Requirement of zinc transporter ZIP10 for epidermal development: Implication of the ZIP10–p63 axis in epithelial homeostasis

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Skin tissues, in particular the epidermis, are severely affected by zinc deficiency. However, the zinc-mediated mechanisms that maintain the cells that form the epidermis have not been established. Here, we report that the zinc transporter ZIP10 is highly expressed in the outer root sheath of hair follicles and plays critical roles in epidermal development. We found that ZIP10 marked epidermal progenitor cell subsets and that ablating *Zip10* caused significant epidermal hypoplasia accompanied by down-regulation of the transactivation of p63, a master regulator of epidermal progenitor cell proliferation and differentiation. Both ZIP10 and p63 are significantly increased during epidermal development, in which ZIP10-mediated zinc influx promotes p63 transactivation. Collectively, these results indicate that ZIP10 plays important roles in epidermal development via, at least in part, the ZIP10–zinc–p63 signaling axis, thereby highlighting the physiological significance of zinc regulation in the maintenance of skin epidermis.

zinc transporter | ZIP10 | skin epidermis | hair follicle | development

The epidermis constitutes a rigid, stratified barrier that protects the body from dehydration and infections (1). In mice, the epidermis begins forming around embryonic day 8.5 (E8.5) (2). At E9.5, epidermal lineages expressing keratin 5 and 14 can be detected in the basal layer and periderm (3). The spinous and granular layers of the mature epidermis begin appearing at E14.5, when hair follicle specification begins (3). From E14.5 onward, epidermal progenitor cells proliferate vigorously to support epidermal development and terminal differentiation. Differentiated, cornified epidermal layers with barrier function are present by E17.5, just before birth (3).

This epidermal development requires the coordinated function of several zinc-binding proteins including enzymes and transcription factors (TFs) to orchestrate the various programs (4, 5). The master epidermal regulator p63 (6) triggers epithelial stratification through the altered balance of expression of its two isoforms, an N-terminal transcriptional activation (TA) domain-containing isoform and a truncated (ΔN) isoform (2, 5). Both isoforms contain a DNA-binding domain (DBD) with a zinc-binding site (6) that incorporates zinc for proper sequence-specific DNA binding (7). Competing metals can alter p63 function (7), implicating the possible requirement of a zinc atom to fine-tune p63 transcriptional activity.

Zinc homeostasis in mammalian cells is tightly regulated by zinc-transporting proteins (8) classified as zinc transporters (ZnTs) or Zrt- and Irt-like proteins (ZIPs) (9). The ZIP family, which has at least 14 members, imports extracellular or luminal zinc into the cytoplasm in mammals (10). ZIP members are expressed in specific tissues and act through rather selective signaling pathways. For example, ZIP13 is expressed mainly in connective tissues and is required for their development, whereas pathogenic ZIP13 mutations are found in a new type of Ehlers–Danlos syndrome (11–14). The intestinal zinc transporter ZIP4 is related to acrodermatitis enteropathica (AE), in which zinc deficiency causes skin sensitization and severe epidermal-barrier dysfunction (15, 16). Loss-of-function *ZIP4* mutations cause a failure in zinc influx through the intestine, resulting in severe skin problems (17, 18). Additionally,

Significance

Although the epidermis of the skin is the first tissue to manifest a zinc deficiency, the mechanisms underlying zinc-mediated epidermal formation are largely unknown. We demonstrated that the zinc transporter ZIP10, which is highly expressed in the outer root shelf of hair follicles, is essential for epidermal formation. Ablating *Zip10* caused epidermal hypoplasia by down-regulating the transcriptional activity of p63, whereas ZIP10-mediated zinc influx promoted p63 transactivation to induce epidermal morphogenesis. Our results establish the physiological relevance of ZIP10 in epidermal development.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo [mRNA sequencing data (accession no. GSE83099) and gene expression microarray data (accession nos. GSE83098 and GSE99204)].

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recent data suggest that ZIP7 fine-tunes endoplasmic reticular condition for supporting protein disulfide isomerase activity in mesenchymal stem cells (19). Although our understanding of the roles of zinc in various cellular phenomena is improving, the molecular relationship between zinc homeostasis and the cells forming the skin epidermis remains largely unknown.

Here, we establish a critical link between ZIP10 and skin development, revealing a molecular mechanism underlying the requirement of zinc for developing the skin epidermis, and the highlight the clinical impact of ZIP10 as a potential therapeutic target for skin diseases.

