***Znf750* regulates skin barrier function by driving cornified envelope and lipid processing pathways**

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**ABSTRACT**

The skin epidermis is a constantly renewing, stratified epithelial tissue that provides essential protective barrier functions. The major barrier is located at the outermost layers of the epidermis, formed by terminally differentiated keratinocytes reinforced by proteins and lipids of their cornified envelope (CE). Disruptions to this differentiation characterize various skin disorders. ZNF750 is an epithelial transcription factor essential for *in vitro* keratinocyte differentiation, whose truncating mutation in humans causes autosomal dominant psoriasis-like skin disease. Here, we utilized an epidermal-specific *Znf750* conditional knockout mouse model to investigate the role ZNF750 plays in epidermal development. We show that deletion of *Znf750* in the developing skin does not block epidermal differentiation completely, suggesting *in vivo* compensatory feedback mechanisms, although it does result in impaired barrier function and perinatal lethality. Molecular dissection revealed ultrastructural defects in the differentiated layers of the epidermis, accompanied by alterations in the expression of ZNF750-dependent genes encoding key CE precursor proteins and lipid-processing enzymes, including gene subsets known to be mutated in human skin diseases involving impaired barrier function. Together, our findings provide molecular insights into the pathogenesis of human skin diseases by linking ZNF750 to a subset of epidermal differentiation genes involved in barrier formation pathways.

**INTRODUCTION**

The mammalian skin epidermis is a constantly renewing, stratified epithelium at the interface between the body and the environment. During epidermal development, a monolayer of epidermal progenitors located at the base of the epidermis undergoes a step-wide differentiation program and gives rise to a stratified and highly specialized tissue that provides essential barrier functions against insults and prevents dehydration (Moreci and Lechler, 2020). The major barrier is provided by terminally differentiated anucleate keratinocytes through their cornified cell envelope (CE), a tough and insoluble structure composed of tightly crosslinked CE precursor proteins sealed by a water-resistant lipid lamellae bilayer (Jonca and Simon, 2023). Disruptions to the process of epidermal differentiation or alterations in barrier formation are characteristic of various common skin diseases, including atopic dermatitis, psoriasis, and epidermal skin cancers (Lopez-Pajares et al., 2013).

 We and others have previously identified the human zinc finger protein 750 (*ZNF750*) as an essential regulator of the *in vitro* keratinocyte differentiation process (Cohen et al., 2012, Sen et al., 2012). ZNF750 acts as a dominant driver of epidermal differentiation downstream of the epidermal master regulator, p63, as evidenced by its ability to partially restore the impaired epidermal differentiation process arrested in mutant p63 keratinocytes (Zarnegar et al., 2012). ZNF750 has also been shown to cooperate with other epidermal factors, such as KLF4 and KDM1A, to induce differentiation genes and to repress progenitor and embryonic genes, respectively (Boxer et al., 2014). In humans, a truncating mutation in *ZNF750* causes autosomal dominant psoriasis-like skin disease (Birnbaum et al., 2006), and several rare *ZNF750* regulatory variants have been shown to be associated with psoriasis (Birnbaum et al., 2011). Here, we investigated the role ZNF750 plays in skin development by utilizing a genetic mouse model in which a *ZNF750* murine homolog, *Znf750* (also known as *Zfp750*), was conditionally deleted in the developing skin epithelium. We found that ZNF750 activity was required for proper skin barrier function and postnatal survival. By combining histological and transcriptional analyses, we elucidated the main ZNF750 downstream pathways and identified a requirement for ZNF750 in controlling the expression of genes encoding both structural proteins and lipid-processing enzymes involved in the formation of the skin barrier.

