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p63 directly induces expression of Alox12, a regulator of epidermal barrier formation

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Abstract

Epidermal development and differentiation are tightly controlled processes that culminate in the formation of the epidermal barrier. A critical regulator of different stages of epidermal development and differentiation is the transcription factor p63. More specifically, we previously demonstrated that p63 is required for both the commitment to stratification and for the commitment to terminal differentiation. We now demonstrate that Δ Np63 α , the predominantly expressed p63 isoform in postnatal epidermis, also plays a role in the final stages of epidermal differentiation, namely the formation of the epidermal barrier. We found that Δ Np63 α contributes to epidermal barrier formation by directly inducing expression of ALOX12, a lipoxygenase which contributes to epidermal barrier function. Our data demonstrate that Δ Np63 α directly interacts with the promoter of *Alox12* in the developing epidermis. Furthermore, we found that the induction of *Alox12* expression by Δ Np63 α depends on intact p63 binding sites in the *Alox12* promoter. Finally, we found that Δ Np63 α can only induce *Alox12* expression in differentiating keratinocytes, consistent with the role of ALOX12 in epidermal barrier formation.

Keywords

p63; Alox12; epidermal barrier; epidermal development

Introduction

The epidermis, the outermost layer of the skin, functions as the body's primary barrier by protecting the organism from dehydration and environmental insults. The epidermal barrier, which is composed of the cornified envelope, the cornified lipid envelope, and extracellular lipids, is formed in a highly reproducible pattern during late stages of embryogenesis (1,2). Postnatally, the epidermal barrier is maintained by a complex epidermal differentiation program, which results in the constant production of the cellular and lipid components of the barrier (3). Compromised barrier function in prematurely born infants or in patients with certain inherited skin diseases often results in dehydration and increased susceptibility to infections (4–6).

Several members of the large family of lipoxygenases (LOX), lipid-peroxidizing enzymes which stereospecifically insert molecular oxygen into polyunsaturated fatty acids (7), have recently been implicated in epidermal barrier formation. Two LOXs which are critical for epidermal barrier formation are ALOX12B (also known as 12R-LOX) and ALOXE3 (also known as eLOX3). Inactivating mutations in *ALOX12B* and *ALOXE3* are found in individuals with non-bullous congenital ichthyosiform erythroderma (NCIE), an ichthyosis characterized by hyperkeratosis and a disruption of the epidermal permeability barrier (8–10). In addition to ALOXE3 and ALOX12B, ALOX12 (also known as platelet-type 12-lipoxygenase [p12-LOX]), is expressed in mouse and human epidermis (11–14). Within mouse epidermis, ALOX12 is expressed in the granular layer and is involved in maintaining the epidermal water barrier as demonstrated by an increase in transepidermal water loss in *Alox12* deficient mice (11). The relatively minor barrier defect observed in *Alox12* deficient mice would suggest that ALOX12 has a minor role in epidermal barrier formation. An alternative explanation could be that compensatory pathways are induced that maintain barrier function in the absence of ALOX12.

In addition to mice with genetic abnormalities in LOXs, epidermal barrier formation is also compromised in mice which lack transcription factors that, directly or indirectly, induce expression of components of the epidermal barrier (3). For example, mice lacking the transcription factor p63 fail to establish an epidermal barrier, causing the mice to die perinatally due to dehydration (15–17). However, since *p63*-deficient mice completely fail to develop an epidermis, it remains unclear whether p63 directly contributes to epidermal barrier formation. This question is further complicated by the finding that alternative promoter usage and differential splicing cause the *p63* gene to be transcribed into six isoforms, each of which may have unique roles during epidermal morphogenesis (18). For example, p63 isoforms containing a transactivation domain (TAp63) are required for the commitment to stratification during early stages of skin development (15). In contrast, p63 isoforms lacking this transactivation domain (Δ Np63) function after the commitment to stratification has occurred, and have important roles in cell adhesion, basement membrane formation, and terminal differentiation (19). Although Δ Np63 isoforms lack the N-terminal transactivation domain, they contain an internal transactivation domain, thus allowing Δ Np63 isoforms to activate gene expression (20,21).

