Protein Palmitoylation by ZDHHC13 Protects Skin against Microbial-Driven Dermatitis

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Atopic dermatitis is a complex chronic inflammatory skin disorder that results from intimate interactions among genetic predisposition, host environment, skin barrier defects, and immunological factors. However, a clear genetic roadmap leading to atopic dermatitis remains to be fully explored. From a genome-wide mutagenesis screen, deficiency of ZDHHC13, a palmitoylacyl transferase, has previously been associated with skin and multitissue inflammatory phenotypes. Here, we report that ZDHHC13 is required for skin barrier integrity and that deficiency of ZDHHC13 renders mice susceptible to environmental bacteria, resulting in persistent skin inflammation and an atopic dermatitis-like disease. This phenotype is ameliorated in a germ-free environment and is also attenuated by antibiotic treatment, but not by deletion of the Rag1 gene, suggesting that a microbial factor triggers inflammation rather than intrinsic adaptive immunity. Furthermore, skin from ZDHHC13-deficient mice has both elevated levels of IL-33 and type 2 innate lymphoid cells, reinforcing the role of innate immunity in the development of atopic dermatitis. In summary, our study suggests that loss of ZDHHC13 in skin impairs the integrity of multiple barrier functions and leads to a dermatitis lesion in response to microbial encounters.


INTRODUCTION

Atopic dermatitis (AD) is a complex chronic inflammatory skin disorder, with an incidence rate of 5–20% in young infants and children worldwide (DaVeiga, 2012; Williams et al., 1999). This skin disorder may persist throughout childhood, and in some instances progress, through a process referred to as “atopic march,” to the development of asthma and allergic rhinitis, significantly affecting the quality of life of patients and their families (Spurgeon and Paller, 2003; Watson and Kapur, 2011). Many genes/loci have been shown to be associated with disease risk or predisposition in the past genome-wide studies (Barnes, 2010), suggesting heterogeneity in the pathogenic mechanism of AD. Recently, there has been increased interest in studying the contribution of both genetic and environmental factors that affect susceptibility to AD and in exploring the possibility that barrier dysfunction is a major cause of AD and other allergic diseases. In support of the latter, genetic studies have revealed that mutations in the filaggrin gene, which encodes a protein component of cornified envelope, are repetitively associated with the risk of AD (Barnes, 2010; Palmer et al., 2006).

Protein S-palmitoylation was one of the first described post-translational protein modifications (Resh, 2006). It plays a very important role in protein-membrane interactions, protein trafficking, protein stability, protein-protein interactions, and signal transduction, all of which have been shown in vitro to regulate cell development and function (Fukata and Fukata, 2010; Greaves and Chamberlain, 2007; Greaves et al., 2009; Linder and Deschenes, 2007; Resh, 2006). Previously, we reported that a null mutation of Zdhhc13, one of 23 DHHC domain-containing palmitoyl acyltransferases in the mammalian genome (Linder and Deschenes, 2004), produced by ethionitrosourea mutagenesis in mice, resulted in multiple severe phenotypes, including amyloidosis, alopecia, dermatitis, and osteoporosis (Saleem et al., 2010). These phenotypes could be attributed in part to destabilization of critical skin barrier components, such as cornifelin, due to the lack of palmitoylation (Liu et al., 2015). In this current study, we further delineated the pathological mechanism underlying the dermatitis phenotype in these mutant mice. Our data clearly suggest that skin barrier dysfunction renders the mouse susceptible to bacterial infection and initiates chronic skin inflammation via innate immunity.

