin lesions in mice with epidermal SIRT1 deletion (cKO). **A**, Gross phenotype of 51- to 64-week-old SIRT1 cKO mice. **B**, Percentage of skin lesion-free mice among SIRT1 WT, cHet, and cKO mice ($n = 15$). **C**, Mouse serum IgE levels in SIRT1 WT, cHet, and cKO mice at 18 months of age. **D**, Representative photomicrographs of HE skin sections from 6-month-old SIRT1 WT, cHet, and cKO mice. Scale bar = 50 μ m. **E**, Numbers of eosinophils detected per high-power field (HPF; $\times 400$ magnification). **F**, Toluidine blue staining of mast cells in skin of SIRT1 WT, cHet, and cKO mice at 6 months of age. Scale bar = 200 μ m. **G**, Scatter chart for numbers of mast cells per low-power field (LPF; $\times 100$ magnification) in SIRT1 WT, cHet, and cKO mouse skin ($n = 3$). **H**, TEWL in 18-month-old SIRT1 WT, cHet, and cKO mice. For Fig 1, C, data are shown as means $\pm$ SEs ($n = 3$). * $P < .05$, t test, compared with the WT group. For Fig 1, E, G, and H, data are shown as means $\pm$ SDs ($n = 3$). * $P < .05$, t test, compared with the WT group.

abundance of K10 or loricrin (Fig 2, E). Electron microscopy showed that the granular layer of the SIRT1 cKO epidermis lacked the large, irregularly shaped keratohyalin (F-granules)³⁰ that contains proFLG compared with SIRT1 WT epidermis, whereas the small round loricrin-containing keratohyalin (L-granules) were present in epidermal granular cells of both WT and SIRT1 cKO mice (Fig 2, F and G), which was similar to the phenotype detected in flaky tail mice, which are deficient in FLG.³¹ These findings indicated that SIRT1 loss inhibits FLG expression *in vitro* and *in vivo*. Further *in vitro* and *in vivo* investigation will elucidate the role of other epidermal differentiation genes in the function of SIRT1 in skin barrier integrity.

SIRT1 deficiency in keratinocytes sensitizes mouse skin to OVA-induced histologic and immunologic response

To determine whether loss of SIRT1 impairs the skin barrier, we tested the hypothesis that skin with SIRT1 loss is permissive to

epicutaneous challenge by protein antigens normally excluded from the skin. To do this, we treated mice with OVA, as described previously (Fig 3, A).¹¹ Allergen challenge induced epidermal acanthosis in SIRT1 cKO mice but not WT mice (Fig 3, B). In SIRT1 cKO mice OVA significantly increased serum IgE levels, but it did not affect IgE levels in WT mice (Fig 3, C). To determine the effect of OVA on skin barrier integrity, we measured TEWL. At the site of the allergen challenge at 6 weeks of allergen challenge treatment, OVA significantly increased TEWL in SIRT1 cKO mice but not WT mice (Fig 3, D). These findings demonstrate that SIRT1 deficiency in the epidermis impairs skin barrier integrity.

SIRT1 regulates FLG expression in a deacetylase-dependent manner

To determine the role of SIRT1 deacetylase activity in FLG expression, we compared the difference in FLG expression in NHEKs and 293T cells transfected with vector (Vec), WT SIRT1,