Results

ZIP10 Is Predominantly Expressed in Epidermal Hair Follicles. Zinc is reportedly enriched in skin areas, especially in hair follicles (20, 21). Although skin epidermis constitutes the primary tissue affected by zinc deficiency, the molecular mechanisms by which zinc contributes to the development of the skin epidermis are poorly understood. To investigate the precise area in which zinc is enriched during embryogenesis, we first analyzed the expression of zinc-induced metallothionein 1 (MT1) mRNA by in situ hybridization using whole sections of E18.5 mice, revealing that *Mt1* was highly expressed in the early hair peg and in organs such as the lung, liver, and intestine (Fig. 1*A*). Next, we analyzed the expression level of the ZIP LIV-1 subfamily, which localizes to the mammalian plasma membrane, to identify zinc transporter(s) that influx zinc in these tissues. Among the ZIP family members, *Zip10* was predominantly expressed in the epithelial tissues, including the outer root sheath (ORS), the lower part of Huxley's layer in the hair follicles, and the tooth germ at E17.5 (Fig. 1*B* and Fig. S1*A*). Immunocytochemistry revealed that the ZIP10 protein was present in the early hair peg during epidermal development (Fig. 1*C*, E17.5). At postnatal day 2, ZIP10 was mainly expressed in the variable components of the hair follicle and was also present in hair matrix cells and in the stable components of the hair follicle (Fig. 1*D*). *Zip4*, a causative gene for AE, was mainly expressed in the intestine as previously reported (22) (Fig. S1*B*). *Zip5* was expressed in the intestine and kidney (Fig. S1*C*), whereas *Zip12* was found in the choroid plexus, medulla oblongata, and spinal cord (Fig. S1*E*). Although *Zip6* was also observed in hair follicles (Fig. S1*D*), it was expressed rather topically such as in the upper hair bulge region (Fig. S1*D*). Moreover, *Zip6*-deficient (KO) mice had no apparent skin abnormalities (Fig. S2). Together, these findings indicated that ZIP10 might serve as a predominant zinc transporter to influx zinc into the epidermis-forming cells, and thus may be involved in the development of the epithelium of the skin and related appendages.

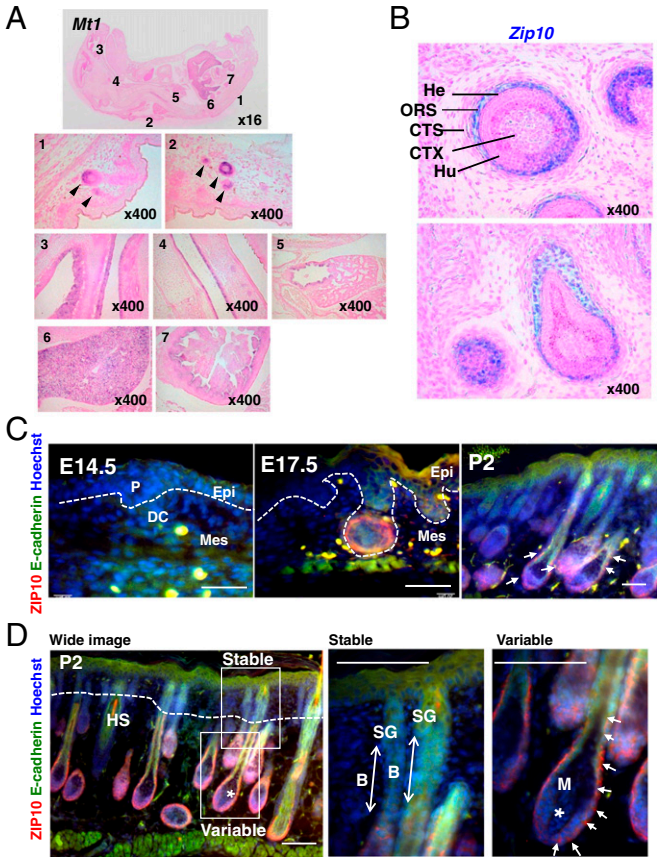

Fig. 1. ZIP10 expression in the epidermis. (*A*) In situ hybridization in the E18.5 mouse embryo showed *Mt1* expression in the (1, 2) dorsal skin, (3) nasal cavity, (4) trachea, (5) lung, (6) liver, and (7) intestine. (*B*) *Zip10* was expressed in the E17.5 mouse embryo in the whiskers. The defined lineages are Henle's (He) and Huxley's (Hu) layer in the internal root sheath, the outer root sheath (ORS), the connective-tissue sheath (CTS), and the cortex of the shaft (CTX). (*C* and *D*) Immunocytochemistry for ZIP10 (red) and E-cadherin (green) in skin from mouse embryos or postnatal day 2 (P2) mice. B, bulge region; DC, dermal condensate; Epi, epithelium; HS, hair shaft; M, hair matrix; Mes, mesenchyme; P, placode; SG, sebaceous gland; *, dermal papilla. (Scale bar, 100 μm.)