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Abbreviations: AD, atopic dermatitis; GF, germ-free; ILC2, type 2 innate lymphoid cells; RIPA, radioimmunoprecipitation assay; SPF, specific-pathogen-free; TEWL, transepidermal water loss; Th2, T helper type 2; TNF-a, tumor necrosis factor-a; WT, wild type

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RESULTS
Deficiency of Zdhhc13 causes cyclic alopecia and AD-like dermatitis
In our previous genome-wide mouse ethynitrosourea mutagenesis study, we identified homozygous mutant mice that had multiple skin phenotypes including cyclic alopecia, ragged hair coat, baldness, wrinkled skin, and acanthosis (Saleem et al., 2010). These phenotypes arose as a result of an A-to-T nonsense mutation in the Arg-452 codon of Zdhhc13; mice harboring this mutation are referred to as Zdhhc13^{g/k} (also known as Zdhhc13^{gkc/gkc-4}) mice (Liu et al., 2015; Saleem et al., 2010). In this study, we also generated Zdhhc13 knockout mice by crossing mice having exon 2 of their Zdhhc13 gene flanked by loxP sites (Zdhhc13^{s/s} mice, created using conventional gene-targeting) with E2A-Cre mice (Supplementary Figure S1a online) resulting in the deletion of Zdhhc13 exon 2 and the creation of a prematurely terminated protein of only 13 amino acids in length (Supplementary Figure S1b). The Zdhhc13 knockout mice (Zdhhc13^{s/s}) were then back-crossed onto the B6 background. Deletion of Zdhhc13 was confirmed both by genotyping (Supplementary Figure S1c) and by reverse transcriptase-PCR with primers designed to detect Zdhhc13 mRNA (Supplementary Figure S1d). The Zdhhc13^{s/s} mice had a very similar phenotype to the Zdhhc13^{g/k} mice with evident cyclic alopecia (Supplementary Figure S1e) and skin hyperplasia (Supplementary Figure S1f). Under specific-pathogen-free (SPF) conditions, the skin manifestations that appeared in mice older than 2 months were alopecia, erythema, and fine scaling (Figure 1a), which induced scratching activity, erosion, and edema (Figure 1b). The clinical severities of the skin lesions in Zdhhc13^{s/s} mice increased with age and, reminiscent of human AD patients, skin became hypertrophied with a leathery bark-like appearance (i.e., lichenification) by approximately 1 year of age (Figure 1c).

There was no apparent gender difference in terms of phenotypic manifestations in Zdhhc13^{s/s} mice throughout the period (data not shown). Increased expression of IL-1β and tumor necrosis factor-α (TNF-α) in Zdhhc13^{s/s} mutant mouse skin further supported the inflamed nature of Zdhhc13-deficient skin (Figure 1d). Skin histology of Zdhhc13^{s/s} mice showed thickening of the epidermis (Figure 1e, Supplementary Figures S2 and S3a online) and both eosinophil (Figure 1f, Supplementary Figure S3b) and mast cell (Figure 1g, Supplementary Figure S3c) infiltration were evident in comparison with wild-type (WT) skin.

Because eosinophils are induced and activated by T helper type 2 (Th2) cytokines and are correlated to the human extrinsic AD phenotype, we monitored Th2-specific and inflammatory cytokine expression in the skin of Zdhhc13^{s/s} mice. When skin mRNA expression in Zdhhc13^{s/s} mice was compared with that in WT littermates, inflammatory cytokines and chemokine expression, namely S100a8, S100a9, and Mcp-1, were elevated starting 1 week after birth (postnatal day [P] 8), and further increased in the second and third weeks of life (P14 and P29) (S100a8 and S100a9) (Figure 1h). A transient increase in Tslp mRNA was observed in the first week, and overt increases in Il-25, Il-4, and Il-13 mRNA were also observed in the second and third weeks (Figure 1h), indicating a predominantly Th2 response. In addition to these Th2-type responses, we also identified other hallmarks of AD, such as elevated skin pH, from 5.0 to 6.0 (Figure 1i) and high serum IgE levels (approximately 400 ng/ml) in Zdhhc13^{g/k} mice (Figure 1j). Taken together, these results suggest that loss of ZDHHC13 induced a strong inflammatory response with many hallmarks of AD (hereafter called AD-like disease).