To determine the role of Δ Np63 α in the epidermis, we previously generated epidermal-specific inducible Δ Np63 knockdown (Δ Np63 i-kd) mice (22). In these mice, a hairpin consisting of sequences specific for Δ Np63 isoforms was placed under the control of the “gene-switch” system. The regulator of the “gene-switch” system (Glp65) is activated by progesterone antagonists such as RU486, and was placed under control of the keratin 14 promoter (K14.Glp65), thus targeting expression of Glp65 to the basal layer of the epidermis. In mice carrying both components of the inducible system, topical treatment with RU486 results in expression of the Δ Np63 hairpin. This hairpin is subsequently processed into Δ Np63-specific siRNAs which degrade endogenous Δ Np63 transcripts. Using this system, we found that downregulating Δ Np63 in the epidermis resulted in hyperproliferation, impaired terminal differentiation, and basement membrane abnormalities (22). Furthermore, most Δ Np63 i-kd mice died after a few days of transgene activation, presumably due to excessive water loss caused by impaired epidermal barrier function.

Although these experiments demonstrated a role of p63 in epidermal barrier formation, it is currently not known how p63 regulates this process. Here, we identify *Alox12* as the first direct target gene of p63 with a role in epidermal barrier formation. We found that, in the developing epidermis, Δ Np63 α interacts with *p63* response elements located within the *Alox12* promoter, and that this interaction is required for the induction of *Alox12*. Furthermore, our data demonstrate that Δ Np63 α can only induce *Alox12* expression in

differentiating keratinocytes, consistent with the role of ALOX12 in epidermal barrier formation.

Materials and Methods

Epidermal-specific inducible $\Delta Np63$ knockdown mice ($\Delta Np63$ i-kd) and administration of RU486

Epidermal-specific inducible $\Delta Np63$ knockdown mice ($\Delta Np63$ i-kd) were previously generated and characterized (22). To downregulate $\Delta Np63$ expression in the basal layer of the epidermis, $\Delta Np63$ i-kd and control newborn mice were treated topically with 1mg/ml RU486 for 5 consecutive days. Skin samples were obtained at day 6. All experiments involving mice were performed with approval from the Institutional Animal Use and Care Committee (IACUC).

Real-time RT-PCR analysis

Total RNA was extracted using RNeasy Kits (Qiagen) and cDNA was synthesized using the High-capacity cDNA Archive Kit (Applied Biosystems). An assays-on-demand TaqMan probe for *Alox12* was also obtained from Applied Biosystems. For PCR amplification of cDNA, we used Taqman Universal PCR Master Mix and the Opticon2 System (MJ Research). The amount of 18S RNA in every sample was used to normalize *Alox12* mRNA levels. The relative amount of each mRNA was determined by the comparative C_T method. Statistical significance was determined using two tailed t-tests.

Western blot analysis

Protein was extracted from skin of newborn $\Delta Np63$ i-kd and control mice after 5 days of topical RU486 treatment. Western blotting was performed with rabbit anti-ALOX12 (Santa Cruz), mouse anti-p63 (4A4; Santa Cruz), and goat anti- β -actin (Santa Cruz) antibodies. After incubation with HRP-conjugated secondary antibodies (Sigma), protein bands were visualized using SuperSignal West Pico Substrate (Pierce).

Primary keratinocyte culture and transduction

Primary keratinocytes were isolated from newborn mice and cultured as previously described (23). Keratinocytes were transduced with adenoviruses encoding $\Delta Np63\alpha$ or GFP at an m.o.i. (multiplicity of infection) of 10. After transduction, keratinocytes were cultured in 50% conditioned media (CM) containing 0.05mM Ca^{2+} . The Ca^{2+} concentration in the media was increased to 0.1mM after 24 hours to induce keratinocyte differentiation. Keratinocytes were harvested for RNA extraction 24 hours after this switch in Ca^{2+} concentration.

