UNIVERSITY OF CALIFORNIA SAN DIEGO

Elucidating the Role of HOPX in Late Epidermal Differentiation

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by

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The Thesis of Selena Ying Chen is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2020

DEDICATION

I dedicate my work to my family and to the advancement of our scientific community.

TABLE OF CONTENTS

Signature Page ……...iii

Dedication ……..iv

Table of Contents ……v

List of Figures ……... vi

Acknowledgements ……...vii

Abstract of the Thesis ……..viii

Introduction ……1

Materials and Methods ……..13

Results ……...19

Discussion ……36

References ……47

LIST OF FIGURES

Figure 1. Microanatomy of the epidermis……4

Figure 2. Generation of three-dimensional *in vitro* organotypic skin cultures to model the human epidermis……..7

Figure 3. *HOPX* is a skin differentiation-expressed gene.…….....…20

Figure 4. HOPX is required for keratinocyte differentiation……..……..…….23

Figure 5. HOPX is necessary but not sufficient for keratinocyte differentiation…..……25

Figure 6. Co-enrichment of ZNF750 and a putative enhancer ~6 kb downstream of the *HOPX* locus……...……28

Figure 7. ZNF750 is necessary and sufficient for HOPX induction……..29

Figure 8. Deletion of the putative enhancer represses HOPX induction…..…….31

Figure 9. HOPX is induced by ZNF750 binding at a downstream enhancer……33

Figure 10. HOPX knockdown affects the late/terminal epidermal differentiation program on a genome-wide scale……...……..35

Figure 11. Model of genetic p63-ZNF750-HOPX relationship in terminal keratinocyte differentiation…….37

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ABSTRACT OF THE THESIS

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The outermost layers of skin consist of stratified epithelia that serve as a barrier and interface to the outside environment. These epithelia are comprised primarily of keratinocytes, which exist as self-renewing progenitors in the innermost layer that differentiate into overlying protective layers. On a transcriptional level, keratinocyte differentiation involves coordinated induction and repression of hundreds of genes, but the genetic regulators governing this process are not fully understood. The homeodomain-only protein homeobox (HOPX) is known to be involved in keratinocyte differentiation, but prior studies disagree whether it promotes or inhibits

this process. Here, we show that HOPX activates skin differentiation in primary human keratinocytes and organotypic epidermal tissue. HOPX expression is induced by the transcription factor ZNF750, which binds an enhancer located ~6 kb downstream of the 3' end of the HOPX gene. Whole transcriptome analysis demonstrates that HOPX controls expression of 589 genes and is a positive regulator of late epidermal differentiation. We propose that HOPX functions within a p63-ZNF750-HOPX pathway to upregulate key proteins required for terminal epidermal differentiation, providing clarity to previous studies showing conflicting results. Future studies will aim to decipher the molecular pathways and mechanisms operating downstream of HOPX that engage the late differentiation program.

INTRODUCTION

The Skin Epithelium

Skin is a complex organ that provides homeostatic regulation and protects the human body against its external environment. The skin serves various roles including thermoregulation, transmission of stimuli, and wound healing. As a barrier, it regulates immune homeostasis and protects against microorganism invasion and mechanical injury (Fitzpatrick, 1993; Simpson, Patel and Green, 2012). Mammalian skin is composed of the hypodermis, dermis, and epidermis. The hypodermis is a layer of fatty subcutaneous tissue in which adipocytes predominantly reside. It serves roles in insulation, as an energy reserve, and provides protection to underlying tissues (Debeer *et al.*, 2013). Overlying the hypodermis is the dermis, a rich extracellular matrix of connective tissue containing collagen, elastin fibers, and mucopolysaccharides. The dermal layer contains the nerve receptors, hair follicles, sebaceous glands, and sweat glands of the skin (Summerfield and Ricklin, 2015). The dermis supports the epidermis, an avascular epithelium that includes tightly packed, stratified layers of squamous keratinocytes (Elias *et al.*, 2002; Proksch, Brandner and Jensen, 2008). The epidermis contains four functionally distinct layers that are each composed of keratinocytes undergoing a different stage of differentiation. The basal layer (stratum basale), directly above the basal lamina, is composed of mitotically active keratinocyte progenitors. Through the terminal differentiation process, keratinocyte progenitors undergo morphological and biochemical changes that eventually result in protective, flattened squames (Fuchs and Byrne, 1994; Blanpain, Horsley and Fuchs, 2007). These keratinocytes migrate outwards from the basal layer into the suprabasal spinous layer (stratum spinosum), granular layer (stratum granulosum), and outermost cornified layer (stratum corneum) of the epidermis (Turksen and Troy, 1998).

Epidermal proliferation and differentiation

Upon commitment to the epidermal lineage, keratinocyte progenitors lose expression of keratins 8 and 18, non-epidermal markers of simple epithelium (Byrne, Tainsky and Fuchs, 1994). They also gain expression of the epidermal keratins, keratin 5 and keratin 14, intermediate filament proteins forming the cytoskeleton of basal epithelial cells and markers of basal cell proliferative potential (Moll *et al.*, 1982; Tyner and Fuchs, 1986; Fuchs and Byrne, 1994). Epidermal proliferation occurs at the basal layer, where keratinocyte progenitors adhere to the underlying basement membrane (Fuchs, 1990).

At the onset of differentiation, basal cells lose mitotic activity and detach from the basement membrane. Cells exit the basal layer and migrate towards the spinous layer, switching off expression of keratin 5 (KRT5) and keratin 14 (KRT14). Across the spinous and granular layers, cells express keratin 1 (KRT1) and keratin 10 (KRT10), proteins that reinforce intercellular adhesion and protect skin against mechanical stresses (Fuchs and Green, 1980; Eichner, Tung-Tien and Aebi, 1986). Spinous keratinocytes also begin expressing involucrin (IVL) and transglutaminase-K (TGMT-K), proteins that later help in the formation of the cornified envelope (Watt and Green, 1981; Warhol *et al.*, 1985). Differentiating keratinocytes in the granular layer synthesize electron-dense protein structures called keratohyalin granules and secrete their protein contents between the granular and cornified cell layers. Larger granules contain profilaggrin, the precursor of filaggrin (FLG), which has roles in aggregating keratin filaments and mediating epidermal water content (Dale, 1985). Smaller keratohyalin granules contain loricrin (LOR), a key protein of the cornified envelope (Mehrel *et al.*, 1990). Cells in the outer granular layer also synthesize ceramides and other lipids that help in creating the insoluble, waterproof barrier of the cornified envelope (Bikle, Zhongjian and Chia-Ling, 2013). Terminally differentiated

keratinocytes in the cornified layer form the cornified cell envelope and eventually become dead, enucleated cells called corneocytes. The cornified cell envelope structure provides the highly rigid, tough outer layering of the epidermis that protects the body from water loss, pathogens, and other physical and chemical insults. In a continuous cycle, dead corneocytes are shed from the surface and replaced by replication and outward differentiation of inner stem/progenitor keratinocytes. This self-renewing ability of progenitors is essential for maintenance of skin homeostasis and response to epidermal injury.

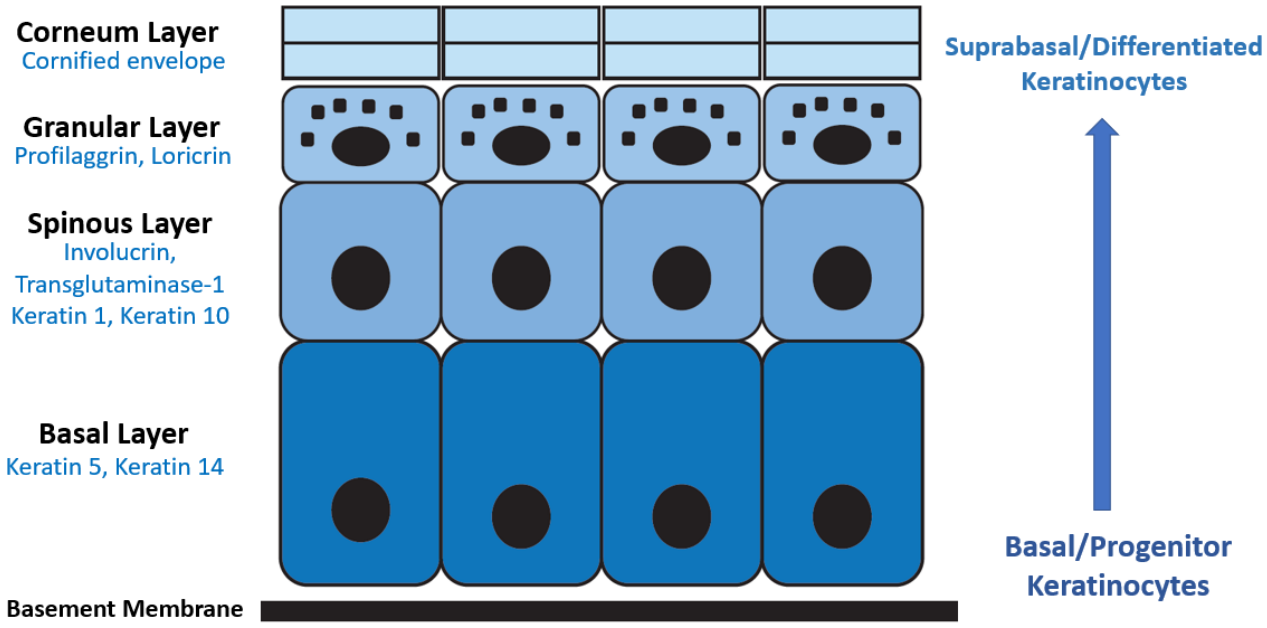


Figure 1. Microanatomy of the epidermis. The epidermis contains four functionally distinct layers, including the stratum basale, the stratum spinosum, the stratum granulosum, and the stratum corneum. The stratum basale is formed by basal keratinocytes residing above the basal lamina, and marked by production of keratins 5 and 14. Keratinocytes undergoing differentiation move upwards to the stratum spinosum, where keratins 1 and 10, and some proteins of the cornified envelope, involucrin and transglutaminase-I, are expressed. At the stratum granulosum, keratinocytes synthesize keratohyalin granules containing profilaggrin and loricrin. At the outer granular layer, structural proteins and lipids involved in formation of the cornified envelope are synthesized, which contribute to waterproofing of the skin barrier. At the corneum layer, the cornified envelope structure is formed.

Induction of keratinocyte differentiation by calcium

Calcium is the major regulator known to control keratinocyte differentiation *in vivo* and *in vitro*. Within the epidermis, a steep, nonlinear calcium gradient spans from the basal layer to the cornified layer, with the highest concentration of calcium in the granular layer (Boyce and Ham, 1983; Menon, Grayson and Elias, 1985). *In vivo* studies demonstrate a role of the calcium gradient in controlling the stepwise differentiation of keratinocytes and the expression of differentiation markers like loricrin and involucrin, although the molecular mechanisms involved are less clear (Elias *et al.*, 2002). Much that is known about calcium-regulated differentiation of keratinocytes comes from *in vitro* studies. Modulating the calcium concentration in culture medium strongly alters keratinocyte proliferation and differentiation (Hennings *et al.*, 1980), such that cells cultured in low calcium (<0.10 mM) remain proliferative and do not stratify. Alternatively, keratinocytes switched to higher extracellular calcium (>.10 mM) are induced to differentiate, undergoing morphological changes and activating pathways involved in the formation of desmosomes, adherens junctions and tight junctions. These intracellular signaling pathways promote intercellular adhesion and epidermal differentiation by activating kinase and phospholipase targets that in turn upregulate intracellular free calcium and PKC activity (Hennings *et al.*, 1980; Bikle, Zhongjian and Chia-Ling, 2013).