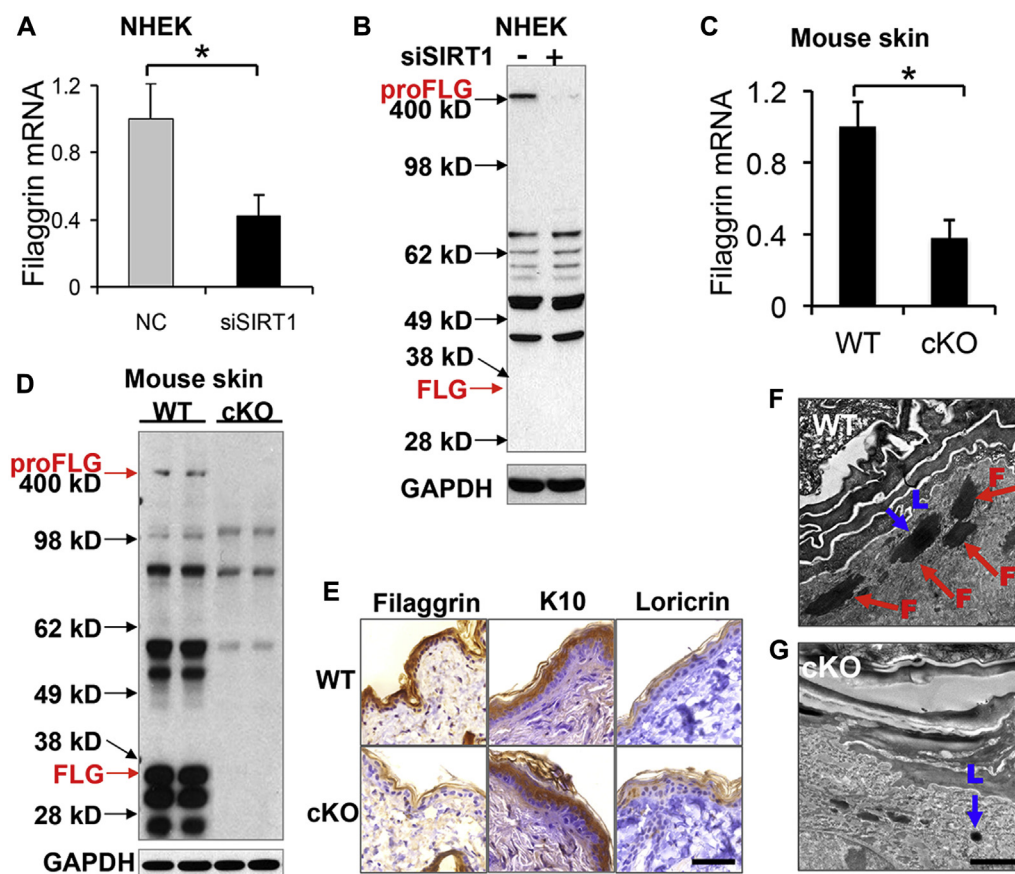


FIG 2. Reduced FLG expression in keratinocytes and mouse skin with SIRT1 loss. **A**, FLG mRNA levels in undifferentiated NHEKs transfected with NC or siRNA targeting SIRT1 (siSIRT1). Data are shown as means $\pm$ SEs from 3 independent experiments. * $P < .05$, t test, compared with the NC group. **B**, FLG protein levels in NHEKs transfected with NC or siSIRT1 as in Fig 2, **A**. **C**, FLG mRNA levels in 6-month-old SIRT1 WT and cKO mouse skin. **D**, proFLG and filaggrin monomer (FLG) protein levels in skin from 6-month-old SIRT1 WT and cKO mouse skin. **E**, Immunohistochemical staining of FLG, K10, and loricrin in skin of 6-month-old WT and SIRT1 cKO mice. Scale bar = 50 μ m. **F** and **G**, Electron microscopic analysis of WT (Fig 2, **F**) and SIRT1 cKO (Fig 2, **G**) epidermis from 6-week-old mice. Red arrows indicate F-granules (F), and blue arrows indicate L-granules (L). Scale bar = 2 μ m. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

and inactive mutant SIRT1 H355Y (Mut).³² Compared with vector-transfected NHEK/293T cells, WT SIRT1 transfection increased proFLG protein abundance, whereas mutant SIRT1 had no effect (Fig 4, **A** and **B**). WT SIRT1 reduced the level of acetylated p53, whereas Mut SIRT1 had no effect, confirming loss of the deacetylase activity of Mut SIRT1 (Fig 4, **A** and **B**). FLG mRNA concentration was significantly increased in SIRT1 WT-transfected 293T cells but unaltered in mutant SIRT1-transfected cells (Fig 4, **C**). These findings indicate that the deacetylase activity of SIRT1 is required for its regulation of FLG. Because SIRT1 is known to deacetylate p53 and thus inhibit its transcriptional function of preventing apoptosis induced by DNA damage,³³ we investigated whether p53 plays a role in FLG regulation. ProFLG protein abundance remained unaltered in NHEKs transfected with siRNA targeting p53 (Fig 4, **D**). Taken together, these observations indicate that SIRT1 promotes FLG expression through its deacetylase activity independent of p53.

