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Esophageal squamous cell dysplasia and delayed differentiation with deletion of *Klf4* in murine esophagus

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Abstract

Background & Aims—*Klf4* (*Krippel*-like factor 4; GKLF) is a DNA-binding transcriptional regulator highly expressed in skin and gastrointestinal epithelia, specifically in regions of cellular differentiation. Homozygous null mice for *Klf4* die shortly after birth from skin defects, precluding their analysis at later stages. The aim of this study was to analyze the function of *Klf4* in keratinocyte biology and epithelial homeostasis in the adult by focusing on the squamous lined esophagus.

Methods—Using the *ED-L2* promoter of Epstein-Barr virus to drive *Cre*, we obtained tissue specific ablation of *Klf4* in the squamous epithelia of the tongue, esophagus, and forestomach.

Results—Mice with loss of *Klf4* in esophageal epithelia survived to adulthood, bypassing the early lethality. Tissue-specific *Klf4* knockout mice had increased basal cell proliferation and a delay in cellular maturation; these mice developed epithelial hypertrophy and subsequent dysplasia by 6 months of age. Moreover, loss of *Klf4* *in vivo* was associated with increased expression of the pro-proliferative *Klf5*, and *Klf4* downregulated *Klf5* both transcriptionally and post-transcriptionally. Using gene expression profiling, we also showed decreased expression of critical late-stage differentiation factors and identified alterations of several genes important in cellular differentiation.

Conclusions—*Klf4* is essential for squamous epithelial differentiation *in vivo* and interacts with *Klf5* to maintain normal epithelial homeostasis.

Keywords

Klf4; esophageal epithelium; differentiation; dysplasia

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Maintenance of normal esophageal epithelial homeostasis requires cell differentiation and desquamation at the luminal surface to be matched by cell proliferation^{1–5}. Esophageal keratinocyte proliferation normally occurs in the basal layer and cells differentiate as they migrate through the suprabasal and superficial layers. Dysregulation of the normal balance of proliferation and differentiation may underlie disorders such as gastroesophageal reflux disease, which affects up to 44% of the US population⁶ and esophageal cancer, the 6th most common cause of cancer death worldwide⁷. Moreover, the processes of keratinocyte differentiation in the esophagus have much in common with those in other squamous tissues, such as the interfollicular skin and the oropharynx, making the esophagus an outstanding model to understand the processes of squamous epithelial homeostasis and disease in general^{2–4, 8}.

Krüppel-like factors (KLFs) are DNA-binding transcriptional regulators which play critical roles in the control of proliferation, differentiation, development, and carcinogenesis in a number of tissues^{9, 10}. Among these, *Klf4* (previously known as GKLf or EZF) is highly expressed in differentiating cells of the gastrointestinal tract, including the suprabasal and superficial layers of the esophagus, as well as the skin¹¹. This distribution suggests that *Klf4* may function in the switch from proliferation to differentiation in stratified squamous epithelia. In vitro, *Klf4* overexpression inhibits proliferation and promotes differentiation of esophageal keratinocytes¹². Furthermore, *Klf4* transcriptionally activates *keratin 4*, a marker of keratinocyte differentiation^{13, 14}. *KLF4* is reportedly downregulated in a number of human epithelial cancers, including esophageal, colorectal, gastric, and bladder carcinomas^{15–18}. However, in other contexts, *KLF4* may promote tumorigenesis¹⁹.

Several animal studies have provided information about the role of *Klf4* in epithelial cells. Many of these studies have focused upon or been limited to the role of *Klf4* during embryogenesis, in part due to the early lethality of *Klf4* null mice, which die on postnatal day 1 from skin barrier defects²⁰. *Klf4* null mice have abnormal differentiation of skin and of goblet cells in the colon, confirming a function for *Klf4* in differentiation of a number of epithelial types^{20, 21}. In a contrasting experiment, ectopic expression of *Klf4* using a tetracycline inducible *keratin 5* promoter produces accelerated epidermal differentiation in the embryo²². To understand the role of *Klf4* later in life, we utilized the *Cre-loxP* system to obtain tissue-specific deletion of *Klf4* in the glandular epithelium of the stomach²³. Loss of *Klf4* in gastric glands results in a four-fold increase in cellular proliferation and alterations in the normal differentiation pathways of the stomach. Conditional deletion of *Klf4* in the eye also results in increased proliferation but with fewer epithelial layers, due to corneal fragility, and loss of conjunctival goblet cells²⁴. These studies highlight the similarities and differences of *Klf4* function in various types of epithelia.