ZIP10 Marks Epidermal Progenitor Cell Subsets. To characterize the ZIP10-positive ($ZIP10^+$) cells in the hair follicles, we sorted the $ZIP10^+$ and $ZIP10^-$ cells and performed mRNA sequencing (Fig. 2*A* and Table S1). *Mt1*, *Mt2*, and *Mt4*, members of the Mt family that are indicators for intracellular zinc levels (8), were up-regulated in the $ZIP10^+$ cells, suggesting that zinc was enriched in these cells (Fig. 2*B*). Of the differentially expressed genes (DEGs) identified, 1,763 were up-regulated and 1,108 were down-regulated in $ZIP10^+$ cells compared with $ZIP10^-$ cells (Table S2 *A* and *B*). The genes up-regulated in $ZIP10^+$ cells were mainly involved in processes related to epidermal development, such as DNA repair, telomere maintenance, and the cell cycle (Fig. 2*C* and Table S3 *A* and *B*). Given that these epidermal processes are critically governed by p63 (23), a master TF for epithelial development, we hypothesized that there might be an overlap between genes that depend on ZIP10 and p63. In fact, a significant number of the DEGs (23%, $P = 1.70 \times 10^{-3}$) between $ZIP10^+$ and $ZIP10^-$ cells were known p63 targets despite comparable *TP63* expression levels in both cells (24) (Fig. 2 *D* and *E*). Moreover, a comparative analysis of gene expression changes between ZIP10-dependent and p63-dependent genes showed a significant correlation (Fig. S3). These results support our hypothesis that ZIP10 and p63 may tightly cooperate for epidermal formation.

Hair follicles contain diverse stem cell subsets that contribute to epidermal homeostasis and regeneration (25). We next investigated the expression of epidermal stem cell marker genes (25, 26) in $ZIP10^+$ cells. *Lgr6*, *CD34*, and *Lgr5* were highly expressed, whereas less *Gli1* was detected in the $ZIP10^+$ cells (Fig. 2*E*), indicating that ZIP10 marks $lgr6^{high}$ and gli^{low} epidermal progenitor cell subsets. Serial stem cells commit to becoming progenitor cells to regenerate the epidermis when the hair cycle restarts or the epidermis is injured (25). Therefore, we tested whether shaving the skin, which stimulates epidermal regeneration, could induce *Zip10* expression. In situ hybridization analysis revealed that *Zip10* was significantly induced in newly generated hair follicles (Fig. 2 *F* and *G*). Together, these data indicated that ZIP10 is functionally associated with the generation and regeneration of the skin epidermis in vivo.

***Zip10*$^{flox/flox}$*keratin14-Cre* Mice Have Epidermal Hypoplasia.** To elucidate the role of ZIP10 roles in the epidermis, we crossed *Zip10*$^{flox/flox}$ mice with *keratin14* (K14)-*Cre* transgenic mice to generate *Zip10*$^{flox/flox}$*keratin14-Cre* (*Zip10*^{K14}) mice. *Zip10*^{K14}