**RESULTS**

***Znf750* conditional knockout (cKO) mice exhibit impaired skin barrier function and perinatal lethality**

To gain insights into the *in vivo* role ZNF750 plays in epidermal differentiation and skin development, we generated *Znf750*-floxed mice carrying LoxP sites flanking exon 2 and the coding region of exon 3 that encompass the entire *Znf750* coding sequence, resulting in a *Znf750* conditional ready targeted allele (Supplementary Figure S1a). Next, to conditionally ablate ZNF750 in the developing skin epidermis, we crossed *Znf750*-floxed mice with *Krt14-Cre* mice, in which Cre recombinase is expressed under keratin 14 promoter that is active in embryonic epidermal progenitors starting at embryonic day (E) 12 (Dassule et al., 2000) (*Krt14-Cre*; *Znf750*flox/flox = *Znf750* cKO). We confirmed the successful deletion of *Znf750* at the mRNA level by performing reverse transcription quantitative PCR (RT-qPCR) and fluorescence *in situ* hybridization against Znf750 transcripts (Figure 1a-b and Supplementary Figure S1d-e). Newborn *Znf750* cKO mice displayed several physical abnormalities, including the absence of abdominal milk spot (despite apparently normal palate development), open eyelids, a shiny skin appearance, and early postnatal lethality (Supplementary Figure S1b-c), suggesting defects in epidermal development or barrier function.

 To determine whether the perinatal lethality evident in *Znf750* cKO newborns could be the result of an impaired skin barrier, we first examined the “outside-to-inside” skin barrier function using a toluidine blue dye exclusion assay. We found that the skin barrier in *Znf750* cKO mice at E17.5 failed to exclude dye, whereas it was excluded in control mice; however, several days later, at P0, the skin barrier of *Znf750* cKO mice displayed an apparently normal dye-exclusion function (Figure 1c). In contrast, the “inside-to-outside” skin barrier function required for preventing water loss and dehydration was impaired in *Znf750* cKO mice at P0, as indicated by a significant increase in trans-epidermal water loss (TEWL), as well as a subsequent dramatic reduction in initial body weight within 10 hours from birth (Figure 1d-e). In line with this impaired barrier function, newborn *Znf750* cKO mice died within 12 to 16 hours after birth. Taken together, these results indicate that during skin development ZNF750 plays a crucial role in the establishment of epidermal barrier functions essential for postnatal survival.

**Loss of ZNF750 results in a delayed epidermal differentiation program**

ZNF750 is considered to act as a driver of the *in vitro* epidermal differentiation program in human keratinocytes, and its loss has been shown to impair the induction of epidermal late differentiation genes, including important granular layer molecular markers such as filaggrin (FLG) and loricrin (LOR) (Boxer et al., 2014, Cohen et al., 2012, Sen et al., 2012). In line with previous *in vitro* studies, our histological analysis of murine *Znf750* cKO epidermis at E17.5 revealed a dramatic delay in the epidermal differentiation process (Figure 2a). The expression of the early differentiation marker keratin 10 (KRT10) in *Znf750* cKO epidermis was comparable with that seen in control mice epidermis, but there was impaired induction of the late differentiation markers LOR and FLG (Figure 2b-d). However, during the later stages of development, *Znf750* cKO epidermis formed terminally differentiated cornified layers and expressed early and late differentiation markers at levels that were comparable with those observed in control epidermis (Figure 2e-h). These observations are in line with the delayed acquisition of the outside-to-inside barrier mentioned above (Figure 1c). Further analysis of the epidermis revealed no significant differences between control and *Znf750* cKO epidermis in either cell proliferation or apoptosis, as determined by Ki67 and activated caspase 3 (CASP3), respectively (Supplementary Figure S2a-c). As differentiated granular layers (stratum granulosum, SG) and terminally differentiated cornified layers (stratum corneum, SC) were formed in *Znf750* cKO epidermis, we concluded that the loss of skin barrier function in newborn *Znf750* cKO mice was not due to an arrest in the epidermal differentiation program.