In this study, we used the *ED-L2* promoter of the Epstein-Barr virus, previously shown to target expression to squamous epithelia of the tongue, esophagus and forestomach^{25, 26}, to produce tissue specific gene ablation of *Klf4*. This approach allowed us to investigate the role of *Klf4* in esophageal epithelial homeostasis while bypassing the early lethality of *Klf4* null mice, resulting from *Klf4* loss in the skin²⁰. Mice with loss of *Klf4* in esophagus developed epithelial hypertrophy, with increased proliferation, altered cell morphology, and evidence of delayed cellular maturation. Similar changes were seen with *Klf4* loss in tongue and with patchy deletion of *Klf4* in the skin of the ventral neck. *Klf4* conditional null mice also developed squamous cell dysplasia by 6 months of age. Thus, expression of *Klf4* is essential for normal squamous epithelial differentiation and homeostasis in vivo.

Methods

ED-L2-Cre/*Klf4*^{loxp/loxp} mice

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania. Mutant mice were homozygous for floxed *Klf4* and hemizygous for the *Cre* transgene. All mice used for the experiments were on a mixed genetic background. For all experiments with *ED-L2-Cre/Klf4*^{loxp/loxp} mice, sex-matched littermate *Klf4*^{loxp/loxp} mice lacking the *Cre* transgene served as controls. Additional details are provided in the supplementary methods.

Cell Culture and Treatment

The isolation and culture of mouse primary esophageal keratinocytes have been described elsewhere²⁷. Cells were cultured and infected or transfected and reporter assays or quantitation were performed as described in the supplementary methods.

Immunohistochemistry/Immunofluorescence/Western blots

Immunohistochemistry, immunofluorescence, and Western blots were performed using standard protocols. For descriptions of the protocols and antibodies used, see the supplementary methods.

RNA analyses

RNA was extracted from esophagi or primary esophageal keratinocytes using the RNeasy Mini Kit (Qiagen, Valencia, CA) following manufacturer's instructions. Reverse transcription was performed with random hexamers and SuperScript II Reverse Transcriptase (Invitrogen). Quantitative real-time PCR (qPCR) analysis was performed in triplicate using an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) and SyBr Green PCR master mix (Applied Biosystems). The TATA box binding protein gene (TBP) was used as the internal control. We used GeneChip Mouse Expression Arrays MOE430A v2 (Affymetrix, Santa Clara, California) for microarray analyses and identified differentially expressed genes using a fold change cutoff of greater than or equal to 2.0 and a false discovery rate of 5%, as described¹². Microarray results have been deposited in GEO under the accession number GSE17447.

Transmission Electron Microscopy

Ultrathin (~80nm) sections of the trans-sectional plane of each esophagus were examined with a FEI Tecnai-T12 transmission electron microscope operated at 80kv. Digital images were captured with a Gatan camera at a resolution of x2048. Additional details are provided in the supplementary methods.

Results

To generate tissue-specific recombination in esophagus, we utilized the EBV *ED-L2* promoter, which targets expression specifically to the tongue, esophagus, and forestomach, but not the skin^{25, 26}. Using *Gt(ROSA)26*^{tm1Sor} reporter mice²⁸, we demonstrated that *ED-L2/Cre* directed Cre-mediated recombination in esophageal epithelia. While no β -galactosidase expression was seen in esophageal tissues of mice containing *Gt(ROSA)26*^{tm1Sor} without the *ED-L2/Cre* transgene (Fig. 1A), *ED-L2/Cre;Gt(ROSA)26*^{tm1Sor} littermates (Fig. 1B) had staining throughout the epithelia. Staining patterns were similar in proximal and distal esophagus. Expression was mosaic in the epithelia of the tongue and forestomach (not shown), and Cre-mediated recombination was demonstrated in two independent lines.