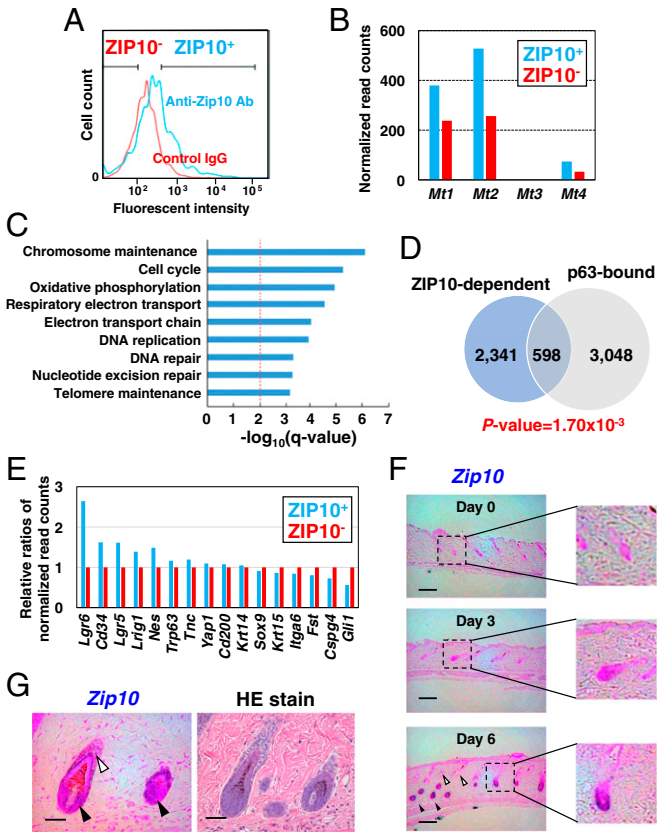

Fig. 2. ZIP10 marks *lgr6*^{high} and *gli*^{low} hair follicle progenitor cells. (*A*) ZIP10^{+} cells from the hair follicles of 2-wk-old WT mice were sorted by flow cytometry using an anti-ZIP10 antibody. (*B*) Normalized read counts of *Mt1*, *Mt2*, *Mt3*, and *Mt4* in ZIP10^{+} and ZIP10^{-} cells. (*C*) Biological processes represented by the up-regulated genes in ZIP10^{high} cells. Bars represent enrichment scores, given as $-\log_{10}(q \text{ value})$, for the pathways shown. The red dotted line indicates the cutoff of q value = 0.01. (*D*) Overlap in gene sets between the ZIP10-dependent genes and the previously identified p63 target genes (24). (*E*) Relative ratios of normalized read counts of known epidermal stem cell markers in ZIP10^{+} compared with ZIP10^{-} cells. (*F*) In situ hybridization revealed that shaving induced *Zip10* expression in the hair peg (filled arrowheads: early stage; open arrowheads: aged) and (*G*) that *Zip10* was induced in the ORS (open arrowhead) and the hair matrix (filled arrowheads) after shaving.

newborn mice had scarlet skin with thin and feeble hair (Fig. 3*A*) and died within a few days, possibly owing to total body dehydration (27) or for undefined reasons. The reduced *Mt1* expression in the hair follicles of *Zip10*^{K14} newborn mice implied the zinc deficiency compared with control mice (Fig. 3*B*). X-gal staining revealed that the skin barrier function was substantively impaired; in particular, the ventral skin shows severe dysgenesis in comparison with the dorsal skin, indicating that epidermal proliferation and differentiation does not progress properly from the middle developmental stage at E10–E14 (Fig. 3*C*). Indeed, hematoxylin and eosin (H&E) staining and in situ hybridization analysis using epidermal differentiation markers indicated that the loss of ZIP10 caused epidermal dysgenesis (Fig. 3*D*). The dorsal skin of *Zip10*^{K14} mice undergoes epidermal stratification, although each epidermal layer appears atrophic with thinning of the granular layer compared with control epidermis (Fig. 3*D*). The ventral skin of *Zip10*^{K14} mice contains no spinous or granular layer, as in the epidermis of the early developmental stage (Fig. 3*D*). Notably, the fact that *keratin 14* is comparable in both mutant and control epidermis (Fig. 3*E*) supports that the epidermal formation is normally initiated, whereas the previous results indicate that progression from the middle developmental stage is impaired. For example, low expression of differentiation markers such as *filaggrin* and *keratin 10* illustrate the defects of epidermal differentiation (Fig. 3*E*), revealing that ZIP10 has a critical role in epidermal development.

ZIP10 Supports p63 Functions During Epidermal Development.

ZIP10 physically and functionally correlates with p63 function. Given that ZIP10-dependent gene expression level nicely correlates with p63-mediated gene expression (Fig. 2*D* and Fig. S3) and the epidermal hypoplasia in *Zip10*^{K14} mice was recapitulated in the abnormality caused by the loss of *Trp63* (28), we next investigated the functional association between ZIP10 and p63. The *Trp63* gene encodes the p63 protein, a zinc-binding TF that marks epidermal progenitor cells and regulates the cellular programs that form the epidermis (29, 30). In the *Zip10*^{K14} epidermis, the number of p63-positive (p63^{+}) epidermal progenitor cells was significantly reduced in both the hair follicle and the interfollicular region, and hair follicles were sparse (Fig. 4*A*) as the *Trp63*-null mice (31). In addition, whereas p63 localizes to the nucleus in the normal epidermis, it localized to the cytosol in the *Zip10*^{K14} epidermis (Fig. 4*B*). Notably, the importance of p63 in epithelial cell proliferation was also demonstrated by using thymus-specific *Trp63*-deficient (*Trp63*^{FoxN1}) mice (30), in which we observed that *Zip10* was highly expressed in