Chromatin immunoprecipitation

For chromatin immunoprecipitation experiments, embryonic skin was isolated from 20 E14.5 or E15.5 embryos and collected in D-PBS on ice. Tissue samples were homogenized and fixed in 1% formaldehyde. Cross-linking was stopped by adding a final concentration of 0.125M glycine. After washing, cells were resuspended in 1ml lysis buffer (1% SDS and 10mM EDTA) and sonicated. Chromatin was immunoprecipitated with 3 μ g of anti-p63 α (H129; Santa Cruz) or anti-K14 antibodies. Immunoprecipitated samples were used to perform PCR with primers surrounding *p63RE-Alox12*: FW 5'-TAG CTG ATC CAA ACG CAC AC and RV 5'-GGG CCA GGT CCA AAA CTT TA. *GAPDH* was amplified as control to confirm equal genomic DNA content in the immunoprecipitated samples using primers FW: 5'-CCA ATG TGT CCG TCG TGG AT and RV: 5'-TGC TGT TGA AGT CGC AGG AG.

Reporter gene assays

A 450bp fragment of the *Alox12* promoter containing a putative p63 response element (*p63RE-Alox12*) was cloned into the pGL3-basic vector (Promega) using primers: FW 5'-GTG TTT GGG GAC CAC AGA T and RV 5'-GGC TCC CTC TGG CCT CTC A. Mutations in *p63RE-Alox12* were introduced using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's instructions. The sequences of the *p63* binding site were changed from CAGG to CCCG and from CTAG to CTCC.

Keratinocytes were nucleofected using Amaxa Biosystems Nucleofector®II and the Amaxa Kit for Primary Mammalian Epithelial Cells (1×10^6 cells per cuvette, program M5). Keratinocytes were nucleofected with reporter constructs (1µg), a $\Delta Np63\alpha$ expression construct (3µg), and a *pCMV-βgal* plasmid (0.5µg). Keratinocytes were plated on collagen IV-coated plates in 50% CM containing 0.05mM Ca^{2+} and incubated at 37°C. After 16 hours, keratinocytes were washed and fed with 50% CM containing 0.1mM Ca^{2+} . Cells were harvested 36 hours later and analyzed for luciferase and β-galactosidase activity using Dual-Light® System (Applied Biosystems) and Dynex Technologies Revelation MLX luminometer. Average reporter gene activities and standard deviations were determined based on three independent experiments. Statistical significance was determined using two tailed t-tests.

Results

Alox12 is a putative $\Delta Np63\alpha$ target gene

To identify novel $\Delta Np63\alpha$ target genes, we performed microarray analyses and compared gene expression profiles of $\Delta Np63$ i-kd and control skin of newborn mice that were treated for 5 days with RU486. We previously reported that IκB kinase-α (*Ikkα*) was downregulated in $\Delta Np63$ i-kd skin and determined that IKKα is a direct target gene of $\Delta Np63\alpha$ in the developing epidermis (22). Since IKKα functions downstream of $\Delta Np63\alpha$, genes that are downregulated in both $\Delta Np63$ i-kd skin and in *Ikkα*^{-/-} skin are unlikely to be direct $\Delta Np63\alpha$ target genes. To identify $\Delta Np63\alpha$ target genes that function independent of IKKα, we also performed microarray analyses on skin RNA isolated from *Ikkα*^{-/-} and control mice (24). By performing a comparative analysis of the two sets of microarray data, we identified *Alox12* as a transcript that was significantly downregulated in $\Delta Np63$ i-kd skin, but not in *Ikkα*^{-/-} skin. Real-time RT-PCR analysis and Western blot analysis confirmed that *Alox12*/ALOX12 expression was downregulated in $\Delta Np63$ i-kd skin, when compared to control skin (Fig. 1A, B). Interestingly, expression levels of *Alox3* and *Alox12b* were unchanged in $\Delta Np63$ i-kd skin (data not shown). Together with the finding that downregulating $\Delta Np63$ in human keratinocytes results in reduced ALOX12 expression in organotypic cultures derived from these cells (25), these data suggest that *Alox12* is a target gene of $\Delta Np63$ in the skin.