To produce deletion of *Klf4* in esophageal epithelia of adult mice, we crossed the *ED-L2/Cre* transgenic mice with *Klf4^{loxP/loxP}* mice²³. We documented *Klf4* gene ablation by PCR (data not shown) and demonstrated the targeted deletion of *Klf4* by performing immunohistochemistry. Staining for Klf4 revealed expression in the skin of the back of both littermate control (Fig. 1C) and *ED-L2/Cre;Klf4^{loxP/loxP}* mice (Fig. 1D). While staining was seen in esophageal suprabasal cells in control mice (Fig. 1E), Klf4 expression was absent in esophageal epithelia of *ED-L2/Cre;Klf4^{loxP/loxP}* mice (Fig. 1F), confirming Cre-mediated deletion of *Klf4* in the esophagus.

ED-L2/Cre;Klf4^{loxP/loxP} mice were born at appropriate Mendelian ratios and survived into adulthood. Grossly, the esophagi and other organs appeared normal, with the exception of the skin of the face and ventral neck, as detailed below. Heterozygous mice had no overt phenotype. Up to one month of age, esophageal epithelia of *ED-L2/Cre;Klf4^{loxP/loxP}* mice appeared histologically normal. However, compared to littermate controls (Fig. 2A–B), esophageal epithelia of 1 month-old *ED-L2/Cre;Klf4^{loxP/loxP}* mice (Fig. 2C–D) had evidence of hypertrophy with altered keratinocyte morphology. This phenotype was more evident in *ED-L2/Cre;Klf4^{loxP/loxP}* mice at 3 months of age (Fig. 2G–H), compared to littermate controls (Fig. 2E–F). Changes were even more dramatic at 6 months of age. Compared to littermate controls (Fig. 2I–J), esophageal epithelia of 6 month-old *ED-L2/Cre;Klf4^{loxP/loxP}* mice (Fig. 2K–L) showed phenotypes ranging from hyperplasia to moderate dysplasia, with loss of surface keratinization, and an apparent delay in keratinocyte maturation. No evidence of invasion was seen in the *ED-L2/Cre;Klf4^{loxP/loxP}* mice, although there were many epithelial buds pushing into the lamina propria.

Even though *Klf4* was not generally deleted in skin, some patchy deletion occurred most predominantly on the skin of the face and ventral neck (Fig. S1). While this was not sufficient to cause lethality, allowing us to study the effects of *Klf4* loss on esophagus in adults, these lesions in the skin generally necessitated euthanasia of *ED-L2/Cre;Klf4^{loxP/loxP}* mice by 6 months of age for humane reasons. We observed crusted skin lesions and moistness of the skin of the ventral neck starting at 3 months of age. No lesions were observed in littermate controls. To rigorously investigate the effect of *Klf4* in other squamous epithelia, the histology of tongue, forestomach, and skin was analyzed (Fig. S2), and the pattern of *Klf4* deletion in these tissues was confirmed by immunohistochemistry (Fig. S3). Evidence of hypertrophy was also seen in the tongue and the skin on the ventral neck, where *Klf4* was deleted in *ED-L2/Cre;Klf4^{loxP/loxP}* mice. No differences in histology were seen in the skin of the back, where *Klf4* was not deleted. In addition, no significant changes were observed in the forestomach of *ED-L2/Cre;Klf4^{loxP/loxP}* mice, likely due to incomplete *Klf4* deletion in this tissue.

As the phenotype was well-established by 3 months of age and mice were euthanized by 6 months of age, we chose to focus on the 3 and 6 month time points for further experiments. To examine the effects of *Klf4* deletion at the ultrastructural level, we performed transmission electron microscopy on esophageal epithelia from 3 month-old mice. In littermate controls (Fig. 3A), cells progressing from the basal to suprabasal layers reoriented and underwent stratification. In contrast, the initial layers of suprabasal cells in *ED-L2/Cre;Klf4^{loxP/loxP}* mice (Fig. 3B) did not undergo stratification, with these cells instead maintaining a longitudinal orientation. Basal cells of *ED-L2/Cre;Klf4^{loxP/loxP}* mice also demonstrated nuclear distortion, suggesting an expansion of proliferative cells, and superficial cells had increased nuclear to cytoplasmic ratios (Fig. 3C–D; Fig. S4) compared to controls, suggesting incomplete differentiation.