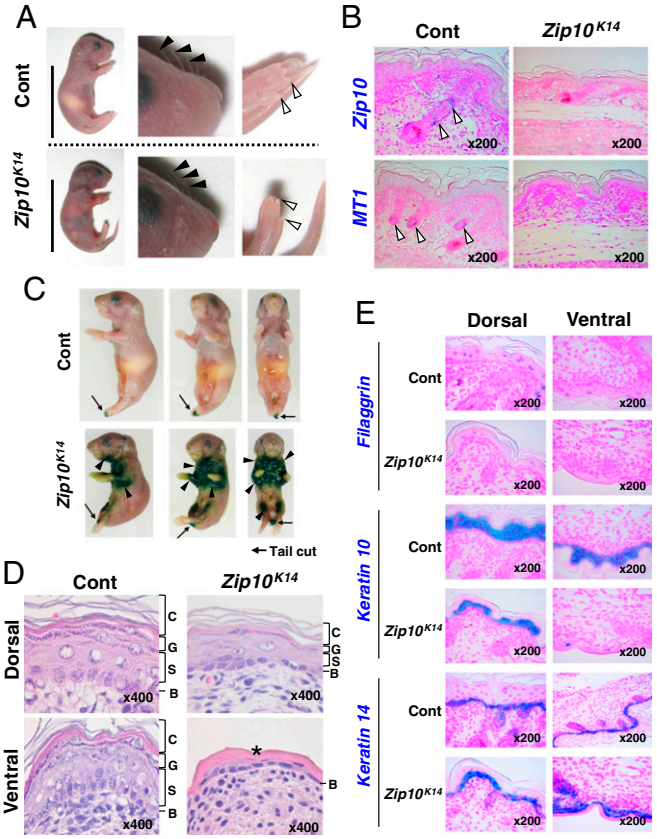

Fig. 3. *Zip10*$^{flox/flox}$*K14-Cre* mice show polar epidermal hypoplasia. (*A*) P1 *Zip10*^{K14} mice had thin whiskers (filled arrowheads) but normal limb development (open arrowheads). (Scale bar, 2 cm.) (*B*) In situ hybridization showed that *Zip10* and *Mt1* were expressed in the skin from P1 control (Cont) (open arrowheads) but not P1 *Zip10*^{K14} mice. (*C*) Skin barrier analysis by X-gal staining revealed that the skin barrier was impaired in P1 *Zip10*^{K14} mice (filled arrowheads); arrows indicate a tail cut. (*D*) H&E staining revealed dorsal epidermal hypoplasia and ventral embryonic epidermis (asterisk) in P1 *Zip10*^{K14} mice. B, basal layer; C, cornified layer; G, granular layer; S, spinous layer. (*E*) In situ hybridization showed reduced *filaggrin* and *keratin 10* expression but normal *keratin 14* expression in *Zip10*^{K14} mice.