Since $\Delta Np63\alpha$ is the predominantly expressed p63 isoform in newborn mouse epidermis, the reduced expression of ALOX12 in $\Delta Np63$ i-kd skin is most likely due to the reduction of $\Delta Np63\alpha$ expression. To test whether $\Delta Np63\alpha$ can induce *Alox12* expression, we overexpressed $\Delta Np63\alpha$ in primary keratinocytes using an adenovirus (*Ad-ΔNp63α*). Keratinocytes were cultured under proliferating conditions (0.05mM Ca^{2+}) or under conditions that promote differentiation (0.1mM Ca^{2+}), and *Alox12* expression levels were determined using Real Time RT-PCR. Interestingly, we found that whereas $\Delta Np63\alpha$ did not affect *Alox12* expression in keratinocytes cultured under low Ca^{2+} conditions, *Alox12* expression was induced by $\Delta Np63\alpha$ in keratinocytes cultured under high Ca^{2+} conditions (Fig. 1C). Surprisingly, baseline *Alox12* transcript levels were comparable in keratinocytes cultured under low or high Ca^{2+} conditions. Since ALOX12 protein is only produced in

differentiating keratinocytes, these data suggest that ALOX12 protein is either not produced or not properly stabilized in undifferentiated keratinocytes.

***Alox12* expression during epidermal development coincides with terminal differentiation**

To further understand the role of *Alox12* in the epidermis, we determined the expression pattern of *Alox12* during skin morphogenesis. To this end, we performed Real-Time RT-PCR analysis for *Alox12* on skin RNA isolated from ICR embryos beginning at embryonic day (E) 10.5 through term. We found that *Alox12* transcript levels were first detectable at E13.5, while robust expression was observed starting at E14.5 (Fig. 1D). *Alox12* remains expressed at later developmental stages and postnatally, consistent with previous reports [Fig. 1D and (11,14)].

Δ Np63 α interacts with the *Alox12* promoter in embryonic skin

We next asked whether Δ Np63 α induces *Alox12* expression by binding to a *p63* response element (*p63-RE*) in the promoter region of *Alox12*. Although *p63-RE* remain poorly defined, it is generally accepted that p63 interacts with degenerate *p53-RE* (26,27). However, the amount of degeneracy that allows for p63 binding remains unclear (28,29). To identify regulatory elements within the *Alox12* promoter, we initially compared a 10kb genomic region upstream of the mouse and human *Alox12* genes using the Consite software (<http://asp.ii.uib.no:8090/cgi-bin/CONSITe/consite>). These comparisons led to the identification of a conserved region within the *Alox12* promoter, which contained a putative *p63-RE*. This *p63-RE* (*p63RE-Alox12*) is located approximately 170bp upstream from the first exon of *Alox12* (Fig. 3A) and is conserved in the human *ALOX12* promoter.

To determine whether Δ Np63 α physically interacts with *p63RE-Alox12*, we performed chromatin immunoprecipitation (ChIP) assays. Since *Alox12* expression levels are high at E14.5 and E15.5 (Fig. 1D), we isolated chromatin from skin samples obtained at these developmental stages. To immunoprecipitate protein-DNA complexes, we used anti-p63 α or control (anti-K14) antibodies. Since Δ Np63 α is the predominant p63 isoform expressed in E14.5 and E15.5 skin, DNA fragments immunoprecipitated with the anti-p63 α antibody most likely correspond to fragments that are bound by Δ Np63 α . PCR amplification of these DNA fragments with primers surrounding *p63RE-Alox12*, revealed an increased recovery of promoter fragments containing *p63RE-Alox12* (Fig. 2), demonstrating that Δ Np63 α directly interacts with the promoter of *Alox12* in E14.5 and E15.5 embryonic skin.