To analyze whether these apparent defects of cell maturation in *Klf4* conditional null mice resulted in increased proliferation, we pulse-labeled cells in S-phase with 5-bromo-2-

deoxyuridine (BrdU) and determined the proliferative index by counting the number of BrdU-labeled cells per 100 basal cells. Compared to controls (Fig. 4A), *ED-L2/Cre;Klf4^{loxP/loxP}* mice (Fig. 4B) showed an increased number of proliferating cells, with changes in proliferation confined to the basal layer. A statistically significant 1.9-fold increase (Fig. 4C) in proliferating cells was seen in *ED-L2/Cre;Klf4^{loxP/loxP}* mice at 3 months of age, compared to littermate controls ($p=0.006$). We next investigated whether *ED-L2/Cre;Klf4^{loxP/loxP}* mice had altered apoptosis in esophageal epithelia by TUNEL staining. Compared to littermate controls (Fig. 4D), which had only rare apoptotic cells in esophageal epithelia, *ED-L2/Cre;Klf4^{loxP/loxP}* mice had increased apoptosis at 3 months of age (Fig. 4E). This may represent an attempt to balance hyperproliferation and apoptosis to maintain homeostasis, as has been described in other epithelia including skin²⁹. By counting the number of TUNEL positive cells per section (Fig. 4F), we confirmed a 2.7-fold increase in apoptosis in *ED-L2/Cre;Klf4^{loxP/loxP}* mice compared to controls ($p=0.001$). Nonetheless, this increased apoptosis was insufficient to prevent the progressive epithelial hypertrophy seen in *ED-L2/Cre;Klf4^{loxP/loxP}* mice.

To characterize changes in the proliferation-differentiation equilibrium in *ED-L2/Cre;Klf4^{loxP/loxP}* mice, we stained 3 month-old mice for keratin 14, which normally marks undifferentiated cells in the basal layer, and keratins 4 and 13, markers of keratinocyte differentiation³⁰. While keratin 14 was localized to the esophageal basal layer of control mice (Fig. 5A), the region of keratin 14 staining was expanded to include at least the first several layers of suprabasal cells in *ED-L2/Cre;Klf4^{loxP/loxP}* mice (Fig. 5B), consistent with delayed differentiation of keratinocytes in the suprabasal layer. In control mice, keratin 13 (Fig. 5C) and keratin 4 (Fig. 5E) were found in band-like patterns throughout the suprabasal and superficial layers. In contrast, *ED-L2/Cre;Klf4^{loxP/loxP}* mice had a marked shift in keratin 13 expression towards the lumen with a loss of the normal band-like pattern (Fig. 5D) and nearly complete extinction of keratin 4 expression in the suprabasal layer (Fig. 5F), further indicating a delay in keratinocyte maturation with *Klf4* loss.

The closely related KLF family member *Klf5* binds to the same cis-regulatory elements as *Klf4* and has been shown to have pro-proliferative properties in a number of contexts¹¹. Moreover, transgenic expression of *Klf5* in esophagus results in increased basal cell proliferation¹². However, while *Klf5* has been shown to transcriptionally regulate *Klf4* *in vitro*³¹, similar regulation of *Klf5* by *Klf4* has not previously been reported. To evaluate whether *Klf5* might contribute to the phenotype of *ED-L2/Cre;Klf4^{loxP/loxP}* mice, we examined the expression of *Klf5* in esophageal epithelia. As shown in Figure 6A, nuclear *Klf5* staining was seen only in basal cells in esophagus of 3 month-old control mice but was found in both basal and suprabasal layers of *ED-L2/Cre;Klf4^{loxP/loxP}* mice (Fig. 6B). Consistent with this, *Klf5* mRNA levels in esophageal epithelial cells isolated from *ED-L2/Cre;Klf4^{loxP/loxP}* mice were increased by 50% compared to control mice (Fig. 6C). To determine whether *Klf5* was a direct transcriptional target for *Klf4*, we examined the 5' regulatory region of *Klf5* for putative *Klf4*-binding sites, using the computational program TESS³² and identified a putative *Klf4* binding site ~600bp from the translation start site. By ChIP assays in mouse primary esophageal keratinocytes, we demonstrated binding of *Klf4* to *Klf5* between -601 to -419 (Fig. 6D). To confirm that *Klf4* functionally inhibited *Klf5*, we transfected mouse primary esophageal keratinocytes infected with pBabe-puro control or pBabe-*Klf4* with a luciferase reporter containing the 1 kb region immediately upstream of the *Klf5* translational start site. Expression of *Klf4* by pBabe-*Klf4* resulted in a 53% decrease in activity (Fig. 6E). Moreover, *Klf4* overexpression resulted in a similar suppression of *Klf5* mRNA (Fig. 6F), confirming that *Klf5* is transcriptionally repressed by *Klf4*. At the protein level, *Klf5* downregulation by *Klf4* was even more dramatic (Fig. 6F), suggesting that *Klf4* may also exert effects on post-transcriptional regulation of *Klf5*.