***Znf750* cKO mice display ultrastructural defects in the granular and cornified layers of the epidermis**

Ultrastructural analysis of skin collected from *Znf750* cKO and control newborn mice showed irregular organization in the SG and SC layers of the *Znf750* cKO epidermis (Figure 3a). To corroborate these alterations, we performed transmission electron microscopy (TEM) analysis, which further showed that darkly stained keratohyalin granules (KG) mainly consisting of profilaggrin (Hoober and Eggink, 2022) were dramatically reduced in *Znf750* cKO epidermis compared with control epidermis (Figure 3b and c). Moreover, instead of being distributed horizontally across the SG layers, KGs in *Znf750* cKO epidermis were atypically abundant around the disintegrating nuclei of terminally differentiating transition cells (Figure 3b). In addition, unlike the typical presence of consistently dark KGs that interacted with an organized network of keratin filaments observed in control epidermis, KGs in *Znf750* cKO epidermis had a bicomponent appearance with darker and lighter content and altered keratin filament organization (Figure 3c and Supplementary Figure S3a). Across the SC layers of *Znf750* cKO epidermis there were regions with dense, abnormal stratification of corneocytes (Supplementary Figure S3b), suggesting that the CE structure may have been impaired. Our analysis of FLG and LOR – two major components of the CE (Jonca and Simon, 2023) – demonstrated a reduction in FLG mRNA and protein levels in *Znf750* cKO epidermis compared with control epidermis, whereas LOR levels were comparable with those in control epidermis (Figure 3d and Supplementary Figure S3c-d). Further analysis of *Znf750* cKO corneocytes demonstrated significantly reduced CE thickness and increased susceptibility to physical stress induced by sonication (Figure 3e-f and Supplementary Figure S3e-f).

Next, we examined the secretion of lamellar bodies (LBs), which contain the lipids required for the formation of the lipid lamellae bilayers that provide the permeable-barrier functions (Elias, 2012). The secretion of LBs at the SC–SG interface was increased in *Znf750* cKO epidermis, although nascent LBs were not prominent in the cytosol of the uppermost SG cells (Figure 3g and Supplementary Figure S3a, g, and h), suggesting a near-total secretory response of these cells in an attempt to compensate for the impaired barrier and increased TEWL in *Znf750* cKO epidermis, as previously described upon barrier disruption (Menon et al., 1992). LB-derived lipid lamellae bilayers at the SC layers did form in *Znf750* cKO epidermis, but displayed a disorganized appearance with non-lamellar gaps, suggesting incomplete lipid-processing (Figure 3h-i).

Finally, we performed lanthanum perfusion studies to examine the tight-junction barrier function at the SG layers. This showed that there were functional tight junctions in *Znf750* cKO epidermis that prevented lanthanum traces from reaching the SC layers (Supplementary Figure S3i).

**The epidermal transcriptional regulatory network is maintained in newborn *Znf750* cKO epidermis**

To determine the transcriptional changes upon loss of epidermal ZNF750, we performed RNA sequencing (RNA-seq) on P0 control and *Znf750* cKO epidermis samples. We observed a total of 343 differentially expressed genes (DEGs) in Znf750 cKO versus control epidermis; 207 DEGs that were downregulated and 136 that were upregulated (Figure 4a and Supplementary Table S1). These somewhat subtle alterations in gene expression, together with our observation that the epidermal differentiation process in *Znf750* cKO epidermis was initially delayed at E17.5 but eventually progressed (Figure 2a-2h), led us to hypothesize that the loss of ZNF750 could be partially compensated for by other epidermal transcription factors (TFs). To examine this possibility, we obtained chromatin immunoprecipitation sequencing (ChIP-seq) data for ZNF750 target genes in differentiated human epidermal keratinocytes (Boxer et al., 2014) and performed ChIP-X transcription factor enrichment analysis using the ChIP-X enrichment analysis (ChEA3) tool, which identifies putative binding of TFs to a given set of target genes based on publicly available ChIP-seq experiments (Keenan et al., 2019). Our analysis revealed the significant enrichment of several TFs, including grainyhead-like (GRHL), ovo-like (OVOL), and Krüppel-like factor (KLF) family members (Figure 4b), all of which are known to play important roles in epidermal development and differentiation (Dragan et al., 2022, Hopkin et al., 2012, Jones et al., 2020, Lin et al., 2020, Mlacki et al., 2014, Teng et al., 2007). However, the greatest enrichment was observed for ZBTB7B (Figure 4b), whose role in the epidermis is yet to be elucidated.