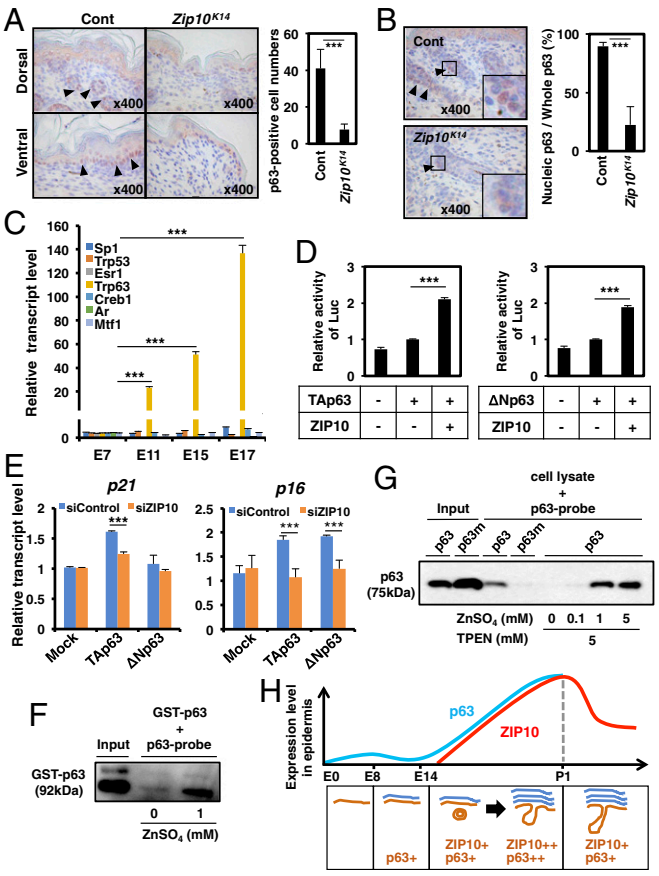

Fig. 4. ZIP10 facilitates p63 transactivation in epidermal development. (*A*) Immunohistochemistry showed fewer p63-expressing cells (brown) in the dorsal and ventral epidermis in newborn (P1) *Zip10*^{K14} compared with control mice, and (*B*) abnormal p63 cellular localization in *Zip10*^{K14} mice. Arrowheads indicate p63-expressing cells. Five different parts of two skin specimens derived from each mouse were analyzed ($n = 5$, $^{***}P < 0.001$). (*C*) Real-time PCR of *Trp63* across embryo development. The data are representative of three independent experiments ($^{***}P < 0.001$). (*D*) ZIP10 overexpression increases the transactivation of the p63 proteins. H1299 cells stably expressing the TAp63 or ΔNp63 protein were transfected with pPG13-Luc for 24 h and subjected to luciferase assay. The data are representative of three independent experiments ($^{***}P < 0.001$). (*E*) *ZIP10* depletion down-regulated p16 and p21 expression in HeLa cells. HeLa cells were treated with siZIP10 for 48 h and then transfected with plasmids encoding p63; the mRNA levels were quantified by real-time PCR. The data are representative of three independent experiments ($^{***}P < 0.001$). (*F*) p63 protein bound to its DNA-binding sequence (p63-probe) was directly modulated by zinc. The GST-tagged recombinant p63 proteins (GST-p63) were mixed with biotinylated p63 binding sequence and avidin-agarose beads. The protein bound to the complex of p63 binding sequence and the avidin-agarose beads were subjected to Western blotting analysis and detected by anti-p63 antibody. (*G*) Cell lysates derived from HEK293 cells transfected with either p63 or p63 mutant (p63m) plasmids were mixed with biotinylated p63 binding sequence and avidin-agarose beads with or without $ZnSO_4$ and TPEN at the indicated concentrations. The protein bound to the complex of p63 binding sequence and avidin-agarose beads was subjected to Western blotting analysis and detected by anti-p63 antibody. (*H*) ZIP10, whose expression is elevated in the progenitor cells at ORS of hair follicles during epidermal morphogenesis, contributes to the activities of elevated p63 by supplying zinc. This allows p63 to properly bind to DNA for initiating the gene expression necessary for epithelium morphogenesis, such as cell proliferation and stratification.

the medulla of the thymus (Fig. S4 *A* and *B*). To determine whether ZIP10 is also essential in the proliferation of progenitor cells, we generated thymus-specific *Zip10*-deficient (*Zip10*^{FoxN1}) mice by crossing *FoxN1-Cre* transgenic mice with *Zip10*$^{flox/flox}$ mice. *Zip10*^{FoxN1} mice had a hypoplastic thymus with increased TUNEL^{+} cells (Fig. S4 *C* and *D*), similar to the thymus in *Trp63*-null (30) and *Zip10*^{K14} mice (Fig. S4*E*). These data indicate that ZIP10 is essential for biological events involving p63 in epithelium tissues.

ZIP10 mediates zinc homeostasis as assessed by molecular and pathway analyses. To reveal the further molecular association between the ZIP10 and p63 related to epidermal formation, we performed microarray analysis with mRNAs isolated from human wild-type (WT) and *ZIP10*-knockdown (KD) keratinocytes. We identified 799 up-regulated and 729 down-regulated genes in *ZIP10*-KD keratinocytes compared with control cells (Fig. S5*A* and Table S2 *C* and *D*). The up-regulated genes in *ZIP10*-KD keratinocytes were involved in the processes of cell death, morphogenesis, and signal transduction (Fig. S5*B* and Table S3 *C* and *D*). The down-regulated genes in *ZIP10*-KD keratinocytes were mainly involved in skin development and keratinization, indicating that *ZIP10* depletion is responsible for disturbing epidermal formation, as was observed in the *Zip10*^{K14} epidermis (Fig. S5*B* and Table S3 *C* and *D*). Based on the fact that zinc homeostasis may affect the transactivation of zinc-dependent TFs such as p63, and to clarify which TFs are responsive to *ZIP10* depletion in keratinocytes, we performed TF enrichment analysis for the down-regulated genes in *ZIP10*-KD keratinocytes using MetaCore software from GeneGo (Thomson Reuters). We identified 13 key TFs (NR3C1, MTF1, CREB1, p63, ESR1, PGR, HIF1A, EPAS1, AR, PPARG, SP1, RBPJL, and p53) that target a significant number of genes [false discovery rate (FDR) < 0.05] (Fig. S5*C*). Among these TFs, SP1, p53, ESR1, p63, CREB1, and AR regulate a significant number of genes involved in epidermal development and homeostasis (Fig. S5*D*). Notably, only *Trp63*, a mouse homolog of the human *p63* gene, is markedly increased during epidermal development (over 100-fold) (Fig. 4*C*). These data indicate that ZIP10 supports the up-regulation of p63 activity to direct epidermal development.