In vitro models for keratinocyte differentiation

Keratinocyte differentiation has been studied over the last thirty years using a variety of culture conditions and cell models. Mono-layered primary keratinocyte cultures are a classic *in vitro* model that is used to study keratinocyte proliferation and differentiation (Borowiec *et al*., 2013). A variety of culture conditions have now been proposed to study keratinocyte differentiation, but high calcium supplementation (>.1 mM) and high cell density are two

conditions classically implemented to induce differentiation *in vitro*. Cell culture models do not form epithelium, however, and can be supplemented by three-dimensional tissue culture models to study epidermal morphogenesis. Such models allow for closer simulation of epidermal differentiation and associated cell-cell interactions, such as those between fibroblasts and keratinocytes. When plated to confluence in three-dimensional cellular constructs, keratinocytes can proliferate into stratified layers. Moreover, keratinocytes that reside in the outermost layer undergo terminal differentiation if exposed to air (Dongari-bagtzoglou and Kashleva, 2006). 3D organotypic skin cultures take advantage of these properties to faithfully recapitulate epidermal differentiation and morphogenesis (Regnier and Darmon, 1989). Fibroblasts are prepared on a bed of human dermal mesenchymal tissue, after which progenitor keratinocytes are seeded and grown for approximately 7-10 days. Cells undergo a similar setting of transdermal feeding and exposure to air that occurs *in vivo*. Keratinocytes in culture will stratify outwards, with the outermost cells differentiating at the air-liquid interface. Ultimately, cells give rise to a fully stratified epithelium that recapitulates the induction of early and late epidermal differentiation markers observed *in vivo* (Regnier and Darmon, 1989). In regenerated epithelial tissue, early differentiation markers such as the spinous differentiation marker keratin 1 are expressed beginning day 2 or day 3 of differentiation. Late differentiation markers like filaggrin and loricrin are expressed farther into the differentiation timeline, around days 4 and 5 (Lopez-Pajares *et al.*, 2015).

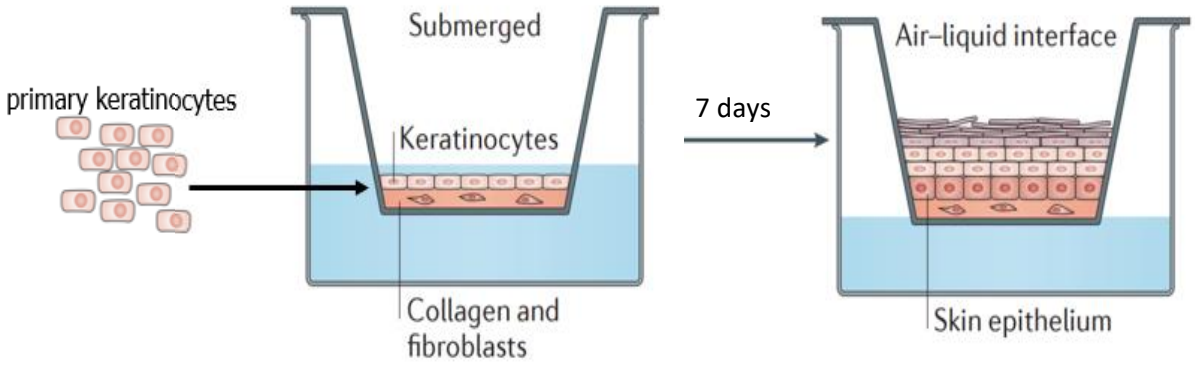


Figure 2. Generation of three-dimensional *in vitro* organotypic skin cultures to model the human epidermis. Primary human fibroblasts and keratinocytes are first grown as monolayer cultures. Next, primary human fibroblasts are seeded on a matrix of collagen, such as devitalized dermis, in a three-dimensional cell culture insert. The progenitor keratinocytes are then seeded and incubated in keratinocyte culture medium. After 72 hours, the insert is lifted, allowing the outer layer of keratinocytes to differentiate at the air-liquid interface. The keratinocyte medium is changed to differentiation medium. Cells are grown at the air-liquid interface over the next 7-10 days to induce epithelial stratification of the dermal and epidermal layers (adapted with permission from Auke Otten).

Regulation of epidermal differentiation

Epidermal homeostasis is mediated by a highly organized network of regulatory elements controlling cell proliferation, differentiation, and barrier formation (Klein and Andersen, 2015). Several regulators of early differentiation have been identified, including the transcription factor p63. p63 is a member of the p53 family of proto-oncogenes and acts as a hallmark regulator of epidermal development and homeostasis. In the context of epidermal differentiation, it is involved in the initiation of epithelial stratification, progenitor maintenance and barrier formation (Koster, 2010). p63 is expressed in all layers of human and murine epithelial tissue and regulates the

induction of both early and late epidermal differentiation genes (Truong and Khavari, 2007; Truong et al., 2006). p63 also controls the expression of key keratins involved in early epidermal stratification, repressing keratins 8 and 18 and activating keratins 5 and 14. Moreover, depletion of p63 in mice resulted in abnormal epidermal growth and loss of the stratum corneum, highlighting its essential role in regulating epidermal differentiation (Su *et al.*, 2009b).

Although p63 is known to control early events of epidermal differentiation, regulators of downstream differentiation are not fully understood. Several transcription factors, such as KLF4 and GRHL3, which control lipid deposition on the cornified envelope, have been implicated in regulating late epidermal differentiation (Segre, Bauer and Fuchs, 1999; Yu *et al.*, 2006). The molecular mechanisms linking p63 with its downstream regulators have also largely remained unclear. In recent years, p63 was shown to bind the ZNF750 promoter, which induces expression of KLF4, GRHL3, and downstream differentiation genes to promote epidermal differentiation (Sen *et al.*, 2012; Lopez-Pajares *et al.*, 2015). Zinc Finger 750 (ZNF750) is a 752 amino acid nuclear protein that encodes an evolutionarily conserved C2H2 zinc finger domain of 60 amino acids at its amino terminus. The C2H2 domain is required for ZNF750-controlled differentiation in human keratinocytes (Sen *et al.*, 2012). In the epidermis, nuclear localization of ZNF750 is absent in undifferentiated basal layers and is increasingly expressed starting at the spinous layer (Birnbaum 2012). The terminal differentiation program is regulated in part via a p63-ZNF750 pathway, in which upregulation of ZNF750 by p63 leads to induction of genes involved in terminal differentiation and barrier formation. Therefore, identifying downstream targets of ZNF750 has great implications for the elucidation of epidermal differentiation.

Homeodomain-Containing Proteins

One class of molecules broadly implicated in differentiation and cell fate decisions are the homeodomain-containing proteins. Members of the homeodomain superfamily contain a 180 bp homeobox sequence that mediates protein-protein interactions and binds DNA (Scott and Weiner, 1984; W. McGinnis *et al.*, 1984; William McGinnis *et al.*, 1984). The homeobox sequence is conserved across vertebrates and encodes the homeodomain structure, which is believed to function by binding at sequence-specific DNA motifs (William McGinnis *et al.*, 1984). Most homeodomains consist of 60 amino acids, a flexible amino terminal tail, and three α-helices surrounding a hydrophobic core, with helices 2 and 3 arranged in a helix-turn-helix motif for binding DNA at the major groove (Laughon and Scott, 1984; Shepherd *et al.*, 1984). Many homeodomains exhibit different amino acid compositions that result in significant variation of DNA binding properties.

The homeobox was first discovered in homeotic genes in *Drosophila melanogaster* (Scott and Weiner, 1984; W. McGinnis *et al.*, 1984) and has since been found in evolutionarily distant animal species, plants, fungi, and single-celled organisms (Cillo *et al.*, 2001; Guyader *et al.*, 2007). Across these organisms, homeodomain-containing proteins are found in both HOX genes and non-HOX genes. Highly conserved genes within the HOX family are distributed in four genomic clusters (Duboule, 2007), whereas non-HOX genes can be found throughout the genome. In animals, homeodomain-containing proteins make up 15-30% of all transcription factors and participate in diverse biological functions, notably induction of developmental programs, cell fate determination, and patterning (Bürglin and Affolter, 2016).

In skin, homeodomain-containing proteins are implicated in regulating epidermal differentiation and growth (Merabet *et al.*, 2009). Several HOX genes demonstrate roles in regulating skin development. This includes the human homeobox genes HOXA4, HOXA5, HOXA7, HOXC4, and HOXB7 which are strongly expressed in the epidermis between week 10 and week 17 of fetal development. HOXA4, HOXA5, and HOXA7 expression are also observed in the granular layer of newborn and adult human epidermis (Stelnicki *et al.*, 1998). Whereas HOXA7 negatively modulates epidermal differentiation by downregulating expression of transglutaminase 1, keratin 10, and involucrin (Celle and Polakowska, 2001), the Hoxb13 gene promotes transglutaminase activity and formation of the cornified envelope in adult epidermis (Mack *et al.*, 2005). Non-HOX genes like Distal-less 3 (Dlx3) are implicated in regulation of epidermal differentiation genes, including expression of filaggrin and loricrin (Morasso *et al.*, 1996). The homeobox proteins MSX-1, MSX-2, and MOX-1 are also expressed in human fetal skin and adult epidermis (Stelnicki *et al.*, 1997).

The Homeodomain-only Protein X, HOPX

The non-HOX protein, homeodomain-only protein X (HOPX), is the smallest member of the homeodomain superfamily that is unique in its inability to bind DNA. In humans, the *HOPX* gene is composed of seven exons and is localized on chromosome 4. HOPX exists as five variant mRNA transcripts that comprise three protein isoforms (National Center for Biotechnology Information data). Isoform a is encoded by the 91-amino acid long mRNA variant 1, isoform b is encoded by the 73-amino acid long mRNA variants 2, 3, and 4, and isoform c is encoded by the 112-amino acid long mRNA variant 5, with a C-terminal sequence distinct from isoforms a and b.

Studies have shown HOPX to be an important regulator of proliferation and differentiation across tissues and cell types. Otherwise denoted NECC1, LAGY, OB1, and Cameo, HOPX is

thought to act primarily as a cofactor, varying in function according to its direct protein interactions. It was first discovered in 2002 in cardiac tissue (Chen *et al.*, 2002; Shin *et al.*, 2002). In the heart, HOPX balances the role between cardiomyocyte proliferation and differentiation through regulation of transcriptional targets of serum response factor (SRF) and histone deacetylase 2 (HDAC2) (Kook *et al.*, 2003). HOPX binding to SRF reduces SRF binding to DNA and therefore transcription of SRF-controlled genes. Meanwhile, binding of HOPX to HDAC2 is critical for GATA4 regulation in murine cardiac progenitor cells (Trivedi *et al.*, 2010).

In the lungs, HOPX controls pulmonary maturation by co-regulation of gene expression downstream of the transcription factors GATA6 and Nkx2.1 (Yin *et al.*, 2006). In skeletal muscle, HOPX interacts with EPC1 (enhancer of polycomb 1) to potentiate transcription of proteins that promote muscle differentiation (Kee *et al.*, 2007). HOPX was also shown to modulate differentiation in trophoblast cells by binding to SRF (Chen *et al*., 2002), as well as differentiation of choriocarcinoma stem cells into syncytiotrophoblasts (Asanoma *et al.*, 2003). Moreover, HOPX was found to modulate epithelial lens fiber cell terminal differentiation, a process of differentiation with similarities to keratinocyte terminal differentiation (Vasiliev, Rhodes and Beebe, 2007). Viewed globally, a common functional theme is that HOPX controls the balance between proliferation and differentiation, though it does so by varying pathways in different tissues.