Δ Np63 α activates a reporter construct containing *p63RE-Alox12*

To determine if Δ Np63 α induces *Alox12* expression by interacting with *p63RE-Alox12*, we performed luciferase reporter gene assays. To this end, we cloned a 450bp fragment of the *Alox12* promoter containing *p63RE-Alox12*, into a luciferase reporter vector. Since Δ Np63 α functions in keratinocytes, luciferase reporter assays were performed in mouse primary keratinocytes. After nucleofection with the reporter constructs, keratinocytes were cultured in media containing a high (0.1mM) concentration of Ca²⁺ to maintain the cells in a differentiating state. Consistent with our finding that Δ Np63 α can induce endogenous *Alox12* expression only in keratinocytes cultured under high Ca²⁺ conditions, we found that Δ Np63 α could activate the *Alox12* reporter in keratinocytes cultured under high Ca²⁺ conditions (Fig. 3B). To determine if activation of the *Alox12* reporter was dependent on *p63RE-Alox12*, we mutated the core sequences of *p63RE-Alox12* in the context of the *Alox12* reporter construct (*p63RE-Alox12-Mut*) (Fig. 3A). We found that Δ Np63 α was unable to activate *p63RE-Alox12-Mut*, demonstrating that Δ Np63 α -mediated *p63RE-Alox12* activation is dependent on the interaction of Δ Np63 α with *p63RE-Alox12* (Fig. 3B). Taken together, these data suggest that in E14.5 and E15.5 embryonic skin Δ Np63 α induces *Alox12* expression by directly interacting with the *Alox12* promoter.

Discussion

Since the cloning of p63 in 1998, many putative target genes of Δ Np63 α have been identified. These target genes include genes required for keratinocyte adhesion, keratinocyte proliferation, keratinocyte terminal differentiation, and basement membrane formation (19). Intriguingly, Δ Np63 α induces these target genes at different developmental stages, suggesting that induction of target gene expression by Δ Np63 α requires transcriptional co-activators which are specifically expressed at the appropriate developmental time points. Although some co-activators of TAp63 isoforms have been identified (30,31), co-activators that cooperate with Δ Np63 α during epidermal development remain to be identified. The earliest known gene induced by Δ Np63 α during epidermal morphogenesis is PERP, a desmosomal component which is critical for cell adhesion in the epidermis (32). PERP is induced by Δ Np63 α shortly after surface epithelial cells have adopted an epidermal fate, a process which occurs around E9.5 in mice. Subsequently, at E11.5, Δ Np63 α induces a critical component of the embryonic basement membrane, FRAS1 (22). When commitment to terminal differentiation occurs, around E15.5, Δ Np63 α induces the expression of IKK α , which is required for cell cycle withdrawal during spinous layer development (22,33). Finally, Δ Np63 α also synergizes with Notch signaling components to induce expression of the terminal differentiation marker keratin 1 (34). Although other putative Δ Np63 α target genes have been identified, most of these were identified using cell lines derived from tissues where p63 does not normally function, such as liver and osteosarcoma-derived cell lines. Therefore, it remains to be determined whether these genes are regulated by p63 during epidermal development and/or differentiation.

We now add *Alox12*, which encodes a LOX involved in epidermal barrier formation, to the list of genes induced by Δ Np63 α (11). During epidermal development, Δ Np63 α directly interacts with the promoter region of *Alox12*, and this interaction is required for the induction of *Alox12* by Δ Np63 α . Like epidermal terminal differentiation, epidermal barrier formation is a process which is dependent on extracellular Ca²⁺. In the epidermis, the extracellular Ca²⁺ concentration is highest in the granular layer, where components of the epidermal barrier are produced (35). Furthermore, the establishment of the Ca²⁺ gradient in the developing epidermis occurs concurrently with the acquisition of epidermal barrier function (36). The instructive role for Ca²⁺ in epidermal barrier formation was established by the finding that Ca²⁺ is essential for the expression of genes that are required for barrier formation (37–39). Consistent with these findings, we found that Δ Np63 α could only induce *Alox12* expression in keratinocytes that were cultured in media containing high levels of Ca²⁺. Since Δ Np63 α cannot induce *Alox12* expression in keratinocytes cultured under low Ca²⁺ conditions, *Alox12* is likely to represent another target gene of Δ Np63 α which requires transcriptional co-activators for its expression.