Next, we addressed the association between ZIP10, zinc, and p63 (Fig. S6*A*). First, zinc-dependent genes were identified by the comparison of *N*,*N*,*N*′,*N*′-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine (TPEN)-treated and untreated cells, and then ZIP10-dependent genes were obtained from among the zinc-dependent genes by comparing *ZIP10*-KD with WT cells (Fig. S6*B*). We found that 545 genes demonstrated common changes in expression by either TPEN treatment or *ZIP10*-KD, whereas 151 genes commonly down-regulated in TPEN-treated and *ZIP10*-KD keratinocytes (Fig. S6 *B* and *C*), which belong to cluster 4 (C4 in Fig. S6*C*), are thought to be directly regulated by ZIP10-mediated zinc. We further investigated these 151 genes by performing TF enrichment and pathway enrichment analysis (Fig. S6 *D* and *E*). The genes in C4 were found to still be significantly involved in epidermal development (Fig. S6*D*). Furthermore, p63 was indicated as one of the most significant factors targeting the genes in C4 (Fig. S6*E*). Taken together, these data support that ZIP10-mediated zinc homeostasis is crucial for the p63 function during epidermal development.

The ZIP10–zinc signal is required for p63 transactivation. Epidermal progenitor cell proliferation and terminal differentiation progress vigorously from E14.5 to just before birth (23). *Zip10* and *Trp63* expression increased dramatically during this period (Figs. 1*C* and 4*C* and Fig. S7), which prompted us to determine whether ZIP10 could support the p63 activity. We used H1299 cells, which express little detectable p63 protein (32), stably complemented with two p63 isoforms, either TAp63 or ΔNp63. The ectopic expression of ZIP10 up-regulated p63 transactivation (Fig. 4*D*). To investigate whether *ZIP10* depletion could affect the expression of p63 target genes, cell lines with high expression of *ZIP10* were selected (Fig. S8) and then treated with the *ZIP10* siRNA. The siRNA depletion of *ZIP10* down-regulated the expression of p63 target genes (Fig. 4*E*). Similarly, depleting intracellular zinc by chelation with TPEN reduced p63 transactivation, whereas this reduction was rescued by zinc treatment (Fig. S9), suggesting that both ZIP10 and zinc participate in p63 regulation.

Finally, we examined whether zinc directly promotes p63 protein binding to the p63 binding sequence. DNA pull-down assay revealed that zinc addition increased DNA binding of p63 recombinant protein (Fig. 4*F*). Furthermore, ectopically expressed p63 protein in cells efficiently recruited the p63 binding sequence but not p63 mutant protein totally lacking zinc binding sites (16) (Fig. 4*G*). TPEN treatment decreased p63 binding to its target DNA sequence, which was restored by the zinc supplement in a dose-dependent manner (Fig. 4*G*). These results clearly indicated that ZIP10-mediated zinc delivery is critical for proper p63 transactivation.

Discussion

In the present study, we show that the hair follicle-localized zinc transporter ZIP10 is essential for epidermis formation. During epidermal development, ZIP10 supplies zinc to p63 to promote its activity. Therefore, the ZIP10–zinc–p63 signaling axis is indispensable for epidermal morphogenesis (Fig. 4*H*).

ZIP10 Is a Key Regulator of Zinc Homeostasis During Epidermal Development. The involvement of ZIP10 in various systems has been reported as follows: ZIP10 is highly expressed in invasive and metastatic breast cancer cell lines, implying its association with their characteristic features (33). ZIP10 is involved in the epithelial to mesenchymal transition by inhibiting glycogen synthase kinases (GSKs) and down-regulating E-cadherin in the NMuMG cell model, and the zebrafish embryo model in which ZIP10 forms a heteromer with ZIP6 to regulate the embryonic development (34–36). These data suggest that ZIP10 plays crucial roles in these cell lines and fish; however, the in vivo physiological relevance of ZIP10 in mammals is largely unknown. Our recent data using a mouse model reveal that ZIP10 facilitates antiapoptotic signaling during early B-cell development and controls the B-cell receptor signaling strength (37, 38). To investigate another role of ZIP10 in vivo, we assessed *Zip10* expression with in situ hybridization using mouse whole-body sections, demonstrating that *Zip10* was enriched in ORS of hair follicles (Fig. 1*B*). Considering these findings together with the observation that the most down-regulated genes in the *ZIP10*-KD cells belonged to the "epidermal development" group that reflects epidermis-specific events (Fig. S5*B*), we thus focused on the role of ZIP10 in epidermal development. In fact, ZIP10 was highly expressed in the hair follicle bulb and ORS cells (Fig. 1), peaked during epidermal formation, as did p63, and was induced again by shaving (Figs. 1 and 2*F*). The ZIP10 pattern of expression was correlated with that of MT1 (20, 21) (Fig. 1). The zinc-inducible protein MT1, which is observed in hair matrix cells of the bulb and in ORS cells (21) along with ZIP10 (Fig. 1), increases when stimulated with proliferation agents (20, 21), implying that zinc constitutes an important metal ion for the proliferation of epidermal progenitors. Notably, although ZIP1, ZIP6, and ZIP10 were expressed in epidermal cells and ZIP6 was also expressed in hair follicles (Fig. S1), neither *Zip1*-KO nor *Zip6*-KO mice exhibit apparent epidermal abnormalities (39) (Fig. S2). These findings indicate that ZIP10 is a key regulator of zinc homeostasis during epidermal development.