In skin, HOPX has been identified as a key genetic regulator of epidermal differentiation. Within the interfollicular epidermis, HOPX is expressed abundantly in differentiated strata of human and mouse skin (Yang *et al.*, 2010; Obarzanek-Fojt *et al.*, 2011)**.** However, studies have reported conflicting results as to the mechanistic role of HOPX in human keratinocyte differentiation. In one study, overexpression of HOPX in undifferentiated keratinocytes led to increased expression of the terminal differentiation genes loricrin and filaggrin, suggesting its role

in activating barrier proteins during differentiation (Obarzanek-Fojt *et al.*, 2011). Consistent with this model, experimental depletion of HOPX led to reduction of filaggrin expression. However, an independent study reported conflicting results (Yang *et al.*, 2010). In this second study, depletion of HOPX in immortalized HaCaT keratinocytes resulted in upregulation of loricrin. Overexpression of HOPX resulted in the repression of the differentiation-associated transcripts involucrin and loricrin. In contrast to the first study, the results supported a model in which HOPX is a negative regulator of skin differentiation. Therefore, while the association between HOPX and keratinocyte differentiation is established, it is not clear whether HOPX functions as an activator or repressor of this process.

Objectives of this Study

In this context, we sought to define whether HOPX is a positive or negative regulator of epidermal differentiation. Our preliminary data demonstrated that repressing HOPX expression led to reduction of late differentiation genes, indicating that HOPX positively regulates epidermal differentiation. Furthermore, we aimed in this study to understand what factors regulate HOPX, as well as what factors are regulated downstream of HOPX.

MATERIALS AND METHODS

Cell culture and treatment

Normal primary human keratinocytes were isolated from discarded neonatal foreskin from circumcisions and cultured in a 50:50 mixture of 154 Medium and KSFM Medium (Life Technologies, Waltham, MA) with manufacturer supplements, as well as penicillin/streptomycin and amphotericin. Differentiation was induced by plating cells to confluence and supplementing media with 1.2 mmol/L calcium.

Antibodies and reagents

The following commercial antibodies and dilutions were used for immunoblotting: HOPX (Santa Cruz Biotechnology, Dallas, TX; SC-30216) (1:250), HA (Cell Signaling, Danvers, MA; 3724) (1:1,000), ZNF750 (Proteintech, Rosemont, IL; 21752-1-AP) (1:200), b-tubulin (Developmental Studies Hybridoma Bank, Iowa City, IA; E7) (1:1,000e2,000), and b-actin (SigmaAldrich, St. Louis, MO; A1978) (1:1,000e4,000). Secondary antibodies included IRDye 800CW anti-rabbit (Li-Cor Biosciences, 926-32211) (1:10,000), IRDye 680RD anti-rabbit (Li-Cor Biosciences, 925-68071) (1:10,000), IRDye 800CW anti-mouse (Li-Cor Biosciences, 925-32212) (1:10,000), and IRDye 680RD antimouse (Li-Cor Biosciences, 926- 68072) (1:10,000). The following commercial antibodies were used for immunofluorescence staining: LOR (Covance, Princeton, NJ; PRB-145P) (1:500), KRT10 (Abcam, Cambridge, UK; ab76318) (1:1,000e2,000). The secondary antibody was Alexa Fluor 555 anti-rabbit (Invitrogen, Waltham, MA; A21428) (1:500).

Western blotting

Keratinocytes were washed with cold phosphate-buffered saline (PBS), and protein lysate was extracted with RIPA buffer supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland). Protein quantification was performed with a BCA Protein Assay kit (Pierce,

Rockford, IL) following the manufacturer's instructions. Next, 12 mg of protein was resolved on 4%-12% SDS-PAGE gels (Invitrogen) with 2-(N-morpholino) ethanesulfonic acid (i.e., MES) buffer and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies overnight at 4 C, washed three times in PBS-Tween (PBS-T), then incubated with secondary antibodies according to the manufacturer's recommendations (Li-Cor Biosciences). Detection of bands and quantitation was performed with Odyssey Image Analysis on the Odyssey Infrared Imager (Li-Cor Biosciences).

Immunofluorescence staining

Slides were fixed for 10 minutes by using 4% paraformaldehyde (for KRT10) or ice-cold acetone (for LOR), then permeabilized three times in PBST for at least 3 minutes each. Blocking was performed with blocking reagent (PBS-T, 5% goat serum) for 1 hour at room temperature and primary antibodies were incubated in PBS-T and 2% goat serum overnight at 4 C. Slides were washed three times with PBS-T and incubated with secondary antibodies in 0.1% PBS-T and 2% goat serum for 45 minutes at room temperature. Slides were washed three times in PBS, then incubated with Hoechst stain for 2 minutes at room temperature and washed with PBS twice and then with milli-Q water before mounting and visualization.

RNA interference

Short hairpin sequences were as follows: shHOPX: GTCAGCCAGCCCAGCTATTTA; HOPX shB: GCTAGCTGTCCTGCTGTTTAA; shZNF750: GGAAAGCCCTTCAAGTATAAA. Hairpins were cloned into the pLKO.1 cloning vector (available from Addgene, Watertown, MA) for lentiviral production.

Organotypic skin culture

Organotypic skin culture was performed essentially as described (Oh *et al.*, 2013). Briefly, devitalized dermis was sectioned into 1.5x1.5-cm squares. Keratinocytes were seeded on the basement membrane of devitalized dermis and grown at the air-liquid interface for 7 days. Tissue was harvested and bisected: half was sectioned onto glass slides in 7-mm sections, and the other half was harvested for RNA.

Reverse transcription and quantitative real-time PCR

Total RNA was isolated with the Directzol RNA MiniPrep kit (Zymo Research, Irvine, CA), following the manufacturer's instructions. cDNA was synthesized from 500 ng of RNA with the iScript Reverse Transcriptase kit (BioRad), and quantitative PCR performed with iTaq Universal SYBR Green Supermix (Bio-Rad). Real-time quantitative PCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). For analysis of mRNA expression, the following primers were used: HOPX, 5'-CTTGCCTTTGCCTCTTCCAC-3' and 5'-ACAGCCCAGGAA AATGAGCA-3'; loricrin, 5'-CCGGTGGGAGCGTCAAGT-3' and 5'-AGGAGC CGCCGCTAGAGAC-30; LCE3D, 5'-TTTCCTGTGGAAGGTCTGAGC-3' and 5'-TGAGGTCAGGCACAAGCACT-3' ; keratin 1, 5'-GCTGCAAGTTGTCAAGTT-3' and 5'-TACCTCCACTAGAACCCAT-3'; keratin 10, 5'-AGCTATGGAAGTAGCAGCTTTGGTG-3' and 5'-TCTGGCGCA GAGCTACCTCAT-3'; RPL32, 5'-AGGCATTGACAACAGGGTTC-3' and 5'-GTTGCACATCAGCAGCACTT-3'. RPL32 served as the loading control in all quantitative real-time reverse transcriptase qPCR analyses.

Cas9-expressing keratinocyte cell line

To generate a Cas9-expressing keratinocyte cell line, the Cas9-Flag-P2Apuromycin cassette was removed from the pLentiCRISPRv2 plasmid (Addgene, no. 52961) and cloned into the BamHI/

XhoI sites of the pLEX MCS vector (Thermo Fisher Scientific, Waltham, MA). Next, the puromycin resistance cassette was swapped for a blasticidin resistance cassette, yielding the vector pLEX-Cas9-Blast. Lentivirus was generated with pLEX-Cas9-Blast and infected into the clone 103 keratinocyte cell line (Sun *et al.*, 2015). After selection in 2 mg/ml of blasticidin for 3 days, selected keratinocytes were plated at 300 cells per 10-cm plate to allow expansion and isolation of individual keratinocyte clones. After 15-18 days of expansion, cell colonies were isolated by ring cloning and further expanded. Western blot was performed with the FLAG tag to identify candidate clones with the highest levels of Cas9 expression. Clone 10 (herein referred to as 103-Cas9-clone#10) was used for the subsequent CRISPR experiments.

CRISPR-Cas9 enhancer knockout

sgRNAs were designed to generate a loss-of-function deletion of the candidate enhancer downstream of the HOPX gene with the following sgRNA sequences: HOPXenh sgRNA-1, 5'-GAGCCACAGCTTATATTGGCA-3'; HOPXenh sgRNA-2, 5'-GCCTGGGATAGTG ACCCTAAG-3'. The 103-Cas9- clone#10 keratinocytes were infected with lentivirus expressing scramble control sgRNA or HOPXenh sgRNA-1 and -2. Cells were selected with puromycin and differentiated with calcium for 6 days. Genomic DNA was isolated and the target genomic region amplified by using the following primers: HOPX-flank, 5'-TAGACTTTAGACACGCAGCCA-3' and 5'-TTTCCCATGATGGACGTGCTA-3'. Amplicons were resolved on a 1.5% agarose gel.

HOPX and ZNF750 overexpression

The open reading frames of HOPX and ZNF750 were cloned in-frame with a 3' hemagglutinin tag (HA) into the pLEX lentivirus vector (Thermo Fisher Scientific). Keratinocytes were infected with overexpression or empty vector, selected with puromycin, and evaluated for overexpression by Western blot against the HA tag. It was observed that the sgControl/sgEnhancer keratinocytes

infected with empty vector or ZNF750 overexpression displayed impairment in up-regulating differentiation markers, which was attributed to having undergone multiple serial rounds of lentiviral infection and antibiotic selection. Therefore, to assess the impact on HOPX expression, differentiation in these cells was induced by short-term propagation at higher densities before harvesting.

RNA sequencing

Primary keratinocytes were infected with shCTRL or shHOPX in triplicate, selected with puromycin for 2 days, and then differentiated for 6 days in confluent conditions in the presence of 1.2 mmol/L calcium. Total RNA from shCTRL and shHOPX keratinocytes were subjected to RNA sequencing with the BGI-seq platform (BGI-America, Cambridge, MA). Quality control, genome alignment, and DEGs were analyzed with Partek Flow version 7.0 (Partek, St. Louis, MO). All samples had a read length of 100 base pairs with a minimal Phred quality score of 34. Reads were aligned to the human genome hg19 reference index with STAR, version 2.5.3a, with default settings (Dobin *et al.*, 2013), resulting in more than 50 million aligned reads per sample. Aligned reads were quantified to hg19-RefSeq Transcripts, version 87, by using the Partek expectation/maximization method. Raw reads were normalized to the total number of counts per million reads. Normalized counts were analyzed for differential expression with an analysis of variance algorithm. Low-expressed genes were excluded if all samples had fewer than 5 reads. Genes were considered differentially expressed if there was at least a 2-fold difference in expression between control and HOPX knockdown and the false discovery rate was less than 0.01. Gene Ontology biological process analysis of the DEGs was performed with PANTHER (Mi *et al.*, 2013) by using the Fisher exact test.

Band quantitation

Intensity of bands was quantitated with Image Lab Software analysis (Bio-Rad). Channel lanes and bands in the agarose gel were detected, and background signal was corrected. Band intensities were normalized to the amplicon length, and relative quantitation of bands was performed to generate a relative quantitation analysis table. Relative band quantitation was determined by the ratio of the band volume divided by the reference band volume.