SIRT1 loss inhibits activation of AhR

Previous reports indicated that coal tar-mediated activation of the AhR signaling pathway leads to enhanced epidermal

differentiation and epidermal barrier function.⁹ To determine the molecular function of SIRT1, we examined whether SIRT1 regulates AhR target genes obtained from our microarray analysis. Among the genes that were upregulated or downregulated by SIRT1 knockdown by greater than 2-fold, we found that SIRT1 inhibition decreased the expression of CYP1B1 (see Table E1), a known AhR target gene, but had no effect on AhR expression (Fig 5, **A**). Real-time PCR analysis indicated that SIRT1 knockdown significantly decreased CYP1B1 mRNA expression (Fig 5, **B**). Epidermal SIRT1 deletion significantly reduced the CYP1B1 mRNA expression in mouse skin (Fig 5, **C**). Next, we assessed the effect of SIRT1 inhibition on AhR activation using the AhR reporter assay with a luciferase plasmid driven by an XRE (XRE-Luc). The transcriptional activity of the AhR reporter was significantly lower in SIRT1 KO cells than in WT cells (Fig 5, **D**). Immunofluorescence analysis showed that SIRT1 knockdown decreased CYP1B1 abundance (Fig 5, **E**). These findings demonstrate that SIRT1 is required for basal AhR activity in keratinocytes.

To determine the role of AhR in FLG suppression in SIRT1-inhibited cells, we investigated whether AhR activation in SIRT1-inhibited cells restored FLG expression. NHEKs were

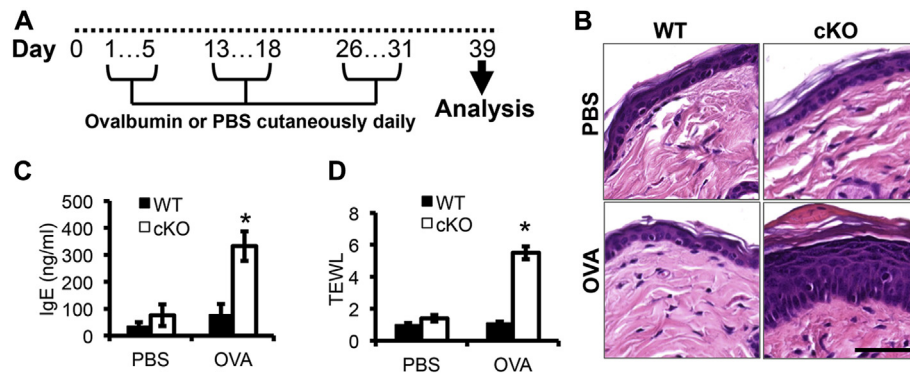


FIG 3. Response to OVA in WT and SIRT1 cKO mice. **A**, Schematic for OVA treatment. **B**, Histology of mouse skin after PBS and OVA treatment as in Fig 3, A. Scale bar = 50 μ m. **C**, ELISA of serum IgE levels in SIRT1 WT and cKO mice after PBS and OVA treatment. **D**, TEWL in SIRT1 WT and cKO mice after PBS and OVA treatment. For all graphs, data are shown as means $\pm$ SEs (n = 4). *P < .05, t test, compared with WT group. The mice were 6 to 8 weeks old.

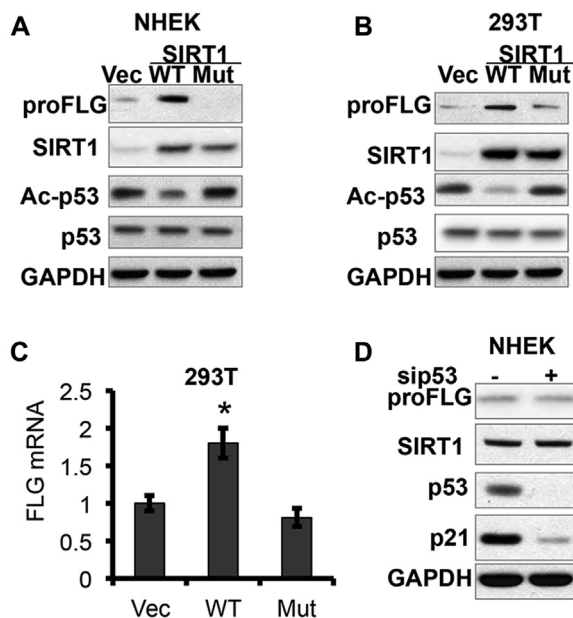


FIG 4. Role of SIRT1 deacetylase activity in its regulation of FLG. **A**, Immunoblot analysis of proFLG, SIRT1, acetylated p53 (Ac-p53), p53, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein levels in NHEKs transfected with SIRT1 vector (Vec), WT, or inactive mutant (Mut) SIRT1 plasmids. **B**, Immunoblot analysis of proFLG, SIRT1, Ac-p53, p53, and GAPDH protein levels in 293T cells transfected with Vec, WT, or Mut SIRT1 plasmids. **C**, FLG mRNA levels in 293T cells transfected with Vec, WT, or Mut SIRT1 plasmids. Data are shown as means $\pm$ SEs from 3 independent experiments. *P < .05, t test, compared with the vector group. **D**, Immunoblot analysis of proFLG, SIRT1, p53, p21, and GAPDH in NHEKs transfected with NC or siRNA targeting p53 (sip53).