Epidermal expression of Zdhhc13 can prevent skin inflammation
Zdhhc13 mRNA expression in the epidermis has previously been confirmed using in situ hybridization (Liu et al., 2015). Here we studied lacZ expression in skin during embryogenesis using Zdhhc13 gene-trap mice (Zdhhc13^{Gt/Gt} mice), wherein the bacterial lacZ gene is under the regulation of the Zdhhc13 promoter (Saleem et al., 2010; Sutton et al., 2013). We found that lacZ expression began at embryonic day (E) 15.5 in both Zdhhc13^{Gt/Gt} and Zdhhc13^{Gt/Gt} mice (Figure 2a, Supplementary Figure S4 online; enlarged image), and the protein was localized in the skin’s stratum granulosum layer at E17.5 (Figure 2a, Supplementary Figure S4). Expression of lacZ diminished at P0 in Zdhhc13^{Gt/Gt} mice (Figure 2a, Supplementary Figure S4), and Figure 2b, Supplementary Figure S5 online, Gt/+ skin with H2O). However, lacZ expression was not completely shut down in skins at birth (Figure 2a) or in young adults (Figure 2b, Supplementary Figure S5, Gt/Gt with H2O) in Zdhhc13^{Gt/Gt} homozygous mice. When the adult skin became dehydrated, such as when the lipid barrier was removed by an acetone/ethanol wash, expression of the gene-trap allele of Zdhhc13 was rapidly elevated in both Zdhhc13^{Gt/Gt} and Zdhhc13^{Gt/Gt} mice, while skins were hyperplastic (Figure 2b, Supplementary Figures S4 and S5). The endogenous Zdhhc13 mRNA was also markedly induced along with the inflammatory cytokine Tslp mRNA after the acetone/ethanol wash (Figure 2c). To further explore whether the skin inflammation is a cell-autonomous phenotype of ZDHHC13 deficiency in keratinocytes, we generated a keratinocyte-specific knockout Zdhhc13 after tamoxifen injection into Zdhhc13^{Gt/Gt},K5-CreERT mice. One month after injection, the conditional knockout mouse skin showed ragged hair in the shaved areas (Figure 2d, right panel), and the histologic sections showed marked hyperplasia and immune cell infiltration (Figure 2e, middle panel). Together, these results strongly suggest that epidermal expression of Zdhhc13 can prevent skin inflammation.

Impaired skin barrier and abnormal filaggrin processing in Zdhhc13-deficient mice
To obtain direct evidence of impairment of skin barrier function, we analyzed skin barrier integrity using a dye permeability assay and a transepidermal water loss (TEWL) assay (Kelleher et al., 2013; Schmitz et al., 2015). In the case of the in situ dye permeability assay, at E16.5 the entire embryo could be stained with either toluidine blue (data not shown) or X-gal (Figure 3a). At E17.5, the dorsal regions of embryo could be stained with either toluidine blue (data not shown) or X-gal (Figure 3a). At E17.5, the dorsal regions of embryo could be stained with either toluidine blue (data not shown) or X-gal (Figure 3a). At E17.5, the dorsal regions of embryo could be stained with either toluidine blue (data not shown) or X-gal (Figure 3a). At E17.5, the dorsal regions of embryo could be stained with either toluidine blue (data not shown) or X-gal (Figure 3a). At E17.5, the dorsal regions of embryo could be stained with either toluidine blue (data not shown) or X-gal (Figure 3a). At E17.5, the dorsal regions of embryo could be stained with either toluidine blue (data not shown) or X-gal (Figure 3a). At E17.5, the dorsal regions of embryo could be stained with either toluidine blue (data not shown) or X-gal (Figure 3a).
determination, adult mice housed in both SPF and germ-free (GF) isolators were tested. Consistent with the results of the dye permeability assay, TEWL was significantly higher in the skin of Zdhhc13k/k mutant mice regardless of whether the animals were kept in an SPF or GF environment (Figure 3b). All these data suggest that the skin barrier is defective in mutant mice.