RESULTS

HOPX is upregulated during late epidermal differentiation

HOPX is expressed abundantly in the differentiated suprabasal layers of murine and human epidermis, however its regulation of the terminal differentiation process is unclear (Yang *et al.*, 2010; Obarzanek-Fojt *et al.*, 2011). We first characterized HOPX expression during early and late keratinocyte differentiation. To establish the dynamics of HOPX expression in a cell culture model of keratinocyte differentiation, primary keratinocyte progenitors were differentiated by plating cells to confluence and supplementing with 1.2 mM calcium. Cells were differentiated for six days and protein lysate was collected on days 0 (undifferentiated keratinocytes), 2, 4, and 6. Western blot demonstrated upregulation of HOPX during later timepoints (days 4-6) of differentiation, in comparison to a marker of early differentiation, keratin 10, which appeared by day 2 (Figure 3b). The expression of key epidermal differentiation markers in cultured keratinocytes resembles that of regenerated epidermal tissue differentiated for a similar time course (Lopez-Pajares *et al.*, 2015). Markers of the spinous layer, like keratins 1 and 10, are induced around days 2 and 3 of differentiation, whereas markers of the outer granular layer and cornified layer, like loricrin and involucrin, appear around days 4 and 5 (Figure 3a). Our data corroborate previous literature demonstrating that HOPX is absent in the undifferentiated basal layers and upregulated in the suprabasal layers of epidermis, suggesting that it plays a role during downstream stages of terminal differentiation. To define this role, we tested HOPX by genetic perturbation of primary keratinocytes and identified its potential transcriptional regulators.

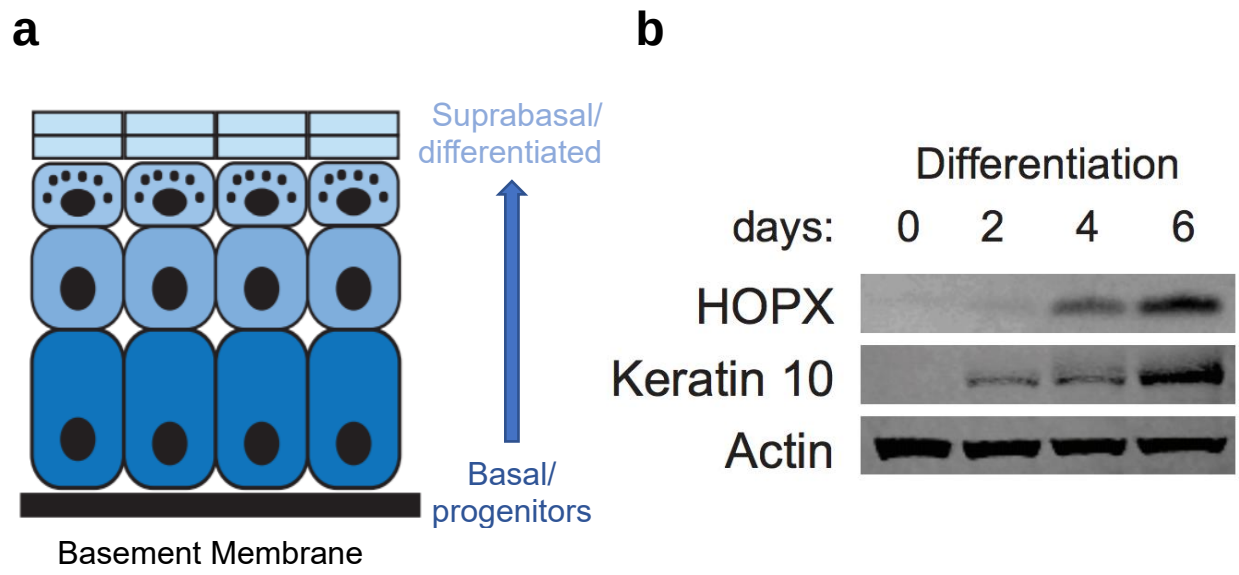


Figure 3. *HOPX* is expressed downstream in terminal keratinocyte differentiation. (a) Graphical summary of induction of early and late differentiation markers in human epidermis. Progenitor keratinocytes detach from the basement membrane and commit irreversibly to the terminal differentiation program, losing mitotic activity. Keratinocytes migrate outwards through the suprabasal spinous, granular, and corneum layers, expressing specific differentiation proteins at each stage of differentiation. Early markers like keratin 10 (KRT10) are expressed during early stages (day 2/3 of *in vitro* differentiation) and late markers like HOPX are expressed downstream (day 4/5 of *in vitro* differentiation). (b) Western blot of HOPX expression during keratinocyte differentiation. Keratinocytes were plated at confluence and differentiated under high calcium conditions for six days. HOPX expression appears around day 4 of *in vitro* differentiation. KRT10 serves as a control for early differentiation (day 2).

HOPX positively regulates genes of late epidermal differentiation

We next assessed the effects of HOPX depletion in cultured keratinocytes differentiated *in vitro*. We hypothesized that depleting HOPX in primary keratinocytes would impair the terminal differentiation program and inhibit expression of key epidermal differentiation genes. We used stable short hairpin knockdown against HOPX (HOPX shA, HOPX shB) in primary neonatal keratinocytes. Monolayer cultures of progenitor keratinocytes were infected with lentivirus containing a scrambled control (shCTRL) or shHOPX construct. Infected cells were then selected and differentiated over a time course of four days, and HOPX expression was measured using quantitative RT-PCR and Western blot. Stable knockdown depleted HOPX mRNA by 98.4% +/- 0.1% (SEM, n=3) compared with the control (shCTRL) and reduced HOPX to undetectable levels on Western blot (Figure 4a).

We then used qRT-PCR to measure the expression levels of canonical early and late epidermal differentiation genes in the context of HOPX depletion. While early differentiation markers such as keratins 1 and 10 were not affected, the expression of the late differentiation markers loricrin (LOR) and late cornified envelope 3D (LCE3D) were diminished (Figure 4b). These genes produce proteins that are critical to form the epidermal barrier and are disrupted in genetic skin diseases, supporting the importance of HOPX in regulating expression of these critical factors. Moreover, these findings support a role for HOPX regulation during the late stages of epidermal differentiation. The results demonstrated that HOPX depletion blocks differentiation and confirmed HOPX as a positive regulator of terminal keratinocyte differentiation.

We next investigated the effects of HOPX depletion on epidermal differentiation in a three-dimensional tissue model using regenerated organotypic skin culture. Human progenitor keratinocytes were again infected with either lentivirus containing a scrambled control or an

shHOPX construct. Independent co-cultures of primary human fibroblasts and selected primary human keratinocytes were grown, then seeded on 1 cm x 1cm square sections of devitalized dermis from human patients and grown at the air-liquid interface for seven days, giving rise to a fully stratified tissue epithelium. On the seventh day of differentiation, the regenerated tissue was sectioned and harvested for RNA.

We performed immunofluorescence to visualize epidermal morphology and observe potential phenotypic changes between control (shCTRL) and HOPX-depleted reconstructed tissue. To detect expression of early and late differentiation markers, we used the spinous layer-expressed differentiation marker, keratin 10, and cornified envelope protein, loricrin. Consistent with results seen in culture, knockdown of HOPX showed no major trend in impact on the expression of keratin 10, but significantly impaired expression of loricrin (Figure 4c). Keratin 10 showed minimal differences in staining across shCTRL and shHOPX tissues, with homogenous expression across the suprabasal stratified layers. In contrast, loricrin expression was significantly repressed in shHOPX but not shCTRL epithelial tissue, with visibly reduced expression in the upper stratified layers. Therefore, our findings in both a cell culture and tissue context demonstrated to us that HOPX depletion blocks differentiation and that HOPX is necessary to activate late/terminal keratinocyte differentiation and form the epidermal barrier.

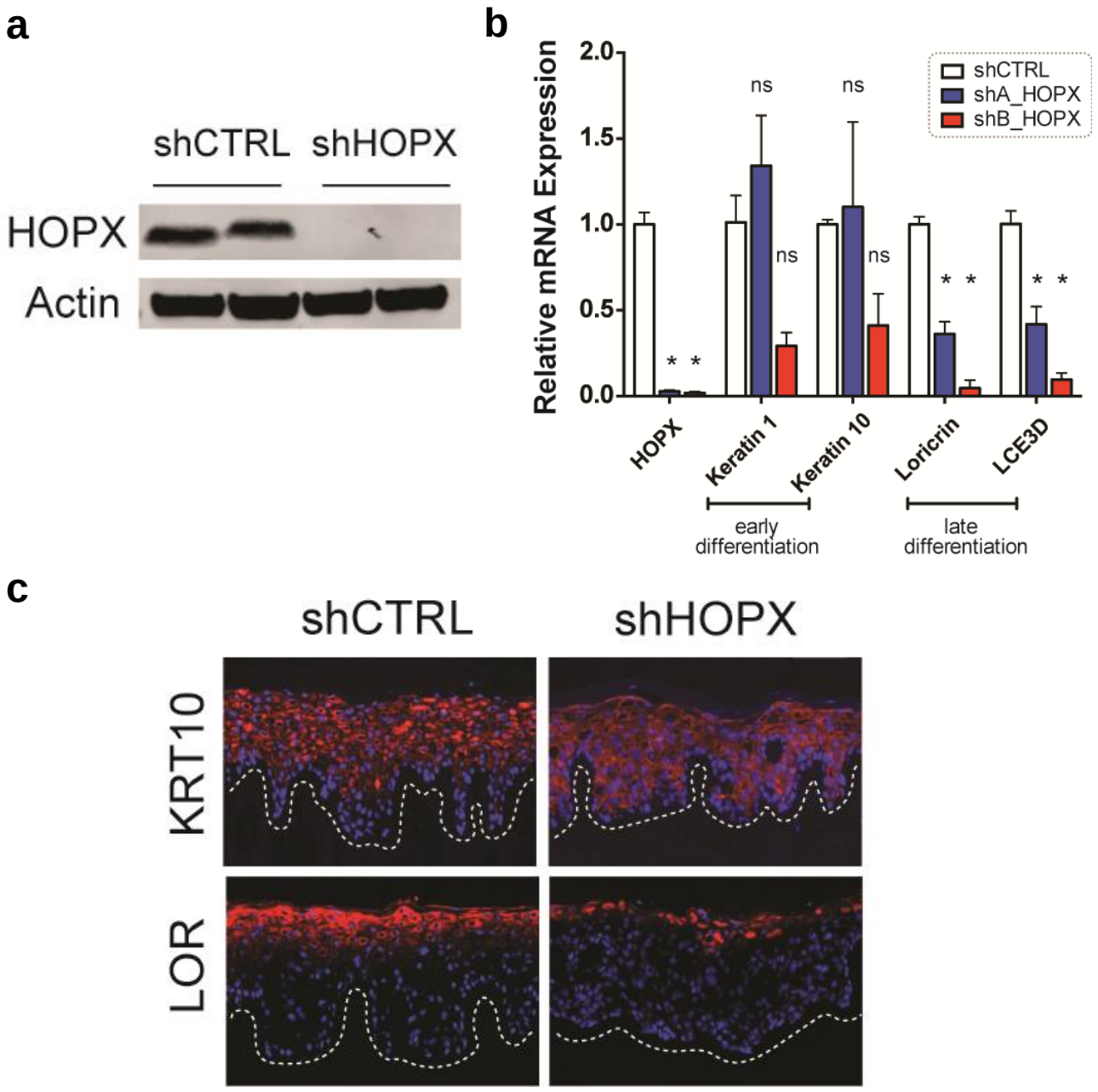


Figure 4. HOPX is required for keratinocyte differentiation. (a) Western blot following short hairpin RNA-induced depletion of HOPX (shHOPX). shCTRL= control. (b) qRT-PCR of differentiation-associated transcripts with HOPX depletion using two independent shRNAs against HOPX. n = 3. Error bars show standard error of the mean. *P < 0.05. ns = not significant. (c) Immunofluorescence staining of control and HOPX-depleted regenerated epithelial tissue. Anti-KRT10 and anti-LOR antibodies were used to stain for KRT10 and LOR expression (red). A Hoescht nuclear stain was also performed (blue).