transfected with NC/siRNA targeting SIRT1 and then treated with the AhR ligand benzo(a)pyrene (BaP). In both NC- and siSIRT1-transfected cells, BaP increased the protein abundance and mRNA expression of both FLG (Fig 5, F and G) and CYP1B1 (Fig 5, F-H). However, SIRT1 knockdown reduced the basal and BaP-induced protein and mRNA levels of FLG and CYP1B1 (Fig 5, F-H). To further confirm the role of BaP-induced FLG expression *in vivo*, we treated SIRT1 cKO

mice with OVA for 6 weeks and then with BaP for 3 or 7 days. BaP increased mature FLG abundance in SIRT1 cKO mouse skin (Fig 5, I) and reduced OVA-induced acanthosis (Fig 5, J) and the IgE level (Fig 5, K). These findings indicate that SIRT1 promotes AhR activity in the regulation of FLG.

AKT activation is involved in AhR regulation of FLG

It has been reported that AhR regulates the phosphatidylinositol 3-kinase/AKT pathway.³⁴ To further determine whether AKT activation plays a role in the AhR regulation of FLG, we measured the effect of BaP on AKT activation and the effect of AKT knockdown on BaP-induced FLG protein concentrations in NHEKs. BaP increased FLG abundance and AKT phosphorylation (Fig 6, A), whereas SIRT1 knockdown reduced basal and BaP-induced AKT phosphorylation (Fig 6, A). Blocking the AKT pathway with small interfering RNA targeting AKT1 (siAKT1) decreased BaP-induced FLG abundance, although it did not affect the CYP1B1 protein abundance (Fig 6, B), implying a distinct regulation of FLG and CYP1B1 by the SIRT1/AhR axis. A similar effect of AKT inhibition on FLG protein levels was detected with siAKT1 or the phosphatidylinositol 3-kinase/AKT inhibitor LY294002 in the keratinocyte HaCaT cell line (see Fig E3, A and B, in this article's Online Repository at www.jacionline.org). These findings indicate that SIRT1-modulated AhR activation regulates FLG through AKT (Fig 6, C), suggesting an indirect mechanism in FLG regulation by the SIRT1/AhR pathway.

SIRT1 protein level is downregulated in human AD and non-AD lesions

To further investigate the specific function of SIRT1 in human dermatitis, we used immunohistochemical analysis to evaluate SIRT1 protein levels in 7 AD and 13 non-AD specimens (spongiotic dermatitis without a history of atopy) compared with 10 sun-protected normal skin specimens. Although some SIRT1 was localized in the cytoplasm, the majority of the protein was localized to the nucleus in epidermal keratinocytes of normal skin (Fig 7, A). SIRT1 levels were reduced (score, 0 or 1) in all 7 AD and all 13 non-AD samples compared with 10% of normal