To visualize any potential impaired skin structure in Zdhhc13k/k mutant mice, we performed an examination of mouse skin at P0 using electron microscopy. We observed an obvious increase in the number of lamellar bodies in the stratum granulosum layer of mutant skin. Moreover, in the mutant mice, many of these lamellar bodies had internal vacuoles (Figure 3c), suggesting a defect in content loading of lamellar bodies. Because genetic mutations in filaggrin are often associated with human AD (Barnes, 2010; O'Regan et al., 2009; Sandilands et al., 2009), we also assessed filaggrin expression in WT and Zdhhc13k/k skin by immunoblotting. We used radioimmunoprecipitation assay (RIPA) lysis buffer, which contained 1% nonidet P-40 and 0.1%
Figure 2. Epidermal expression of Zdhhc13 is essential for maintaining skin barrier integrity. (a) LacZ staining (blue) of Zdhhc13<sup>Gt</sup> and Zdhhc13<sup>Gt/Gt</sup> skin from embryo stage E13.5 to P0. (b) LacZ staining of Zdhhc13<sup>Gt</sup> and Zdhhc13<sup>Gt/Gt</sup> adult skin after washing with acetone/ethanol (A/E) or H<sub>2</sub>O (control). The red background staining in (a) and (b) is nuclear fast red. (c) RT-PCR analysis of Zdhhc13 and Tslp expression in adult WT mouse skin after washing with A/E or H<sub>2</sub>O. Gapdh was used as a loading control and -RNA was used as the negative control. (d) Zdhhc13<sup>f/f</sup>;K5-Cre<sup>ERT</sup> and Zdhhc13<sup>f/f</sup> mice were all injected with TAM at P22, and mice were separated into shaved and unshaved groups. The two left-hand panels show photos of shaved Zdhhc13<sup>f/f</sup> and Zdhhc13<sup>f/f</sup>;K5-Cre<sup>ERT</sup> mice taken immediately after shaving. Photos were taken of the same mice 1 month later (right). Also shown is an unshaved Zdhhc13<sup>f/f</sup>;K5-Cre<sup>ERT</sup> for comparison. (e) Hematoxylin and eosin (H&E) stained back skin sections taken from the same mice 1 month after TAM injection. The bottom panels are higher magnifications of the top panels. Scale bars: 100 µm (e, upper); 50 µm (b and e, lower); 20 µm (a). The dotted lines indicate the border of the epidermis and dermis. RT-PCR, reverse transcriptase-PCR; TAM, tamoxifen; WT, wild type.
SDS, to prepare total cell lysates, and a modified RIPA lysis buffer, containing only 1% nonidet P-40, to prepare soluble protein lysates. Western blot analysis showed that in neonatal mouse skin there was no obvious difference between WT and Zdhhc13<sup>k/k</sup> mice in the expression of total filaggrin proteins or other corneocyte envelope proteins, such as involucrin, or

Figure 3. Impaired skin barrier and abnormal filaggrin processing in Zdhhc13-deficient mice. (a) X-gal penetration assay of E16.5, E17.5, and neonatal whole-mount embryos (WT and Zdhhc13<sup>k/k</sup>). Side and top (back) views are shown for whole embryos and side views only for whole-neonatal mice. Skin sections were cut from the back skin of neonatal mice that had been subjected to the X-gal penetration assay. X-gal precipitation (blue); nuclear fast red (red). (b) Transepidermal water loss (TEWL) measured in the back skin of adult mice raised in specific-pathogen-free (SPF) or germ-free (GF) isolators (SPF: WT/Het n = 14, Zdhhc13<sup>k/k</sup> n = 10; GF: WT/Het n = 14, Zdhhc13<sup>k/k</sup> n = 13). Error bars represent mean ± SEM (**P < 0.001). (c) Electron microscopy of WT (upper panels) and Zdhhc13<sup>k/k</sup> (lower panels) mouse skin at P0. Arrowheads indicate lamellar bodies. The right panels are higher magnifications of the respective left-hand panel. (d) Immunoblotting analysis of filaggrin (Fil), profilaggrin (Profil), keratin 10 (K10), and involucrin (Inv) expression levels in WT and Zdhhc13<sup>k/k</sup> skin lysates at P0. Actin was used as a loading control. RIPA and a modified RIPA (mRIPA) buffers were used for cell lysate preparation. (e) Immunofluorescence microscopy of filaggrin (green) in WT and Zdhhc13<sup>k/k</sup> mouse skin at P2. The integrin α6 staining (red) shows the skin basement membrane that separates the epidermis and dermis. (f) Quantification of filaggrin intensity from images such as (e) using ImageJ software. The measured area is the whole epidermis of the picture excluding the stratum corneum (n = 4 for each of WT and Zdhhc13<sup>k/k</sup>; error bars represent mean ± SEM; *P < 0.05). Scale bars: 20 μm (a and e); 100 nm (c, left); 50 nm (c, right). The dotted lines indicate the border of the epidermis and dermis. RIPA, radioimmunoprecipitation assay buffer; SEM, standard error of the mean; WT, wild type.
other differentiated keratin proteins (e.g., keratin 10, K10) (Figure 3d, RIPA). However, we found a dramatic accumulation of soluble processed filaggrins, which were shorter than the filaggrin monomers (approximately 35 kDa), but not of soluble profilaggrins, filaggrin monomers, keratin 10, or involucrin in Zdhhc13kk mice (Figure 3d). Diffuse immunostaining also indicated a significant accumulation of processed filaggrin in skin sections of mutant mice (Figure 3e and f). These results suggest that the ZDHHC13 protein may be involved in filaggrin degradation and epidermal lipid barrier formation.