Exogenous HOPX does not drive late/terminal keratinocyte differentiation

Having established HOPX as necessary for keratinocyte differentiation, we next sought to determine whether HOPX expression itself was sufficient to drive this process. Using lentiviral transduction, we infected progenitor keratinocytes with an empty vector (EV)-expressing or HOPX-expressing lentiviral cassette and maintained selected cells under proliferative conditions (low calcium and low cell density) to discourage differentiation. After verifying HOPX overexpression by Western blot (Figure 5a), we propagated empty vector (EV) and HOPX overexpressing (HOPX OE) keratinocytes for 6 days. On gross microscopic observation, HOPX OE cells displayed no overt morphologic differences. We harvested total RNA and performed quantitative RT-PCR to measure expression of differentiation genes. This revealed no significant upregulation of early (keratins 1, 10) or late (filaggrin, loricrin, late cornified envelope 3D) differentiation genes with HOPX overexpression (Figure 5b). These data indicated to us that HOPX is necessary, but not sufficient, to promote transcriptional hallmarks of epidermal differentiation.

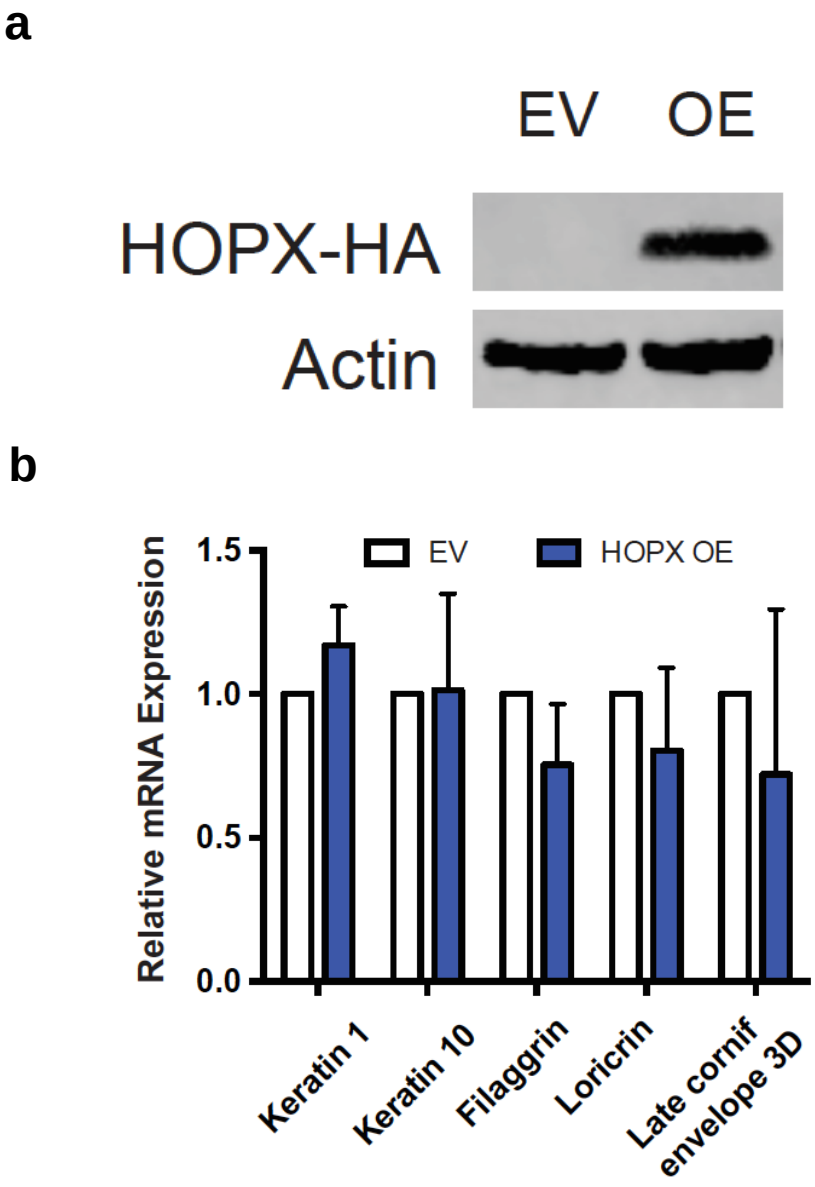


Figure 5. HOPX is necessary but not sufficient for keratinocyte differentiation. (a) Western blot of EV and HOPX-HA overexpression. (b) Quantitative RT-PCR of differentiation-associated transcripts with HOPX overexpression in undifferentiated primary keratinocytes. Keratin 1 and 10 represent markers for spinous layer differentiation. Filaggrin, loricrin, and late cornified envelope 3D (LCE3D) represent markers for late epidermal differentiation. Error bars show standard error of the mean.

HOPX is activated by the transcriptional regulator ZNF750

In keratinocytes, the genetic regulators that control HOPX expression are not known. To gain insight to this process, we used data from the ENCODE consortium to examine the chromatin landscape at the *HOPX* genomic locus. We examined histone 3, lysine 27 acetylation (H3K27Ac NHEK) together with DNase-I hypersensitivity sites (DHS NHEK) in neonatal human epidermal keratinocytes as chromatin marks for candidate enhancers near the *HOPX* locus. This survey found a strong peak located approximately ~6 kb downstream of the 3' end of the *HOPX* gene that showed coincident H3K27Ac and DHS enrichment (Figure 6). To identify candidate transcription factors that bind at this putative enhancer, we examined published chromatin immunoprecipitation-seq (ChIP-seq) data with a focus on transcription factors that are known to affect skin development and differentiation. This analysis identified a binding site for the zinc finger transcription factor ZNF750 overlapping with the H3K27Ac/DHS peak. ZNF750 is a well-defined transcription factor that has a critical role for differentiation in human skin and acts downstream of p63 (Sen *et al.*, 2012). These observations led us to hypothesize that ZNF750 controls HOPX expression by binding an enhancer downstream of HOPX.

To test our hypothesis, we first evaluated whether HOPX expression depended on ZNF750. We generated a short hairpin RNA (shZNF750) to deplete ZNF750 and infected progenitor keratinocytes with lentiviral constructs containing a scrambled control (shCTRL) or shZNF750 construct. Cells were induced to differentiate by plating to confluence and supplementing with high calcium, and ZNF750 knockdown was confirmed by quantitative RT-PCR and Western blot. ZNF750 mRNA and protein levels were reduced by approximately 70% following knockdown (Figure 6a-6b). We next assessed HOPX induction. We found that HOPX was not upregulated in ZNF750-depleted cells, indicating that ZNF750 was required for HOPX

expression (Figure 7a-7b). These findings indicate the necessity of ZNF750 for activation of HOPX during epidermal differentiation.

Next, we aimed to determine whether ZNF750 was itself sufficient to induce HOPX expression in the absence of differentiation. To drive ZNF750 overexpression, we transduced human progenitor keratinocytes with a ZNF750 or empty vector (EV) lentiviral construct. The cells were then maintained under proliferative conditions (low calcium, low cell density) until protein lysate was collected for Western blot analysis on day 5. Under progenitor conditions, ZNF750 and HOPX are both usually not expressed. This was confirmed in EV keratinocytes, which lacked ZNF750 and HOPX protein expression. In contrast, keratinocytes infected with a ZNF750 expressing lentiviral cassette demonstrated ZNF750 overexpression and HOPX induction in the absence of differentiating conditions. Together, these data demonstrated that overexpression of ZNF750 (OE) was sufficient to induce HOPX expression. Collectively, our findings showed that ZNF750 is not only necessary but also sufficient to activate HOPX.

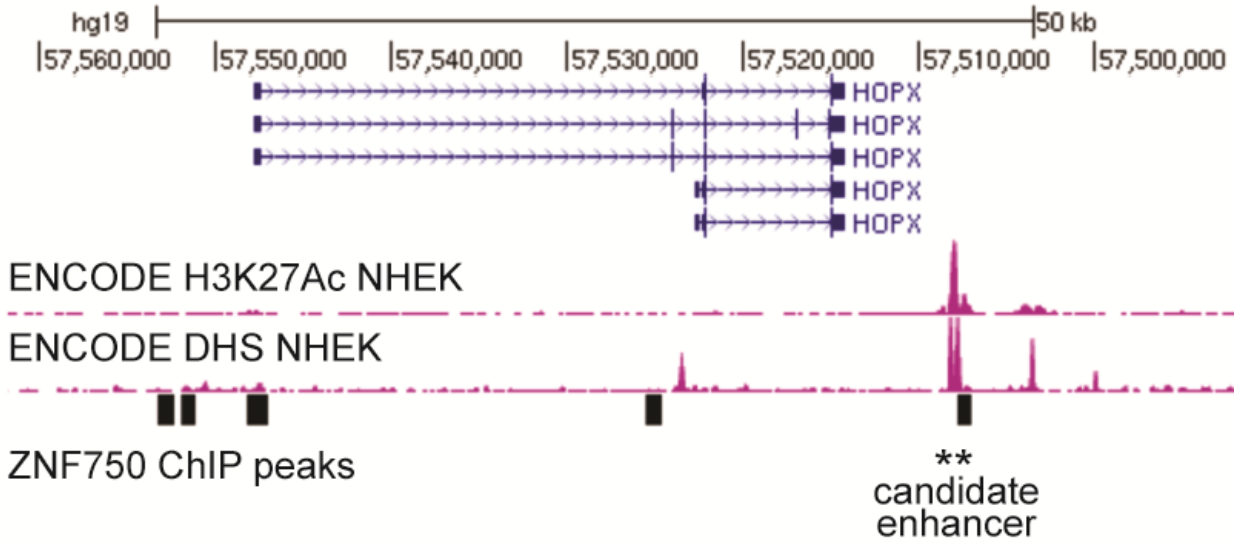


Figure 6. Co-enrichment of ZNF750 and a putative enhancer ~6 kb downstream of the HOPX locus. A candidate enhancer was identified approximately 6 kb downstream of the HOPX locus, which showed strong enrichment for H3K27Ac and DHS marks. Chromatin immunoprecipitation-sequencing tracks around the HOPX genomic locus demonstrated a ZNF750 binding site at this enhancer region. ZNF750 binding sites are represented as black rectangles.