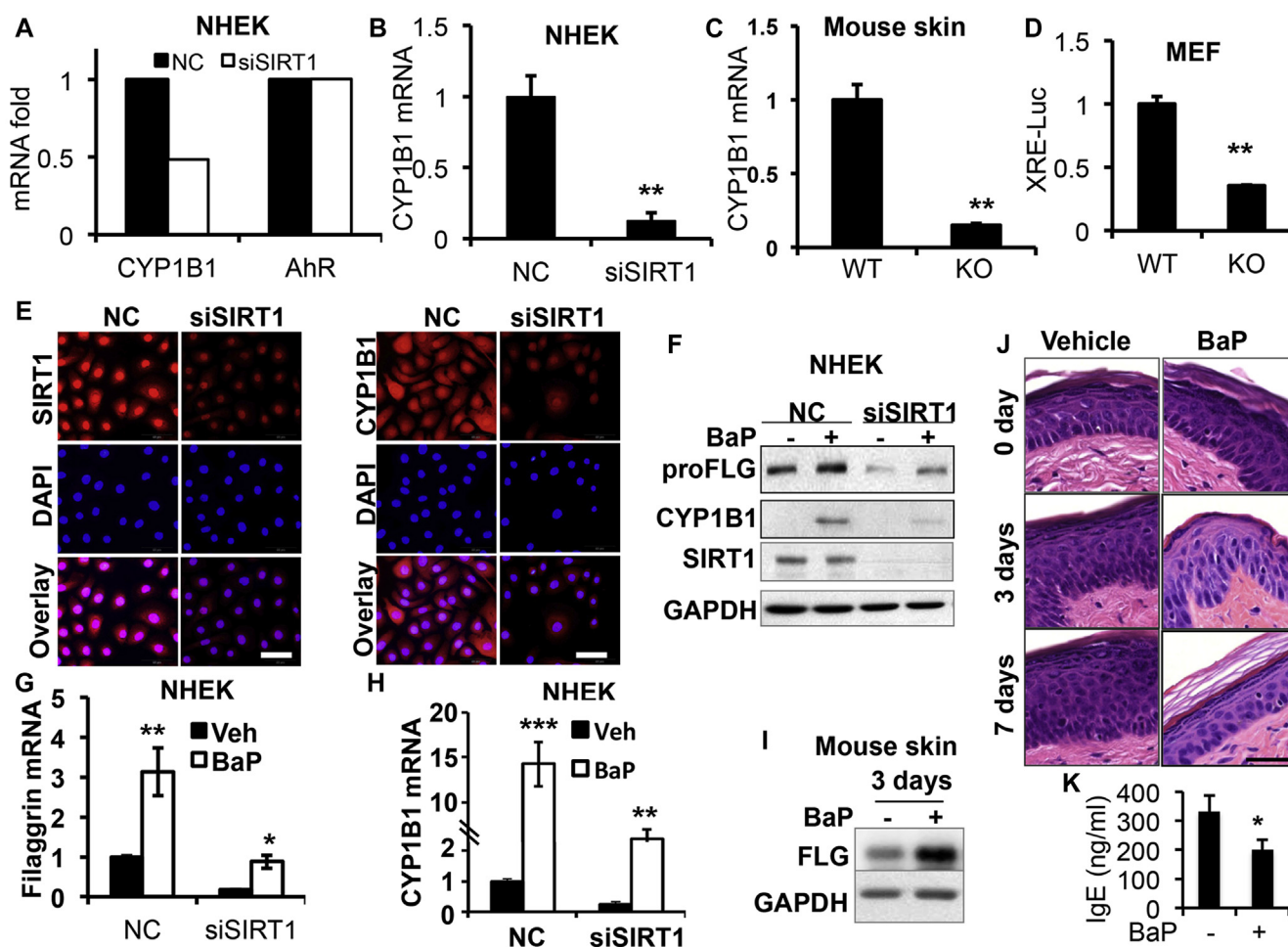


FIG 5. Role of AhR in SIRT1 regulation of FLG. **A**, Gene array analysis of mRNA levels of CYP1B1 and AhR in NHEKs transfected with NC or siSIRT1. **B**, Real-time PCR analysis of CYP1B1 mRNA expression in NHEKs transfected with NC or siSIRT1. **C**, Real-time PCR analysis of CYP1B1 mRNA expression in SIRT1 WT and cKO mouse skin ($n = 3$). **D**, Luciferase reporter assay of AhR reporter activity (XRE-luc) in SIRT1 WT and KO MEF cells. **E**, Immunofluorescence analysis of SIRT1 and CYP1B1 in NHEKs transfected with NC or siSIRT1. 4'-6-Diamidino-2-phenylindole dihydrochloride (DAPI) staining (blue) was used as a nuclear counterstain. Scale bar = 50 μ m. **F**, Immunoblot analysis of FLG, SIRT1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein levels in NHEKs transfected with NC or siSIRT1 and then treated with BaP (10 μ mol/L) for 48 hours. **G**, Real-time PCR analysis of FLG mRNA levels in NHEKs transfected with NC or siSIRT1 and treated with BaP (10 μ mol/L) for 48 hours. **H**, Real-time PCR analysis of CYP1B1 mRNA levels in NHEKs transfected with NC or siSIRT1 and treated with BaP (10 μ mol/L) for 48 hours. **I**, Immunoblot analysis of filaggrin monomer (FLG) and GAPDH protein levels in SIRT1 cKO mice transfected with OVA for 6 weeks, followed by treatment with BaP for 3 or 7 days. **J**, Representative pictures of hematoxylin and eosin staining for SIRT1 cKO mice treated with OVA for 6 weeks, followed by treatment with BaP for 3 or 7 days. **K**, Serum IgE levels in SIRT1 cKO mice treated with OVA for 6 weeks, followed by treatment with BaP for 7 days. Scale bar = 50 μ m. For all graphs, data are shown as means $\pm$ SEs from 3 independent experiments. * $P < .05$, t test, compared with the control (NC or WT) group for Fig 5, A-D, or the vehicle (Veh) group in Fig 5, G-H.

skin samples (1/10; Fig 7, B). This reduction was statistically significant, as analyzed by using the Mann-Whitney test ($P < .005$ between normal skin and AD skin and between normal skin and non-AD skin).