To identify other potential *Klf4* targets in esophageal epithelium, we isolated esophageal epithelial cells from control and *ED-L2/Cre;Klf4^{loxP/loxP}* mice at 3 months of age and performed gene expression profiling. We identified a total of 121 genes which were differentially regulated more than two-fold in esophageal epithelia of *ED-L2/Cre;Klf4^{loxP/loxP}* mice compared to littermate controls (Table S1). Many of these genes are involved in cornified cell envelope formation, keratinization, and keratinocyte differentiation. Among the most highly upregulated genes in esophageal epithelia of the *ED-L2/Cre;Klf4^{loxP/loxP}* mice were those encoding the small proline-rich proteins (*Sprr2d*, *Sprr2f*, *Sprr2g*, *Sprr2h*, *Sprr2i*, *Sprr2j*), which serve as precursors of the cornified cell envelope³³. Consistent with the findings of our microarray analyses, increased expression of *Sprr2d*, *Sprr2f*, *Sprr2g*, *Sprr2h*, *Sprr2i*, *Sprr2j* was reported in the skin of neonatal *Klf4*-null mice³³. Interestingly, decreased expression of late-stage differentiation markers, such as *envoplakin*, *Klk5*, and *late cornified envelope 1A1*, *1A2*, *1B*, and *1D* was also observed in esophageal epithelia of the *ED-L2/Cre;Klf4^{loxP/loxP}* mice.

From the total number of differentially expressed genes, we selected 16 genes for validation by qPCR. These genes were chosen based upon their likely roles in keratinocyte differentiation, implication in clinically relevant diseases involving squamous epithelia, and/or large fold-changes in *ED-L2/Cre;Klf4^{loxP/loxP}* mice versus controls. Of the 16 genes tested, 14 had statistically significant changes confirmed by qPCR which were in the same direction as the microarray results (Table 1). The relevance and importance of a number of these genes are outlined in the discussion below. One additional gene, *Ephb6*, was similarly decreased by qPCR but did not reach statistical significance ($p=0.09$). The final gene, *Lthp1*, showed no change on qPCR and was likely a false-positive on microarray. Thus, the qPCR data confirmed the validity of the gene expression profiling.

Given the importance of the Wnt pathway in controlling cellular homeostasis, the reported link between *Klf4* and β -catenin³⁴, and the identification of several potentially relevant genes by gene expression profiling, we examined whether nuclear localization of β -catenin was altered in *ED-L2/Cre;Klf4^{loxP/loxP}* mice versus controls. Unlike in the intestine, *Klf4* does not appear to play a role in canonical Wnt signaling, based on the lack of changes in nuclear β -catenin (Figure S5). However, loss of *Klf4* does appear to alter both β -catenin levels and localization. This finding may be more important with respect to the role of β -catenin as a structural protein at the adherens junctions³⁵. Moreover, we cannot exclude an effect of *Klf4* on non-canonical Wnt signaling. Of note, Wnt5a (see Table 1) is involved in non-canonical Wnt signaling and has been implicated in tumor progression, although the evidence as to whether Wnt5a has a tumor-promoting or tumor-suppressing role is conflicting³⁶.

Discussion

Under normal conditions, the adult tissue architecture is maintained by a careful balance between the rates of cell production and loss, which is in turn regulated by distinct patterns of transcriptional control. Diseases of the esophagus are among the leading causes of morbidity and mortality in the United States and throughout the world^{37, 38}, and in the esophagus and other tissues, disruption of the factors governing squamous epithelial homeostasis may lead to uncontrolled cell proliferation, inappropriate injury responses, dysplasia, and/or cancer⁵. While a number of important pathways have been identified for the development and differentiation of the skin⁴, many details remain to be elucidated, and less is known about the regulatory pathways in the squamous epithelia of the esophagus.