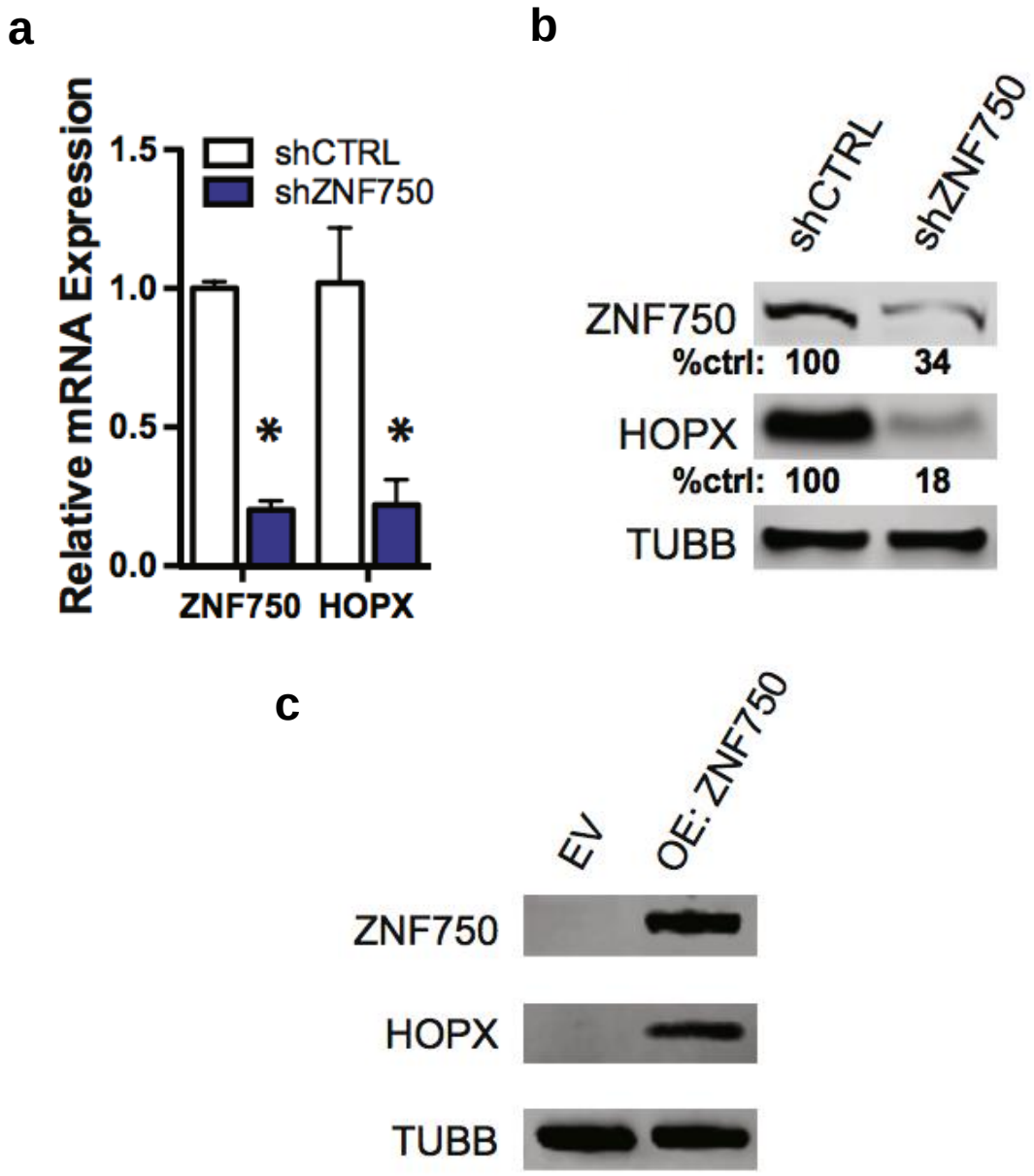


Figure 7. ZNF750 is necessary and sufficient for HOPX induction. (a) qRT-PCR of control and ZNF750-depleted (shZNF750) keratinocytes differentiated for 6 days. N=2. Error bars show standard error of the mean. *P < 0.05. (b) Western blot of day 5 differentiated keratinocytes after short hairpin RNA-induced depletion of ZNF750 (shZNF750). Quantitation of band intensity performed with Li-Cor software (Li-Cor, Lincoln, NE). Band intensities were normalized to loading control and shCTRL band intensity. (c) Western blot of keratinocytes transduced to overexpress ZNF750 (OE ZNF750). Keratinocytes were infected with empty vector (EV) or ZNF750-HA.

Transcriptional activation of HOPX is regulated by a putative enhancer 6 kb downstream of the *HOPX* locus

We next wanted to specifically test whether ZNF750 binds to the candidate downstream enhancer and contributes to HOPX induction. To do this, we used CRISPR/Cas9-mediated genomic editing to delete the candidate enhancer and test whether this affected HOPX induction. We designed two short guide RNAs (sgRNAs) flanking the H3K27Ac/DHS and ZNF750 binding site (which we have denoted as sgEnhancer) and infected them into Cas9-expressing keratinocytes (schematized in Figure 8a). We then used PCR of genomic DNA to assess for successful targeted deletion. sgControl and sgEnhancer keratinocytes with an intact enhancer region were approximately 800 bp, and sgEnhancer keratinocytes with successful knockout of the enhancer region were detected by a reduced band size of 287 bp (Figure 8b).

Using this approach, we successfully deleted the enhancer element in ~75% of pooled keratinocytes. We made three attempts to isolate individual sgEnhancer clones but were unsuccessful in propagating primary keratinocyte clones long enough to perform downstream experiments. Therefore, we compared pooled sgEnhancer keratinocytes against keratinocytes infected with scrambled control sgRNAs (sgControl). Differentiating these keratinocytes at confluence and under high calcium conditions, we assessed HOPX mRNA expression levels and found that sgEnhancer keratinocytes showed a ~50% reduction in HOPX induction. These findings indicated to us that ZNF750 interacts with the candidate enhancer downstream of HOPX to control HOPX induction in the terminal differentiation program.

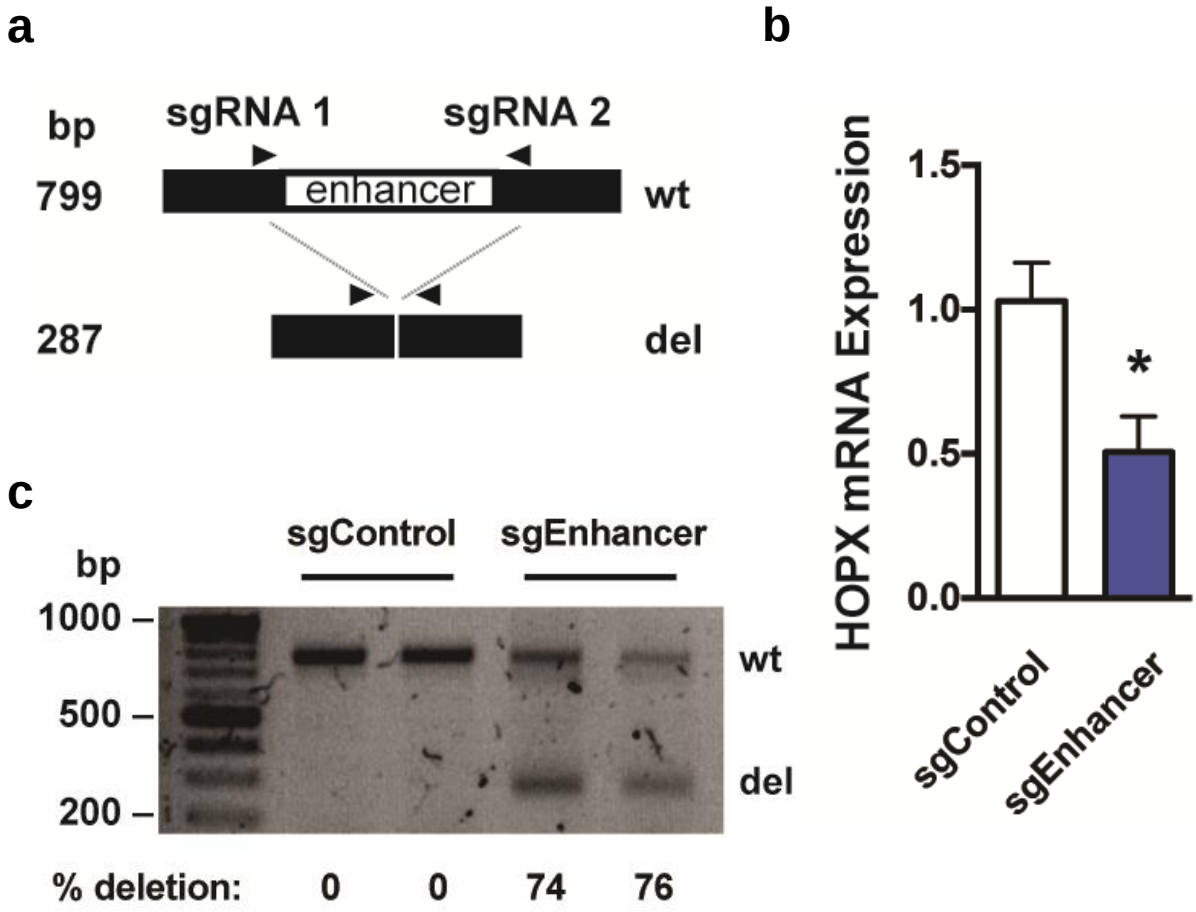


Figure 8. Deletion of the putative enhancer represses HOPX induction. (a) Scheme of CRISPR/Cas9-mediated enhancer deletion and detection by PCR assay. (b) PCR assay to detect HOPX enhancer deletion. Cas9 was introduced into keratinocytes along with sgRNAs flanking the candidate enhancer (sgEnhancer) or scramble control guides (sgControl). Quantitation of band intensity was performed with Bio-Rad (Hercules, CA) Image Lab software analysis. Band intensities were normalized to amplicon length to determine relative deletion efficiency. (c) qRT-PCR of HOPX expression in control and enhancer-deleted pooled differentiated keratinocytes. n = 3. Error bars show standard error of the mean. *P < 0.05.

ZNF750 mediates HOPX induction through binding at the putative enhancer

Finally, we performed ZNF750 overexpression experiments in sgEnhancer keratinocytes to establish that repressed HOPX expression was indeed associated with loss of ZNF750-induced transcriptional activation at the enhancer site. To address this possibility, we first transduced sgEnhancer and sgControl keratinocytes with empty vector (EV) or ZNF750 (ZNF750-FHH OE) lentivirus and grew them under proliferating conditions (low calcium, low density). We then compared HOPX protein expression in each pool of cells on days 2, 4, and 6 of propagation by Western blot. HOPX expression appeared in ZNF750-treated sgControl keratinocytes on day 4 and was upregulated on day 6, complementing our ZNF750 overexpression experiments in progenitor keratinocytes (Figure 7c). Some HOPX expression also appeared in ZNF750-overexpressed sgEnhancer keratinocytes on day 4 and was unchanged on day 6. We repeated this experiment and observed similar results. We then passaged these cells for 32 days and monitored HOPX induction over this time course. As observed on day 6, HOPX protein expression was not appreciably increased in ZNF750-overexpressed sgEnhancer keratinocytes on day 32. The modest induction of HOPX on day 4 of sgEnhancer keratinocytes and its subsequent fixed expression during propagation suggested to us that while ZNF750 also interacts with other enhancer elements to transcriptionally activate HOPX, this candidate enhancer element is a critical site at which ZNF750 binds to regulate HOPX induction.

An immortalized cell line undergoing three sequential infections was generated to achieve our EV/ZNF750-overexpressed control or enhancer knockout keratinocytes, which may delay or weaken the differentiation process. Consequently, we overexpressed ZNF750 in newly differentiated sgControl versus sgEnhancer keratinocytes to monitor effects on HOPX induction. Following ZNF750 overexpression, HOPX expression was induced in sgControl keratinocytes but

not in sgEnhancer keratinocytes (Figure 9). Band quantitation of ZNF750 and HOPX protein levels showed that ZNF750 induced an approximately two-fold increase in HOPX induction in sgControl cells. No increase in HOPX induction was observed in sgEnhancer cells. Together, these experiments demonstrated that HOPX is a downstream activation target of the transcription factor ZNF750, which acts, at least in part, through binding an enhancer downstream of HOPX.