DISCUSSION

In this study we have shown that SIRT1 regulates skin barrier integrity and promotes FLG expression. Epidermal SIRT1 deletion causes AD-like skin lesions in older mice and sensitizes mice to epicutaneous allergen challenge. FLG regulation by

SIRT1 requires the deacetylase activity of SIRT1 and the AhR/AKT pathway by SIRT1 (Fig 6, C). Compared with normal human skin, SIRT1 is downregulated in human AD and non-AD specimens. Our results demonstrate the critical role of SIRT1 in skin barrier function and protecting the skin against allergen challenge.

SIRT1 plays vital roles in multiple physiologic and pathologic contexts in aging, metabolism, and cancer.^{15,19} The role of SIRT1 in skin diseases and function is just now beginning to be understood. Our recent studies have delineated the critical role of SIRT1 in UV-induced skin cancer and injury.²³ Recent microarray

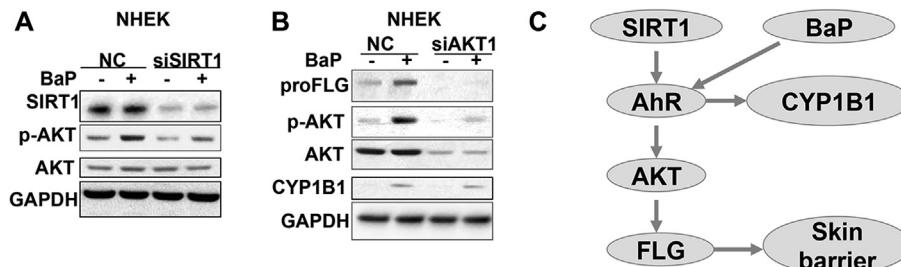


FIG 6. Role of AKT in AhR regulation of FLG. **A**, Immunoblot analysis of FLG, CYP1B1, phosphorylated AKT (p-AKT), AKT, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in NHEKs treated with BaP (10 μ mol/L) for 48 hours. **B**, Immunoblot analysis of FLG, p-AKT, AKT, CYP1B1, and GAPDH in NHEKs transfected with siRNA targeting AKT1 (siAKT1) or NC and then treated with vehicle or BaP (10 μ mol/L). **C**, Schematic for the role of SIRT1 in regulating skin barrier function.

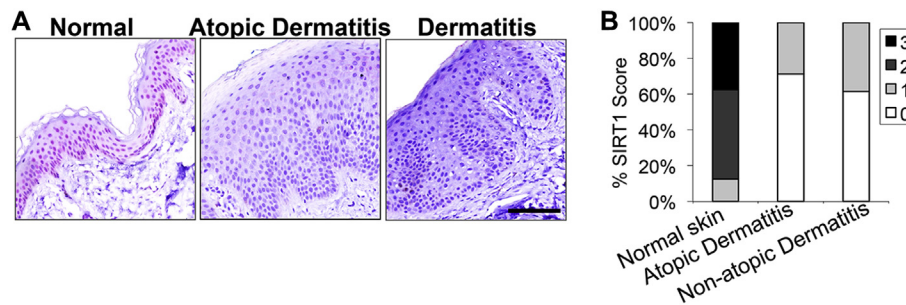


FIG 7. SIRT1 protein levels in human normal, AD, and non-AD skin. **A**, Alkaline phosphatase-anti-alkaline phosphatase red staining for SIRT1 protein levels in human normal, AD, and non-AD skin. Scale bar = 100 μ m. **B**, Percentage (in stacked column format) for each score of SIRT1 expression.

studies have shown that SIRT1 promotes keratinocyte differentiation *in vitro* and suggested the role of several structural proteins and enzymes, including FLG.²⁴ Our findings in the current study indicate that SIRT1 regulates FLG expression, but not K10 or loricrin expression, in its deacetylase-dependent mechanism.