Paradoxically, unlike ALOX12, the expression of Δ Np63 α declines during epidermal differentiation. However, consistent with previous reports (40), we found that differentiating keratinocytes still contain ~25% of the amount of Δ Np63 α protein that is present in undifferentiated keratinocytes (unpublished observations). This level of Δ Np63 α protein is clearly functional since it cooperates with Ca²⁺ to induce *Alox12* expression in differentiating keratinocytes, where Δ Np63 α and ALOX12 are co-expressed.

In summary, we have identified a novel role for Δ Np63 α in directly inducing the expression of *Alox12*, a gene involved in epidermal barrier formation. Our finding that Δ Np63 α only induces *Alox12* expression in primary keratinocytes that are cultured under differentiating conditions is consistent with the known role for ALOX12 in epidermal barrier formation.

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Figure 1. $\Delta Np63\alpha$ induces *Alox12* expression in mouse skin

(A) Real Time RT-PCR analysis for *Alox12* on skin RNA isolated from three newborn $\Delta Np63$ i-kd and three control littermates after 5 days of topical RU486 treatment. Error bars represent standard deviations of data obtained from three mice per genotype ($p < 0.05$). (B) Western blot analysis for ALOX12 and $\Delta Np63\alpha$ on protein extracts isolated from newborn $\Delta Np63$ i-kd and control skin after 5 days of topical RU486 treatment. β -Actin (ACTB) was used as a loading control. (C) Real Time RT-PCR analysis for *Alox12* on RNA isolated from primary keratinocytes transduced with adenoviruses expressing $\Delta Np63\alpha$ (*Ad-ΔNp63α*) or GFP (*Ad-GFP*) as a control. Keratinocytes were cultured in media containing 0.05mM Ca^{2+} to maintain the keratinocytes in a proliferative state or in 0.1mM Ca^{2+} to induce

differentiation. All expression values were normalized to *Ad-GFP* transduced keratinocytes cultured under low Ca^{2+} conditions. Error bars represent standard deviations of data obtained from three individual cultures of keratinocytes per condition (low Ca^{2+} cultures: $p=0.22$; high Ca^{2+} cultures: $p<0.05$). (D) Real Time RT-PCR for *Alox12* on RNA isolated from embryonic skin at different developmental stages. Each embryonic skin was processed individually, except for E10.5–E12.5 embryos, for which several individual skins were combined and processed as one sample. RNA was isolated from three independent samples per developmental stage. The *Alox12* expression level at E13.5 was arbitrarily set to 1. C_t values between E10.5 and E12.5 were >32 , and were considered background. Error bars represent standard deviations of data obtained from three independent samples ($p<0.05$ for E13.5–E14.5, E14.5–E15.5 and E15.5–E16.5). For (A), (C), and (D), mRNA expression levels were normalized to the amount of *18S* mRNA in each sample.

Figure 2. p63 directly interacts with the *Alox12* promoter

Chromatin immunoprecipitation (ChIP) analysis on E14.5 and E15.5 mouse skin using an anti-p63α antibody and primers surrounding *p63RE-Alox12*. Anti-K14 antibody was used as a negative control and primers specific for *GAPDH* were used as loading control.

Figure 3. $\Delta Np63\alpha$ transactivates a reporter construct containing *p63RE-Alox12*

(A) A putative *p63RE* was identified in the *Alox12* promoter (*p63RE-Alox12*) using Consite (<http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite>). To identify *p63RE*, we searched for *p53* binding sites (>65% conservation of core sequence) which were located within a region of the promoter that was at least 75% conserved. The core sequences of *p63RE-Alox12* are underlined. Mutations generated in *p63RE-Alox12* are indicated in italics and underlined with a wavy line. (B) Reporter constructs containing *p63RE-Alox12* and *p63RE-Alox12-mut* were nucleofected into primary keratinocytes with or without a $\Delta Np63\alpha$ expression vector. Keratinocytes were cultured in high Ca^{2+} media to induce differentiation. Error bars represent standard deviations of three independent experiments ($p < 0.05$).