Using our RNA-seq data and RT-qPCR, we measured the expression of these TFs and found that most of them maintained comparable levels of expression between *Znf750* cKO and control epidermis, with the expression of some TFs such as Grhl3 and Ovol2 even increasing upon the loss of ZNF750 (Figure 4c and Supplementary Figure S4a). We also examined the expression of additional key epidermal TFs, such as p63 and Klf4 (Koster et al., 2004, Mills et al., 1999, Segre et al., 1999), as well as other TFs that have been predicted to co-occupy cis-regulatory elements with ZNF750 in epidermal differentiation (Kim et al., 2021). Each of them showed similar expression trends between *Znf750* cKO and control epidermis (Figure 4c and Supplementary Figure S4a). These data indicate that the activity of ZNF750 in driving epidermal differentiation *in vivo* could be partially compensated for by other epidermal TFs.

**ZNF750 is an essential regulator of genes related to skin barrier functions**

To gain insights into the molecular mechanisms underlying the skin barrier defects observed in *Znf750* cKO epidermis, we subjected the ZNF750-dependent DEGs (Figure 4a and Supplementary Table S1) to Gene Ontology (GO) analysis using the DAVID tool (Huang da et al., 2009, Sherman et al., 2022). Interestingly, both upregulated and downregulated DEGs were enriched for GO terms related to epidermal development and keratinization (Figure 5a and Supplementary Figure S5a). Genes upregulated in *Znf750* cKO epidermis included cornified envelope (CE) components such as small proline-rich protein 1b (Sprr1b), Sprr2d, and involucrin (Ivl) (Supplementary Figure S5b), whose upregulation may serve as a compensatory mechanism in an attempt to rescue the skin barrier defect, as previously described (Koch et al., 2000, Kypriotou et al., 2012, Presland et al., 2000, Utsunomiya et al., 2020). Other upregulated genes included Krt6a and Krt6b, which are known to be induced upon barrier breach (Paladini et al., 1996, Takahashi et al., 1998), thus providing further molecular confirmation for the observed barrier defects in *Znf750* cKO epidermis.

Next, we focused on the genes that were significantly downregulated in *Znf750* cKO epidermis (Figure 4a and Supplementary Table S1). GO analysis revealed enrichment in terms related to epidermis development and keratinization (Figure 5a), including genes composing CE, such as *Sprr1a*, *Sprr4*, *Cdsn*, *Scel*, and *Lce1* family members, as well as keratinization genes, such as *Casp14* and *Prss8*, which are required for the processing of CE precursor proteins (Figure 5a and 5b). We also observed enrichment for terms related to lipid metabolism, including several cytochrome P450 *Cyp2* family members, as well as the lipoxygenase (LOX) genes *Aloxe3* and *Alox12e* (Figure 5a and 5b) that play a central role in the processing of barrier lipids required for proper formation of the cornified lipid envelope (CLE) and the lipid lamellae (Elias et al., 2008). Interestingly, we also observed enrichment for genes related to the innate immune response and leukocyte-mediated immunity (Supplementary Table S1). We validated our RNA-seq data using RT-qPCR for several of the keratinization and lipid metabolism genes, and we confirmed their significant reduction in *Znf750* cKO epidermis (Figure 5c). Notably, a subset of these ZNF750-dependent genes, including *Cdsn*, *Casp14*, *Prss8*, and *Aloxe3* (Figure 5c; blue), are known to be mutated in human conditions that involve impaired barrier functions and/or have been shown to be essential for epidermal barrier functions in genetic mouse models (Hoste et al., 2013, Kirchmeier et al., 2017, Krieg et al., 2013, Leclerc et al., 2009, Leyvraz et al., 2005, Mashima and Okuyama, 2015, Oji et al., 2010, Shamseldin et al., 2023).