Environmental bacterial pathogens cause skin inflammation in Zdhhc13kk mice

The similarity of the AD-like disorder of Zdhhc13kk mice to the human extrinsic type of AD prompted us to explore whether external microbial factors also play an important role in this skin inflammation model. First, we investigated the earliest time-point that showed signs of keratinocyte hyperplasia in Zdhhc13kk mutant skin by immunohistologic examination of skin at different postnatal time-points using antibodies against various epidermal differentiation markers (Supplementary Figure S6 online). In neonates (i.e., P2), we did not observe differences between WT and mutant skin. As a reference, reductions in the stratum spinosum and stratum granulosum layers in WT skin started at P8, continued at P14 and, by the time of weaning (i.e., P22), WT skin had only two cell layers (Figure 1e, Supplementary Figure S2). However, Zdhhc13kk skin still had five to six layers of cells that highly expressed all markers at P22 (Figure 1e; also see Supplementary Figure S3 for details). Consistent with this observation, cells in the stratum basale of mutant skin remained highly proliferative, as indicated by high Ki67 positive staining, whereas WT cells were mostly Ki67 negative and quiescent (Figure 4a, upper panels). At P22 mutant skin showed high levels of signaling through the NF-kB pathway because p-IKK was upregulated in the upper skin layer (Figure 4a, middle panels). Subsequently, expression of keratin 6, a marker of skin inflammation, appeared in mutant skin at P14 (Supplementary Figure S6) and became very prominent at P22 (Figure 4a, bottom panels, and Supplementary Figure S3). Furthermore, the dermis area was heavily infiltrated with various immune cells, such as mast cells (Figure 1g), eosinophils (Figure 1f), macrophages, and

Figure 4. Mutation of Zdhhc13 results in skin inflammation and recruitment of immune cells into the dermis. (a) Immunostaining of Ki67, p-IKK, and K6 (green) in back skin of WT and Zdhhc13kk mice at P22. (b) Immunostaining of macrophage (F4/80), CD3, CD4, and CD8 (green) in the back skin of WT and Zdhhc13kk mice at P22. DNA, blue. Scale bars: 20 μm. The dotted lines indicate the border of the epidermis and dermis. WT, wild-type.
CD3/CD4 cells (Figure 4b). Mutant skin became hyperproliferative (i.e., K14 and K10 overexpression at P14) within 2 weeks after birth, before the inflammatory responses.