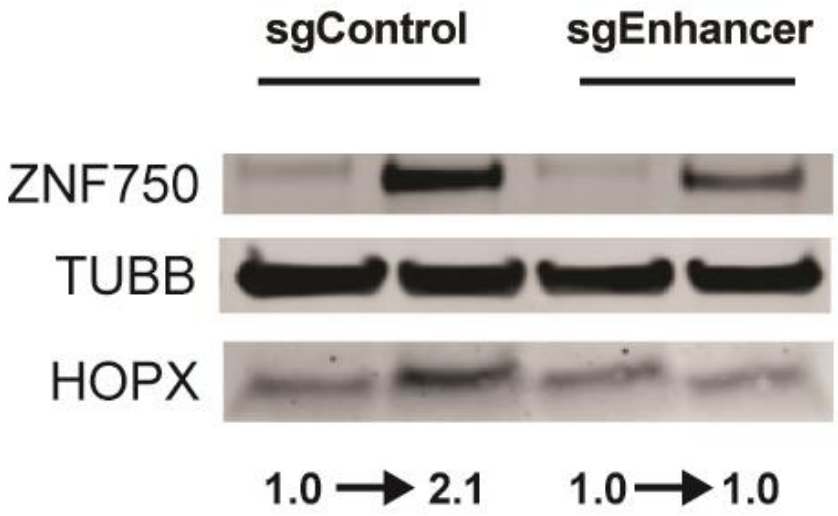


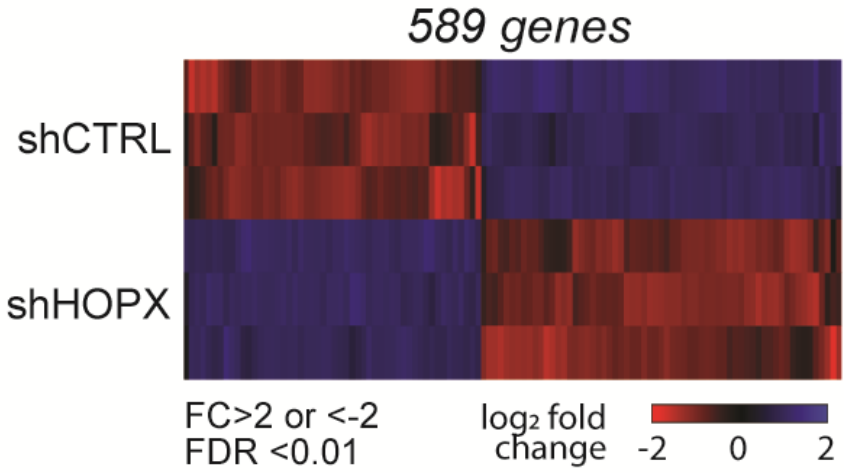
Figure 9. HOPX is induced by ZNF750 binding at a downstream enhancer. Western blot of differentiating sgControl and sgEnhancer keratinocytes transduced to overexpress an empty vector (EV) or ZNF750 construct. From left to right: sgControl cells transduced with EV-expressing lentiviral cassette, sgControl cells transduced with ZNF750-expressing lentiviral cassette, sgEnhancer cells transduced with EV-expressing lentiviral cassette, sgEnhancer cells transduced with ZNF750-expressing lentiviral cassette. Beta-tubulin (TUBB) was used as a loading control. Quantification was performed with LiCor software.

Genome-wide Transcriptional Impact

From a two-dimensional cell culture and three-dimensional tissue context, depletion of HOPX negatively disrupted terminal differentiation. These results indicated that HOPX lies within a genetic pathway known to activate keratinocyte differentiation. To further substantiate our findings, we also assessed the genome-wide transcriptional impact of depleting HOPX.

We knocked down HOPX expression in primary keratinocytes using stable short hairpin RNAs (shHOPX) and induced *in vitro* differentiation of shCTRL and shHOPX cells by seeding at confluence and maintaining with 1.2 mM calcium supplementation. We then performed RNA-sequencing of day six differentiated shCTRL and shHOPX keratinocytes (data deposited at NIH GEO GSE125152). We set a Log2 fold change as the threshold with an ANOVA/false discovery rate of less than 0.01. Under these parameters, we found that HOPX depletion led to a change in expression of 589 differentially expressed genes (DEGs) (Figure 10a)**.** Next, we performed Gene Ontology analysis using PANTHER classification to characterize those differentially expressed genes most affected by HOPX knockdown. Our data showed that the pathways most significantly affected were involved in processes relating to late epidermal differentiation, with genes enriched in the processes of cornification**,** keratinocyte differentiation, keratinization**,** and epidermal cell differentiation (Figure 10b). At a genome-wide level, these findings further supported the context of HOPX as an essential regulator to drive the late epidermal differentiation program.

a



b

GO Term	# of genes	Bonferroni corrected p-value
cornification	23	1.1×10^{-8}
keratinocyte differentiation	30	4.1×10^{-6}
keratinization	26	3.3×10^{-5}
epidermal cell differentiation	30	1.0×10^{-4}

Figure 10. HOPX knockdown affects the late/terminal epidermal differentiation program on a genome-wide scale. (a) RNA sequencing of HOPX-depleted keratinocytes. Heatmap representing the differentially expressed genes (DEGs) associated with HOPX depletion. DEG threshold was assigned as fold change greater than 2 or less than 2, with a calculated false discovery rate of less than 0.01. (b) Gene Ontology enrichment analysis of DEGs altered by HOPX depletion with PANTHER classification.

DISCUSSION

In this study, we used complementary methods, including whole transcriptome analysis and gain and loss of function analysis, to elucidate the role of HOPX in terminal keratinocyte differentiation. The divergent data in the previous two reports may be explained by their use of different cell systems. Whereas the first report demonstrated a positive role of HOPX in epidermal differentiation using primary human keratinocytes (Obarzanek-Fojt *et al.*, 2011), the second report (Yang *et al.*, 2010) demonstrated a negative role of HOPX in HaCaT cells, an immortalized cell line that exhibits variability based on passage number. HaCaT cells are also reportedly delayed in differentiation and continue to proliferate even under differentiation-inducing conditions, such as supplementing culture medium with high calcium (Micallef *et al.*, 2008). The different properties exhibited by primary versus immortalized keratinocytes further complicated comparisons made between their studies on early and late epidermal differentiation.

Our study provides clarity to the previous two conflicting studies and expands upon them by identifying transcriptional regulators of HOPX. We showed for the first time that the nuclear factor ZNF750 interacts with a critical enhancer site downstream of the *HOPX* locus to transcriptionally activate HOPX. Our findings are collectively characterized in a p63-ZNF750-HOPX pathway in which HOPX functions to upregulate key proteins required for terminal epidermal differentiation (Figure 11).

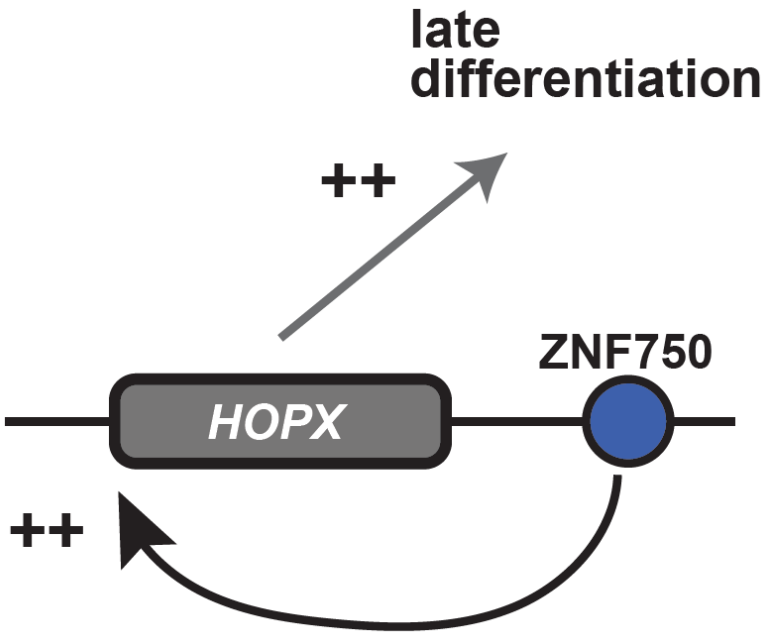


Figure 11. Model of genetic p63-ZNF750-HOPX relationship in terminal keratinocyte differentiation. p63 drives induction of the transcription factor ZNF750, an epidermal differentiation regulator that activates expression of downstream differentiation genes. We define a role for ZNF750 in binding an enhancer site downstream of the *HOPX* locus to promote HOPX transcriptional activity. Our proposed model confirms a positive role for HOPX in regulating the terminal differentiation program in human epidermis. Downstream, HOPX activates key proteins required for late epidermal differentiation, including those involved in pathways of cornification, keratinocyte differentiation, and keratinization.

This work complements and contrasts against the prior studies by the use of regenerated organotypic skin cultures, allowing for evaluation in a three-dimensional tissue context. Overall, our organotypic skin models agree with past *in vivo* HOPX knockout mouse models, which reported normal epidermal morphology and impaired mRNA expression of terminal differentiation proteins (Obarzanek-Fojt *et al.*, 2011). However, some discrepancies between our studies were also observed. Although our shHOPX regenerated tissue ultimately gave rise to fully stratified epithelium, we noticed some abnormal keratinocyte morphology in both cell and tissue cultures following HOPX depletion. This indicated to us that HOPX is potentially important but not essential for processes involved in stratification. Our cell and tissue culture models also showed concomitant downregulation of loricrin mRNA and protein levels, unlike HOPX knockout mouse models that reported loss of loricrin mRNA but not protein expression. One explanation may be that compensatory mechanisms supporting HOPX and loricrin depletion exist in mouse and human epidermis and are suppressed or absent *in vitro*. These distinctions in findings may also be explained by our use of different tissue models, namely an *in vivo* murine skin model and an *in vitro* human organotypic skin model. Mouse knockout models incorporate the gene knockout from the start of development, and thus better capture phenotypes of development. However, this also allows time for compensation by other genes for the function of the lost gene. This is what might have been observed in the HOPX-knockout mouse models. Global HOPX knockout in mouse models may also adversely affect tissues and organs where HOPX is not expressed, thereby affecting the overall phenotypes observed. In our study, we performed rapid and acute shRNA-mediated knockdown, a method that best captures phenotypes of post-natal homeostasis. Under these acute conditions, our *in vitro* models limited the opportunity for genetic compensation by alternative pro-differentiation mechanisms, indicating that HOPX is indeed required for

downstream terminal differentiation genes. Given these idiosyncrasies, repeating HOPX/ZNF750 loss of function assays in *ex vivo* human organ cultures and in HOPX knockout mice would help to further clarify whether these results can be recapitulated under live conditions.

While many of the genetic regulators of epidermal differentiation have yet to be uncovered, one network involves binding by the master transcription factor p63 to ZNF750, which in turn induces terminal expression of several downstream targets, including KLF4, OVOL1, and GRHL3. Our work expands this model and identifies HOPX as another target regulator of terminal differentiation which acts downstream of ZNF750. While our knockdown experiments demonstrated that HOPX is necessary to drive late differentiation, overexpression of HOPX in the absence of differentiation demonstrated that HOPX is not sufficient to drive this process. ZNF750 was shown to act directly downstream of p63 and controls most but not all p63-dependent differentiation genes (Sen *et al.*, 2012). In similar fashion, HOPX and other ZNF750 targets likely each control a subset of ZNF750-dependent differentiation genes. The subset of genes regulated by HOPX alone thus may not be sufficient to drive the late epidermal differentiation program. Additionally, it was previously reported that HOPX overexpression in normal human keratinocytes differentiated for six days showed significant upregulation of the late differentiation proteins FLG and LOR compared to control keratinocytes (Obarzanek-Fojt *et al.*, 2011). Given these findings, HOPX may not be a major player in the induction of downstream differentiation genes but rather in the upregulation of their expression post-transcriptionally. One potential mechanism may be that HOPX interacts with partner proteins to increase mRNA stability of transcriptionally activated genes. HOPX might also upregulate terminal epidermal differentiation via epigenetic modifications. In skeletal muscle, HOPX was shown to modulate differentiation by interacting with enhancer of polycomb 1 (Epc1) (Kee *et al.*, 2007). In epidermis, HOPX may similarly interact

with chromatin remodeling proteins, potentially in a feedback loop, to increase chromatin accessibility of transcriptionally active genes. It is also important to note that our *in vitro* experiments were performed acutely in primary human keratinocytes, whereas normal epidermal turnover occurs approximately every 6-8 weeks in humans and approximately every 8-10 days in mice. Therefore, there is also substantial interest in testing HOPX overexpression in the absence of differentiation in both *in vivo* and *ex vivo* conditions. These future studies may clarify whether enforced HOPX expression can compensate for the loss of terminal differentiation genes and/or accelerate epidermal barrier formation. One such measure might include overexpressing HOPX in ZNF750-depleted keratinocytes to determine if HOPX can rescue the expression of ZNF750-dependent differentiation genes.