At the molecular level, our findings indicate that SIRT1 modulates AhR activity, which regulates AKT activation and thus promotes FLG expression (Fig 6, C). As one of the critical regulators of the skin barrier, AhR is a cytoplasmic transcription factor in xenobiotic detoxification; on ligand binding, AhR translocates to the nucleus³⁵ and binds to the DNA motifs known as XREs.³⁶ AhR activation by coal tar induces FLG expression and skin barrier repair.³⁷ As one of the oldest treatments for AD, coal tar is found to promote epidermal differentiation by activating AhR.³⁷ We found that SIRT1 positively regulates AhR activation. SIRT1 loss in keratinocytes and mouse skin inhibited basal and ligand-induced AhR activity. AhR ligand increased FLG expression in control and SIRT1-inhibited keratinocytes, indicating that SIRT1 fine tunes AhR activation. SIRT1 uses its deacetylase function to regulate FLG. However, we did not detect the acetylation of either AhR or its partner, aryl hydrocarbon receptor nuclear translocator (ARNT; data not shown). It is possible that histone deacetylation¹⁹ mediates the effect of SIRT1 on AhR activity and FLG expression. Further investigation is needed to elucidate the mechanism of AhR regulation by SIRT1. Nevertheless, our results imply that the combination of SIRT1 activation and AhR/AKT activation might maximize skin barrier repair and the therapeutic effect on AD.

Both AhR and ARNT have been shown to regulate keratinocyte differentiation and barrier function.^{37,38} AhR activation by coal

tar promotes keratinocyte differentiation and the skin barrier.³⁷ ARNT promotes the activation of epidermal growth factor receptor (EGFR)/extracellular signal-regulated kinase to induce the expression of FLG and other epidermal structure proteins.³⁸ Our study showed that AKT activation is required for AhR ligand-induced expression of FLG but not CYP1B1 expression (Fig 6, C). Given that AKT is the downstream pathway of EGFR, it is possible that noncanonical AhR activation activates the EGFR/AKT pathway to regulate FLG expression, further underscoring the critical role of AhR in skin barrier function.

In addition to regulating FLG expression, other functions of SIRT1 might play a role in the development of AD-like skin lesions. For example, SIRT1 inhibits activation of the proinflammatory transcription factor NF- κ B by acetylating its subunit p65,^{39,40} and topical inhibition of NF- κ B has been shown to improve AD in mice.⁴¹⁻⁴³ SIRT1 activators and inhibitors have been shown to modulate airway inflammation in an OVA-challenged mouse model.^{44,45} However, it remains unclear whether SIRT1 regulation of NF- κ B has a role in either AD or airway inflammation. It is possible that the epithelial barrier is involved in the effect of SIRT1 modulators in airway inflammation in the mouse model of asthma, as it is in AD. Indeed, we note that mice with epidermal SIRT1 deletion had AD-like lesions only at an older age, suggesting a critical role of SIRT1 loss in skin inflammation. On the other hand, NF- κ B might play a role in FLG regulation by SIRT1 because TNF- α has been shown to suppress FLG expression.⁴⁶ Further studies are needed to define the molecular mechanisms in the role of SIRT1 in both the skin barrier and the airway epithelial barrier.

In human dermatitis tissue SIRT1 downregulation can involve multiple mechanisms. For example, in flaky tail mice harboring a homozygous frameshift FLG mutation,^{11-14,31} loss of FLG reduced SIRT1 expression.⁴⁷ SIRT1 and FLG might form a positive feedback loop in maintaining the availability of both proteins and thus skin barrier function. Loss of one leads to suppression of the other. In addition, AD-associated cytokines might also contribute to SIRT1 downregulation. Future investigation can elucidate the potential regulation of SIRT1 expression by critical cytokines involved in AD pathogenesis and FLG regulation, including but not limited to TNF- α , IL-4, or IL-13.^{46,48-52}

In summary, we have demonstrated that SIRT1 promotes FLG expression and is required for skin barrier function. Loss of SIRT1 predisposes mice to susceptibility to epicutaneous allergen challenge and causes late-onset AD-like skin inflammation. SIRT1 enhances the activation of AhR and AhR/AKT-induced FLG expression. Our findings might provide new molecular insights into the regulation of skin barrier integrity and the pathogenesis of AD and open up new opportunities for developing targeted mechanism-based strategies for AD prevention and therapy.

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Clinical implications: SIRT1 deficiency inhibits FLG expression, predisposes mice to spontaneous development of AD-like lesion, sensitizes mouse skin to epicutaneous allergen challenge, and is associated with human dermatitis.