To investigate the possibility that microbial infection after birth leads to AD-like skin disorders, we rederived Zdhhc13kn/k mice into a GF isolator to avoid any possible microorganism infection after mice were born. Intriguingly, with lack of exposure to germs, Zdhhc13kn/k mice appeared to be healthy in terms of appearance, size, body weight (Figure 5a, left and right panels), and bone density (Figure 5b), and did not produce the overt hair phenotype and skin inflammation (as indicated by histologic comparison of GF samples with SPF).
samples, Figure 5c). Return of GF mice to an SPF vivarium before weaning rendered mice susceptible to the baldness phenotype, but they were resistant to baldness if this was done after weaning (Figure 5d; this phenomenon was also seen in K5-Cre specific knockout Zdhhc13 mice without shaving, Figure 2d). To further test this hypothesis of a microbial-driven phenotype, we applied a bacitracin-neomycin antibiotic ointment over the entire bodies of neonates twice a day, from birth to P21, in an SPF vivarium. Although the antibiotic-treated mice were small and weak, the hair coats of the treated mutant mice were normal (Figure 5e), and a histologic study indicated that, in local skin regions, there were no signs of keratinocyte hyperplasia, K6 induction, or p-IKK activation in the treated skin (Figure 5f).

Skin phenotypes of Zdhhc13-deficient mice are an innate immune-specific disorder

There are many studies suggesting that the Th2 immune response plays a crucial role in initiating and exacerbating the inflammation severity of AD (Brandt and Sivaprasad, 2011). To explore the essential role of adaptive immunity in this skin inflammation phenotype, we generated Rag-1−/−;Zdhhc13−/− double-mutant mice, which lack T and B lymphocytes, to examine the effect on skin phenotype. Surprisingly, the Rag-1−/−;Zdhhc13−/− mutation did not rescue the alopecia or epidermal hyperplasia (Figure 6a). Accordingly, double-mutant skin still had substantial mast cell accumulation (Figure 6b), suggesting that the Th2 immune response is dispensable, and an innate immune response may be more important in skin inflammation. Because we had already

![Figure 6](image_url)

**Figure 6.** AD-like disease of Zdhhc13-deficient mice is an innate immune-specific disorder. (a, b) Mouse photos and back skin H&E (a) and toluidine blue (b) staining for mast cells of skin sections from Rag-1−/− and Rag-1−/−;Zdhhc13−/− double-mutant mice. (c, d) Photos (left) and H&E staining of back skin sections (right) for Tnf−/− and Tnf−/−;Zdhhc13−/− double-mutant mice (c) and Il1r1−/− and Il1r1−/−;Zdhhc13−/− double-mutant mice (d). (e) Skin IL-33 levels measured in the back skin of adult mice. (f) The total number of ILC2s present was calculated in 6 cm² of back skin. The ILC2s were CD45+ cell-gated with Lin− and ST2+. (e) Zdhhc13−/− and Zdhhc13−/− (n = 6); Rag-1−/− and Rag-1−/−;Zdhhc13−/− (n = 3). (f) n = 4 per condition. Data in (e) and (f) represent mean ± SEM (*P < 0.05; ***P < 0.001). Scale bars: 50 μm. The dotted lines indicate the border of the epidermis and dermis. AD, atopic dermatitis; H&E, hematoxylin and eosin; ILC2s, type 2 innate lymphoid cells; SEM, standard error of the mean.
demonstrated high TNF-α and IL-1β expression in inflamed skin, the role of inflammatory cytokines was also investigated by cross-breeding Zdhhc13<sup>Δ<sub>k</sub></sup>k mice with TNF-α (Figure 6c) or IL1R1-deficient mice (Figure 6d). Additional loss of TNF-α or IL-1β signaling in Tnf<sup>−/−</sup>;Zdhhc13<sup>Δ<sub>k</sub></sup>k or Il1r1<sup>−/−</sup>;Zdhhc13<sup>Δ<sub>k</sub></sup>k mice also did not protect skin from inflammation and epidermal hyperplasia, suggesting that either TNF-α and IL-1β have overlapping action in this inflammation condition, or the involvement of an unknown innate immune cytokine(s) which plays a more critical role in triggering epidermal inflammation than TNF-α and IL-1β.