Finally, we showed that ectopic expression of *Znf750* in mouse primary keratinocytes was sufficient for the induction of a subset of these ZNF750-dependent genes, without affecting the undifferentiated state of the cells, as evidenced by the levels of the early differentiation marker Krt1 (Figure 5d). Taken together, our data indicate that ZNF750 plays a prominent role in regulating the expression of genes whose products either comprise or process both the lipid and protein components of the CE.

**DISCUSSION**

In this study, we report the generation of a genetic mouse model that was utilized to determine the *in vivo* role ZNF750 plays in epidermal development. We show that the epidermal loss of ZNF750 in murine skin leads to ultrastructural defects in the differentiated layers of the skin epidermis, which compromises skin barrier functions and results in perinatal lethality. Transcriptional analysis of DEGs in *Znf750* cKO epidermis demonstrated that ZNF750 controls the expression of structural protein precursors of the CE, as well as key enzymes involved in the formation of the CE and the barrier lipid lamellae. These main findings illustrate the essential role that ZNF750 plays in epidermal development and point to the key molecular pathways by which ZNF750-specific activity in the epidermis regulates skin barrier functions, thus providing a molecular link to the psoriasis-like skin condition in humans caused by autosomal dominant heterozygous ZNF750 mutation (Birnbaum et al., 2006).

 The epidermal differentiation program is a finely tuned, stepwise program orchestrated by the p63 master regulator and its downstream targets (Candi et al., 2006, Koster et al., 2007, Miroshnikova et al., 2019, Truong et al., 2006). ZNF750 acts as a central downstream target of p63 that promotes terminal epidermal differentiation, and its enforced expression has been shown to be sufficient for partial restoration of the differentiation process in p63-deficient or mutant keratinocytes (Sen et al., 2012, Zarnegar et al., 2012). ZNF750 regulates epidermal differentiation through cooperation with other transcriptional regulators or by driving the expression of downstream epidermal TFs; the silencing of ZNF750 in primary human keratinocytes or organotypic epidermis impairs the epidermal differentiation program (Boxer et al., 2014, Cohen et al., 2012, Sen et al., 2012). Interestingly, our data showed that the epidermal differentiation program was only partially affected in mice lacking epidermal ZNF750. Although initially delayed in *Znf750* cKO epidermis at E17.5, the epidermal differentiation program proceeded later during development and resulted in the formation of a stratified epidermis containing terminally differentiated SC layers. However, the later stages of differentiation, involving the proper expression of cornification genes, were impaired and resulted in reduced barrier function activity. Taking these findings together, we conclude that the *in vitro* loss of ZNF750 resulted in a greater impact on epidermal differentiation in comparison with its loss *in vivo*. It is possible that this discrepancy is due to inherent differences in the regulatory axis between murine and human epidermis, or it could have resulted from the different environment and molecular feedback mechanisms that exist *in vivo* and can compensate for the absence of ZNF750 in the developing skin. Indeed, we observed that the TF regulatory network involved in epidermal differentiation was maintained in *Znf750* cKO epidermis, and the expression levels of epidermal TFs that were enriched at ZNF750 target genes remained largely unaffected. In addition, our analysis demonstrated upregulated expression of Grhl3 and Klf4, both known to play a prominent role in driving the epidermal differentiation program (Klein et al., 2017, Lin et al., 2020, Patel et al., 2006, Segre et al., 1999, Yu et al., 2006), further supporting the notion of partial compensatory mechanisms by other TFs in the absence of ZNF750.