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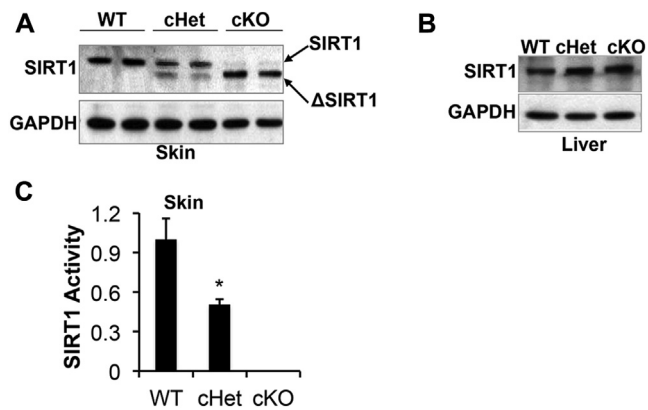


FIG E1. SIRT1 protein level and activity in skin from SIRT1 WT, cHet, and cKO mice. **A** and **B**, Immunoblot analysis of SIRT1 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in skin (Fig E1, **A**) and liver (Fig E1, **B**) from WT, cHet, and cKO mice at 4 to 6 weeks of age. The *upper band* in the SIRT1 blot in Fig E1, **A**, shows the WT SIRT1 protein (SIRT1), whereas the *lower band* shows the nonfunctional truncated protein results from the excision of the conserved Sir2 motif of the SIRT1 catalytic domain (Δ SIRT1). **C**, Fluorometric assay of SIRT1 activity in skin from WT, cHet, and cKO mice ($n = 3$) at 4 to 6 weeks of age. From Ming et al.²³

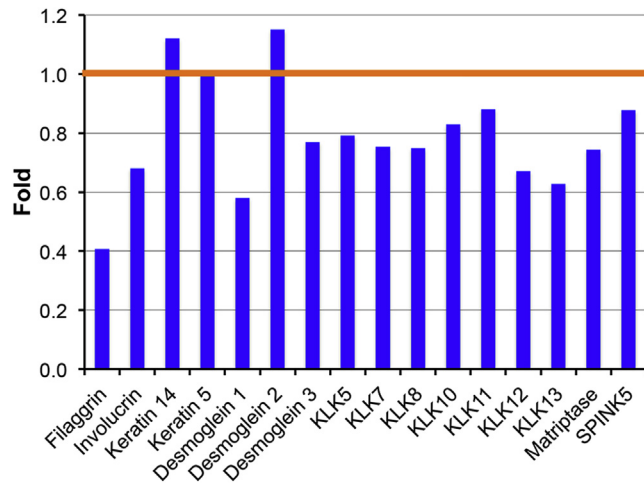


FIG E2. Effect of SIRT1 knockdown on mRNA levels of the epidermal differentiation genes FLG, involucrin, keratin 14, keratin 5, desmoglein 1, desmoglein 2, desmoglein 3, KLK5, KLK7, KLK8, KLK10, KLK11, KLK12, KLK13, matriptase, and SPINK5.

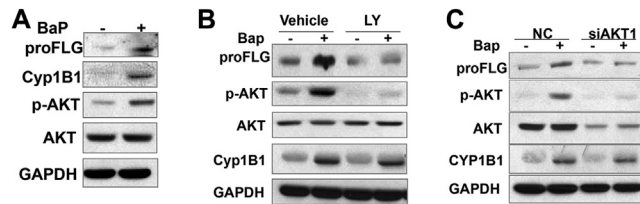


FIG E3. Role of AKT in AhR regulation of FLG. **A**, Immunoblot analysis of FLG, CYP1B1, phosphorylated AKT (*p-AKT*), AKT, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in HaCaT cells treated with BaP (10 $\mu\text{mol/L}$) for 48 hours. **B**, Immunoblot analysis of FLG, *p-AKT*, AKT, CYP1B1, and *GAPDH* in HaCaT cells treated with vehicle, BaP (10 $\mu\text{mol/L}$), LY294002 (LY; 10 $\mu\text{mol/L}$), or the combination of BaP and LY294002. **C**, Immunoblot analysis of FLG, *p-AKT*, AKT, CYP1B1, and *GAPDH* in HaCaT cells transfected with siRNA targeting AKT1 (siAKT1) or NC.

TABLE E1. Genes upregulated or downregulated in siSIRT1 NHEKs compared with NC control cells by greater than 2-fold

Genes	Fold (siSIRT1/NC)
Ribosomal protein L4 (<i>RPL4</i>)	0.33
<i>FLG</i>	0.41
Glycoprotein (transmembrane) nmb (<i>GPNMB</i>)	0.44
Cyclin-dependent kinase inhibitor 1C (<i>p57</i>)	0.46
Lymphocyte antigen 6 complex, locus D (<i>E48</i>)	0.46
<i>MDC1</i>	0.46
<i>CYP1B1</i>	0.48
Lymphocyte antigen (<i>HLA-G3</i>)	0.49
Cyclin L1 (<i>CCNL1</i>)	2.73