ZIP10 Regulates Epidermal Progenitor Cells. We found that ZIP10$^+$ cells highly express *Lgr6* but less *Gli1* (Fig. 2*E*), suggesting that ZIP10 may regulate the function of LGR6$^+$ hair follicle stem cells. Hair follicles contain a heterogeneous pool of stem cells (25) that contributes critically and distinctly to the rebuilding of epidermal structures such as the interfollicular epidermis, hair follicles, and sebaceous glands after a skin injury (40). LGR6$^+$ stem cells can generate all of these cell lineages, whereas the GLI1$^+$ stem cells do not contribute to sebaceous glands (3, 25). *Lgr6* expression appears at E14.5, peaks around postnatal day 7–15, and then gradually decreases (3), thus sharing the time course features of *Zip10* (Fig. 1). Considering that *Zip10*^{K14} mice exhibit severe hypoplasia in stratified epithelia, diminished hair follicles numbers, and that epidermal loss by shaving markedly induces *Zip10* expression in WT hair follicle cells (Figs. 2*F* and 3), ZIP10 activity may support the maintenance of hair follicle stem cells (Figs. 3 and 4).

Involvement of ZIP10 in p63 Transactivation. It is of particular interest that p63 requires ZIP10 for its transactivation (Fig. 4*D*). p63 is a master TF for epidermal development: Ablating p63 severely disturbs the development of stratified epithelia and epithelial appendages and is embryonic lethal (28, 41), suggesting that p63 is crucial for epithelial stem cells. Among p63 isotypes, TAp63 is expressed in early embryonic epidermal development to trigger the commitment to stratification (2, 42), whereas ΔNp63 is predominantly expressed during late epidermal development and is mainly expressed in basal cells in the adult epidermis to maintain the proliferative potential of epidermal progenitor cells (2). Given that *Zip10*^{K14} mice were born with apparent appendages (Fig. 3*A*) and comparable level of *keratin 14* marking epidermal basal cells (Fig. 3*E*), which are not reported on *p63*-null mice (41), these results indicate that p63 is active in the early epidermal developmental stage of ∼E14. However, when stratification occurs, p63 expression is dramatically elevated along with the requirement of ZIP10 for directing zinc homeostasis toward p63, leading to efficient epidermal morphogenesis. The potential exists that MTF-1, a zinc-responsive TF (43), may direct the gene regulation for epidermis formation via ZIP10. However, *MTF1* is comparably expressed during epidermal development (Fig. 4*C*), which is inconsistent with p63 and ZIP10 (Figs. 1*C* and 4*C*), and we could not find significant changes in keratin expression in *MTF1*-KD cells (Fig. S10), suggesting low probability of MTF-1 involvement in gene regulation for epidermis formation.

ZIP10–Zinc–p63 Axis for Epidermal Formation. p63, which belongs to the p53 family, in fact possesses zinc-binding residues that are required for normal transactivation. A crystal structure analysis of p63 revealed many pathogenic mutations in its zinc-binding motif or in positions that could affect zinc binding (6). Zinc binding stabilizes p53 DBD architecture, whereas zinc chelation disrupts it, indicating that zinc is essential for folding the WT conformation for sequence-specific DNA binding (44). Therefore, it might also occur that zinc capturing in the equivalent domains facilitates p63 function. The loss of ZIP10 interferes with p63 functions in keratinocytes but does not activate Caspase-3, unlike its effect in B cells (37) (Fig. S11), indicating that the molecules influenced by ZIP10 may differ in each cell type. Our study thus yields insights into the relevance of ZIP10-mediated zinc signals in regard to p63 function, suggesting that further exploration of the role(s) of ZIP10, p63, and the intermediary molecules will help to understand the pathophysiology of the molecular basis of the epidermis, and of the severe dermatitis caused by zinc deficiency.

In conclusion, we established an essential role of ZIP10 in skin epidermal development and showed that ZIP10-mediated intracellular zinc homeostasis governs epidermal development, at least in part, by supporting p63 transactivation. Our findings provide an insight into the relevance of zinc homeostasis in dermatological processes. Further exploration of the questions that remain unanswered regarding the molecular mechanisms involving the ZIP10–zinc–p63 axis will help elucidate the critical role of zinc homeostasis in skin formation and regeneration.

Materials and Methods

Detailed descriptions of all of the materials and methods are provided in *SI Materials and Methods*. Differences between two groups were analyzed by Student's two-tailed *t* test. All procedures including mouse handling were conducted according to guidelines approved by the RIKEN Institutional Animal Care and Use Committee (K24-007).

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