 In our genetic mouse model, the epidermal-specific deletion of *Znf750* using the *Krt14-Cre* driver impaired outward barrier functions, as evidenced by increased TEWL and a substantial loss of body weight, ultimately resulting in early postnatal lethality. Interestingly, a team conducting a parallel study using a *Znf750*-null mouse model (Butera et al., 2023) reported that epidermal thickness is reduced and both inward and outward barrier functions are impaired upon germline deletion of *Znf750*. In both Butera and colleagues’ and our own studies, the epidermal differentiation program appeared to progress and form cornified layers, yet a large set of overlapping genes involved in the late stages of epidermal differentiation and cornification were misregulated. The source of the partial discrepancy between the two genetic mouse models could stem from either indirect effects caused by the deletion of *Znf750* in other tissues of the constitutive *Znf750* KO mice or be due to the earlier epidermal deletion of *Znf750* compared with our conditional *Krt14*-driven *Znf750* cKO mice. Notably, although we confirmed that *Znf750* was practically deleted by E15.5, prior to the late stages of epidermal differentiation, it is possible that ZNF750 initiates some of its effects early in development before *Krt14-Cre* becomes fully active. This is similar to the case of *Grhl3*, in which a germline deletion in mice results in a more severe epidermal phenotype compared with deletion in the developing epidermis (Gordon et al., 2014, Yu et al., 2006).

By performing a lipid metabolism analysis, Butera and colleagues (2023) further identified alterations in lipid composition in the epidermis of *Znf750* KO mice. This is in agreement with our observation of changes in lipid processing genes, such as LOX and cytochrome 450 genes, which were downregulated in both mouse models. Importantly, using TEM analysis, we identified disruptions in the organization of the lipid lamellae bilayers in *Znf750* cKO epidermis, providing a molecular link between transcriptional alterations and impaired barrier function. In addition, our transcriptional analysis revealed the downregulation of several genes, including *Casp14* and *Prss8*, known to be involved in the processing of FLG and other CE precursors, whose mutations in human and/or mouse models resulted in impaired barrier function (Hoste et al., 2013, Kirchmeier et al., 2017, Leyvraz et al., 2005, Shamseldin et al., 2023). We also observed downregulated expression of genes encoding CE precursor proteins, including Cdsn, Scel, Sprr, and Lce1 family members, some of which were also shown to be downregulated in *Znf750*-null epidermis, suggesting this may also be a conserved ZNF750-dependent regulatory pathway. In line with this finding, our TEM analysis demonstrated that the thickness of CEs in *Znf750* cKO corneocytes was reduced, which can lead to increased susceptibility to physical stress. Thus, while both our study and that of Butera and colleagues (2023) present strong and complementary evidence of major alterations associated with lipid metabolism, here we have expanded the molecular spectrum of the phenotype reported by Butera and colleagues (2023) and provided additional evidence supporting a role for ZNF750 in the formation of the corneocyte CE. Together, these molecular functions are linked to the skin barrier defects observed in *Znf750* cKO epidermis.

 The importance of ZNF750 has been demonstrated by a truncating loss-of-function mutation in humans that causes autosomal dominant psoriasis-like skin disease (Birnbaum et al., 2006), as well as by pathogenic *ZNF750* mutations associated with epithelial cancers (Lin et al., 2014, North et al., 2018, Zhang et al., 2015), where ZNF750 is proposed to play a tumor suppressor role (Bi et al., 2020, Butera et al., 2020, Cassandri et al., 2020, Hazawa et al., 2017, Zhang et al., 2018). Although we did not observe any marked alterations in the skin of heterozygous mice lacking one copy of *Znf750*, it is very likely that the complete deletion of *Znf750* would impact tissue homeostasis of adult mouse skin and/or other epithelial tissues. Here, we have provided direct evidence for the importance of ZNF750 activity *in vivo*, which is required for the proper formation of the skin barrier. Our *Znf750* conditional mouse model provides a future avenue for investigating the role ZNF750 plays in tissue homeostasis of the adult skin and other epithelial tissues.

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