Recently, clinical and experimental observations have suggested that type 2 innate lymphoid cells (ILC2) might regulate the onset of AD (Kim et al., 2013; Salimi et al., 2013). ILC2 cells are activated by the innate cytokine, IL-33, via the ST2 receptor and contribute to increases in type 2 cytokine production (Salimi et al., 2013). To investigate the presence of ILC2 cells in our AD-like mouse model, we analyzed skin IL-33 levels and ILC2 cell numbers. We noted IL-33 was significantly increased in Zdhhc13<sup>Δ<sub>k</sub></sup>k and Rag1<sup>−/−</sup>;Zdhhc13<sup>Δ<sub>k</sub></sup>k mice (Figure 6e), and ILC2 cell numbers were increased in Zdhhc13<sup>Δ<sub>k</sub></sup>k skin (Figure 6f). These data further support that initiation of dermatitis in Zdhhc13<sup>Δ<sub>k</sub></sup>k mice involves innate immunity rather than adaptive immunity.

**DISCUSSION**

In the current study, we report development of a mouse model of human AD wherein genetic deletion of ZDHHC13, an epidermal-specific S-palmitoylation enzyme, results in a variety of phenotypes that are highly reminiscent of severe human AD, including acanthosis, increased scratching, skin redness, together with early-stage dermatitis and late-stage lichenification (Figure 1). Data from several biochemical and immunological measurements also revealed similarities to the human disease, including elevated serum IgE, Th2 cytokine expression, increased skin pH (Figure 1), and immune cell infiltration (Figures 1, 4, and 6). Furthermore, SPF Zdhhc13<sup>Δ<sub>k</sub></sup>k mice manifested skin inflammation beginning at the perinatal stage and developed eczema with 100% penetrance by 3 weeks. Using TEWL measurements, skin barrier impairment was evident in the skin of Zdhhc13<sup>Δ<sub>k</sub></sup>k GF mice (Figure 3); this is reminiscent of the epidermal barrier dysfunction reported in the nonlesion skin of patients with AD (Jakasa et al., 2007). The data also suggest the involvement of ILC2 cells in this skin inflammation, similar to what has been described in filaggrin-deficient mice (Saunders et al., 2015). All these indications are in line with the hallmarks of the extrinsic type of human AD (Peterson and Chan, 2006; Tokura, 2010), which is highly associated with pathogen colonization and an impaired skin barrier (Cork et al., 2006; Elias et al., 2008). Moreover, after rederiving Zdhhc13-deficient mice in a GF isolator we proved that commensal bacteria are the factors that initiate skin infection and inflammation, providing strong evidence to support the hypothesis of extrinsic AD in humans.

In our previous reports, Zdhhc13-deficient mice showed many severe phenotypes, including osteoporosis (Liu et al., 2015; Saleem et al., 2010; Song et al., 2014), and the severity of these phenotypes varied when Zdhhc13-deficient mice were raised in a distinct SPF animal vivarium. In a GF isolator, mutant mice were rescued from most of these inflammatory phenotypes (Figure 5). When both WT and mutant mice were moved back from a GF isolator to an SPF animal room for 1 month, overall bone volume decreased (Figure 5b, 1 month after). However, we noticed that the bone volume of these mutant mice remained higher than that of mutant mice grown in an SPF vivarium (Figure 5b, SPF). Bone loss is prevalent in many chronic autoinflammatory diseases, including rheumatoid arthritis, ankylosing spondylitis, inflammatory bowel disease, and systemic lupus erythematosus (Amarasekara et al., 2015). Patients with AD also have a higher prevalence of low bone density (Haack et al., 2009). Therefore, we suggest that in addition to gut microbiota, chronic skin inflammation may also contribute to bone density homeostasis.

In this study, we found that elevation of the proinflammatory cytokotytes IL-25 and IL-33, as well as ILC2 innate immune cells, is highly correlated with the initiation of Zdhhc13<sup>Δ<sub>k</sub></sup>k skin inflammation. Previously, IL-25 and IL-33 have both been identified as being overexpressed in the skin of human patients and mice with AD (Roediger et al., 2013; Salimi et al., 2013), and both induce Th2 responses in animals (Miller, 2011). Expression of IL-25 and IL-33 in the skin can recruit ILC2 cells, stimulating them to produce high levels of IL-13 (Salimi et al., 2013). Moreover, IL-33 has been suggested to be involved in the pathogenesis of various allergic disorders such as asthma (Kondo et al., 2008) and allergic rhinitis (Haenuki et al., 2012) amongst others, reinforcing the notion that Zdhhc13<sup>Δ<sub>k</sub></sup>k is an AD-like mouse model. The Zdhhc13<sup>Δ<sub>k</sub></sup>k mice reported here might also be used as a mouse model for further study of atopic march.