RNA-sequencing analysis followed by Gene Ontology classification of HOPX knockdown in differentiated human keratinocytes demonstrated that depletion of HOPX most significantly impaired genes involved in cornification, epidermal differentiation, keratinization, and keratinocyte differentiation. Notably, cornification and keratinization are two processes that take place in the stratum corneum and are critical for formation of the epidermal barrier. During keratinization, terminally differentiating keratinocytes produce keratin polypeptides that polymerize into keratin intermediate filaments within the cytoplasm. These structures contribute to the hard, protective outer layer of the stratum corneum and provide resistance against mechanical stress. Cornification marks the final stage of keratinization, in which the outermost differentiating keratinocytes undergo programmed cell death and ultimately give rise to dead, flattened squames in the cornified layer. These cells, denoted corneocytes, compose a majority of the epidermal barrier (Eckhart *et al.*, 2013). These findings validated that the genes most affected by HOPX depletion participate in pathways essential to late stages of differentiation and epidermal

barrier formation, further confirming a role of HOPX in the epidermal differentiation program. Using keratinocyte cultures, organotypic skin cultures, and transcriptome-wide analysis, we were able to establish that HOPX is required to activate essential pathways participating in terminal epidermal differentiation. While the underlying mechanisms are still unknown, our Gene Ontology characterizations bring further insight to potential pathways through which HOPX acts to regulate late epidermal differentiation.

At the cellular level, HOPX localization has been shown to vary according to the stage of differentiation. This is a common feature of several homeobox genes (Stelnicki *et al.*, 1998; Kömüves *et al*., 2000). In keratinocytes, cells induced to differentiate (day 2) showed a majority of HOPX localization in the cytoplasm with some localization of HOPX in the nucleus. More terminally differentiated cells (day 6) showed nearly all cytoplasmic localization of HOPX (Obarzanek-Fojt et al., 2011). HOPX also lacks a nuclear localization signal, and therefore likely interacts by binding directly or indirectly to other proteins in the cytoplasm, some of which may translocate to the nucleus. Given these features of HOPX, we did not perform chromatin immunoprecipitation sequencing experiments to determine HOPX binding sites. We instead analyzed ZNF750 ChIP-seq data and H3K27Ac/DNase I hypersensitivity marks around the *HOPX* locus and identified strong co-enrichment of a ZNF750 binding site with an enhancer region ~6 kb downstream of *HOPX*. Our ZNF750 overexpression experiments in enhancer knockout (sgEnhancer) keratinocytes validated that ZNF750 binds critically at this enhancer site to induce HOPX. In undifferentiated sgEnhancer cells overexpressed with ZNF750, we were surprised to see slight HOPX induction on day 4 of differentiation. ZNF750 likely interacts with other elements to transcriptionally activate HOPX, which may account for the modest induction of HOPX despite the enhancer knockout. On the other hand, control cells continued to gain HOPX expression

whereas sgEnhancer cells did not in the following days. These observations indicated that the enhancer site is likely a critical genomic binding region of ZNF750, and loss of the enhancer markedly attenuated transcriptional activation of HOPX by ZNF750. In addition to uncovering further upstream regulators of HOPX, there is significant interest in identifying the other major regulatory elements that interact with ZNF750 to induce HOPX.

We also note that to achieve our EV/ZNF750-overexpressed sgControl and sgEnhancer cells, we required an immortalized keratinocyte cell line on which we performed three separate infections and propagated over long periods of time. Using an immortalized cell line allowed us to achieve the desired gene edits that otherwise may not have been possible to obtain in primary keratinocytes. However, immortalized cells also pose the risk of showing unique gene expression patterns that are unlike those found *in vivo* (Carter and Shieh, 2010). It would be unsurprising if the triple-infected immortalized cells exhibited some altered cell function and if this contributed to our unexpected findings in the undifferentiated sgEnhancer keratinocytes. Another possible consequence included altered squamous differentiation. HaCaT cells are one spontaneously immortalized human keratinocyte cell line that have been studied in both organotypic skin culture and murine skin models. In both contexts, the cells gave rise to stratified epithelia but differentiated more slowly than normal human keratinocytes (Breitkreutz *et al.*, 1998; Schoop *et al.*, 1999; Boelsma *et al.*, 1999). We considered that the events required to induce epidermal differentiation in our gene-edited cells might also be delayed or suppressed. Therefore, we repeated the ZNF750 overexpression experiments in sgEnhancer keratinocytes differentiated for a day to enhance the differentiation process. Whereas HOPX expression was steadily upregulated in sgControl cells, HOPX expression did not increase in sgEnhancer cells during the differentiation time course. Thus, despite induction of the differentiation program, loss of the enhancer significantly limited the

capacity of ZNF750 to transcriptionally activate HOPX. Our results collectively demonstrated that while other ZNF750 partners involved in regulating HOPX are sufficient to drive some HOPX induction, ZNF750 requires the candidate enhancer site to markedly activate HOPX expression. Investigating other HOPX and ZNF750 partners would be valuable in clarifying the underlying mechanisms and the downstream pathways through which they act. Future studies should include ChIP-sequencing, mass spectrometry, and co-immunoprecipitation pulldown experiments to decipher these molecular pathways that involve HOPX and that engage the late differentiation program.

Clinical Implications of HOPX Regulation in the Epidermis

Our findings also highlight the clinical relevance of HOPX. Proper epidermal homeostasis is critical to human health, and disruption of the epidermal skin barrier underlies diseases such as eczema, psoriasis, and keratinocyte skin cancers, that collectively affect over 20% of the U.S. population (Lopez-Pajares *et al.*, 2013). HOPX was identified as a genetic determinant in epidermal differentiation and psoriasis, highlighting for it a critical role in skin homeostasis. HOPX expression is strongly upregulated in lichen planus (LP), a skin inflammatory disease, and squamous cell carcinoma (SCC), a keratinocyte skin cancer. Patients with lichen planus and squamous cell carcinoma exhibit a well-differentiated epidermis, including a thickened granular layer, where HOPX is expressed. In turn, HOPX expression is absent or markedly suppressed in cutaneous pathologies with more proliferative phenotypes, such as psoriasis and basal cell carcinoma (BCC) (Obarzanek-Fojt *et al.*, 2011). The epidermis is marked by hyperproliferative keratinocytes and a state of under-differentiation, as evidenced by lack of a granular layer. Thus, HOPX expression appears to strongly correlate with the level of differentiation in cutaneous

pathologies, with under-expression of HOPX leading to hyperproliferative phenotypes and over-expression leading to well-differentiated phenotypes.

In many non-skin cancers, HOPX expression is also significantly downregulated or absent, which may contribute to the hyperproliferation of cells and under-differentiation commonly observed in malignant tissues. Suppression of HOPX expression is implicated in breast, lung, esophageal, stomach, and other cancers. While the mechanisms underlying HOPX regulation are still largely unknown, hypermethylation of the HOPX promoter leading to HOPX inactivation have been revealed in lung, esophageal, and gastric cancers (reviewed in Mariotto *et al.*, 2016). Throughout various tissues and cell types, a functional theme of HOPX regulating a balance between proliferation and differentiation is apparent. Our findings highlight further this role of HOPX and add skin to the list of tissues that are critically modulated by HOPX. Although abnormal HOPX expression has been characterized in various cutaneous pathologies, the underlying interactions and mechanisms involving HOPX are still unknown. In skin, a greater number of associational and functional studies on HOPX should be implemented to elucidate its role in normal and diseased states of the epidermis.

In the context of epidermal differentiation, past module mapping studies showed an approximately 40% frequency of correlation between HOPX mRNA expression and epidermal differentiation expression models (Lopez-Pajares *et al.*, 2013). Moreover, a study of the human psoriatic transcriptome mapped HOPX to gene co-expression modules that are highly expressed in epidermis and that in turn map to reported psoriasis susceptibility loci (Li *et al.*, 2014). Other major transcriptional regulators of epidermal differentiation, like ZNF750 and the downstream targets KLF4 and GRHL3 (Sen *et al.*, 2012; Zarnegar *et al.*, 2012)**,** also mapped to psoriasis

susceptibility loci. These data implicate a role of HOPX and other members of the p63-ZNF750 epidermal differentiation pathway in psoriasis.

Psoriasis is a chronic, autoimmune disease characterized by impacted immune function and red, scaly, and/or itchy plaques and lesions of the skin. At the cellular level, it is typified by altered keratinocyte differentiation and pathogenic inflammation that is driven by T cell activation and secretion of proinflammatory mediators (Lowes *et al.*, 2012). The IL-17 and IL-23 signaling pathways are two pathways that have been established in the pathogenesis of psoriasis (Martin *et al.*, 2013). Interleukin (IL)-17 is one major cytokine effector in the IL-17 signaling pathway that participates in the onset and perpetuation of this disease. Human psoriatic skin is characterized by increased IL-17 levels, and reconstructed epidermal models stimulated with IL-17 demonstrated a role of IL-17 upregulation in keratinocyte hyperproliferation, activation of inflammatory genes in terminally differentiated keratinocytes, and induction of genes that are activated by IL-17 *in vivo* (Martin *et al.*, 2013; Chiricozzi, 2014). While HOPX is associated with psoriasis susceptibility, no functional studies exist in this context. Therefore, uncovering potential interactions between HOPX and factors contributing to psoriatic pathogenesis, including IL-17 and other members of the IL-23/IL-17 signaling pathways, may help us further understand how HOPX is downregulated in this diseased state.

Mutations in ZNF750 have also been linked to autosomal dominant forms of psoriasis and disorders that histologically resemble psoriasis, collectively denoted psoriasiform dermatitis. A frameshift mutation resulting in a truncated, 43 amino acid long version of the ZNF750 was first identified in a multigeneration Jewish Moroccan family with seborrhea-like dermatosis. The skin disease was characterized by early onset with 100% penetrance, and patients presented psoriasiform elements including enhanced keratinocyte proliferation, skin inflammation, and

abnormal terminal epidermal differentiation (Birnbaum *et al.*, 2006). Additionally, a c.-625A>C mutation in the ZNF750 promoter region was identified in a multigeneration Chinese family presenting autosomal dominant psoriasis, which reduced ZNF750 promoter activity by 42% and was deemed the causal mutation for the genetic disease (Yang *et al.*, 2008). It would be of great interest to characterize the expression of HOPX and other regulatory targets downstream of these distinct ZNF750 mutations. Our findings highlight the clinical relevance of HOPX as a potential target in disorders like these, where differentiation is impaired. By clarifying the role of HOPX in skin, we may uncover new therapeutics for human skin diseases, like psoriasis and keratinocyte skin cancers, and improve our overall understanding of skin homeostasis.

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