**MATERIAL AND METHODS**

**Mice**

Mutant Zdhhc13<sup>Δ<sub>k</sub></sup>k mice were generated by conventional ethyl nitrosourea mutagenesis and bred as described (Saleem et al., 2010). The Zdhhc13<sup>Δ<sub>k</sub></sup>k mouse was produced from AC0492 embryonic stem cells obtained from the Sanger Institute Gene Trap Resource (Saleem et al., 2010; Sutton et al., 2013). The Zdhhc13<sup>Δ<sub>k</sub></sup>k mouse was generated as described in the Supplementary Materials and Methods online (Supplementary Figure S1). The Cre mouse used to generate the Zdhhc13<sup>Δ<sub>k</sub></sup>k mouse was E2A-Cre (#003724), The Jackson laboratory, Sacramento, CA), and the epithelial-specific knockout of Zdhhc13 was generated using K5-Cre<sup>ERT</sup> mice (Liang et al., 2009), which required a tamoxifen injection (Feil et al., 1996). The Rgs1<sup>−/−</sup> (I002216), Tnf<sup>−/−</sup> (I003008), and IItf<sup>Tm1Kond</sup> (I003018) mice were purchased from The Jackson Laboratory. The primer sequences for mice genotyping are shown in the Supplementary Materials and Methods. GF rederivation of Zdhhc13<sup>Δ<sub>k</sub></sup>k mice was performed in the National Laboratory Animal Center, National Applied Research Laboratories, Taiwan (Chuang et al., 2012).

**β-Galactosidase expression analysis**

β-Galactosidase (LacZ) expression in mouse tissues was detected using X-gal staining (Nagy et al., 2007).
Skin permeability assay (toluidine blue and X-gal penetration assay)
Skin permeability was monitored with toluidine blue and X-gal as described (Hardman et al., 1998). After staining, embryos or neonatal pups were photographed.

Transmission electron microscopy
Aldehyde-fixed skin sections were post-fixed with 0.2% ruthenium tetroxide (RuO4) (Hou et al., 1991; Madison et al., 1988) and ultrathin sections were examined using transmission electron microscopy (Tecnai G2 Spirit TWIN from FEI Company, Hillsboro, OR) and images were captured using a Gatan CCD Camera (794.10.BP2MultiScanTM, Pleasanton, CA).

Skin tissue processing and flow cytometry
Whole skin sheets were incubated in DMEM containing 0.2% collagenase type IV and 0.01% DNase I (Worthington Biochemical, Lakewood, NJ) at 37 °C for 60 minutes. The digested tissue was filtered through a 70-μm mesh to obtain a single cell suspension. Lymphocytes were further enriched by centrifugation on a 33% continuous Percoll (Amersham Biosciences, Piscataway, NJ) gradient. Cells were washed in FACS running buffer (2% fetal calf serum in phosphate buffered saline), stained with fluorochrome-conjugated antibodies, and examined using FACSA Canto (BD Biosciences, San Jose, CA). The antibodies used for ILC2 staining were as follows: CD45, lineage markers (CD3e, CD11b, CD11c, FcεRI, CD19, F4/80, and CD49b), and ST2.

Antibodies used for immunohistochemistry and immunoblotting
Antibodies used for immunohistochemistry or immunoblotting are described in the Supplementary Materials and Methods.

Statistical analysis
Statistical comparisons between groups were performed using the two-tailed Student’s t-test and Microsoft Excel (Microsoft, Redmond, WA); differences were considered statistically significant at P-values <0.05.

Study approval
For animal studies, the protocols were approved by the Institutional Animal Care and Utilization Committee of Academia Sinica (protocol numbers: 11-03-148 and 14-12-764).

Reverse transcriptase-PCR and real-time PCR, skin pH and TEWL measurement, and ELISA
Information describing the above common experiments is provided in the Supplementary Methods.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.12.011.

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