In this study, we examine the role of the transcriptional regulator *Klf4* in esophageal epithelial homeostasis. By utilizing the *ED-L2* promoter to delete *Klf4*, we bypass the early

lethality of *Klf4* null mice²⁰ and are uniquely qualified to evaluate the function of *Klf4* in esophageal epithelial homeostasis in the adult. Loss of *Klf4* in squamous epithelia of the esophagus, as well as tongue and skin of the ventral neck, results in hypertrophy, hyperplasia, defects in differentiation, and dysplasia, all of which occur in the absence of any significant inflammatory response. Previously, transgenic expression of human *KLF4* was reported to induce squamous epithelial dysplasia in the skin³⁹. Our findings do not exclude that *Klf4*, as a critical mediator of the proliferation-differentiation equilibrium, must be carefully titrated and that both too much or too little can result in dysregulation and dysplasia.

Klf4 is down-regulated in esophageal squamous cell carcinoma and in a number of esophageal cancer cell lines^{16, 17, 40}. These observations suggest that *Klf4* may contribute to the initiation and/or progression of esophageal cancer. Unfortunately, due to patchy deletion of *Klf4* in the skin of the ventral neck of *ED-L2/Cre;Klf4^{loxP/loxP}* mice, leading to development of severe skin lesions, we have not been able to age the conditional *Klf4* knockout mice beyond 6 months. Therefore, the functional role of *Klf4* in esophageal carcinogenesis cannot be fully addressed in this study. Our experiments were performed on mice of a mixed genetic background. We are currently crossing *Klf4^{Loxp/Loxp}* mice and *ED-L2/Cre* mice to different inbred strains in an attempt to minimize the effects of this patchy deletion of *Klf4* in the skin and to allow for a more systematic evaluation of the consequence of loss of *Klf4* in the esophagus.

Esophageal epithelia of *ED-L2/Cre;Klf4^{loxP/loxP}* mice have a 1.9-fold increase in proliferation, and we have identified the pro-proliferative *Klf5* as a novel target for *Klf4* *in vitro* and *in vivo*. *Klf5* has been shown to regulate esophageal epithelial proliferation *in vitro* and *in vivo*^{12, 27}, and mice with transgenic expression of *Klf5* in esophagus, like *ED-L2/Cre;Klf4^{loxP/loxP}* mice, have increased proliferation confined to the basal layer of the esophagus. We believe that this is due to the normal repression of *Klf5* by *Klf4* in transit amplifying cells as they exit the esophageal basal layer. Moreover, *Klf4* may compete with *Klf5* for binding to target genes^{31, 41}. Nonetheless, transgenic expression of *Klf5* alone does not alter epithelial differentiation¹², indicating that *Klf4* drives differentiation through other mechanisms. Also of note, the effect *Klf4* on *Klf5* is directly relevant to normal homeostasis but not necessarily to carcinogenesis since *Klf5* may play a different role in cancer than in normal epithelia^{40, 42}. Overall, we suggest that when *Klf4* expression is turned on in the suprabasal layer of the esophagus, *Klf4* represses *Klf5* both transcriptionally and post-transcriptionally and at the same time competes with *Klf5* for binding to the promoters of other key regulatory genes, thus switching cells from the proliferation to differentiation program (Fig. 6H).

Loss of *Klf4* also results in a 2.7-fold increase in apoptosis. While we cannot exclude a direct effect of *Klf4* on apoptosis, these findings are typical of efforts to maintain homeostasis in rapidly renewing epithelia, such as esophagus, intestine, and skin^{2, 29, 43}. Although these numerical changes might seem to suggest that the increase in proliferation is balanced by changes in apoptosis, proliferation is much more common in the esophagus than apoptosis, such that a nearly 3-fold increase in apoptosis does not offset the effects of a 2-fold increase in proliferation. Nonetheless, we cannot exclude that loss of *Klf4* *in vivo* alters the rate of cell migration and that this may also influence the development of epithelial hypertrophy and hyperplasia. Of note, *Klf5* has been shown to promote esophageal epithelial cell migration *in vitro*⁴⁴. Thus, we are currently investigating the role of *Klf4* in cell migration.

Other potential *Klf4* targets identified through our gene expression profiling may also be critical for *Klf4* function in squamous epithelia. For example, envoplakin is a cytoskeletal

linker protein and a component of the epidermal cornified envelope⁴⁵. Epidermis of *envoplakin* null mice has a higher proportion of immature cornified envelopes than that of control mice. Similarly, Lce1A1, Lce1A2, Lce1B, and Lce1D are major cornified envelope components induced during keratinocyte terminal differentiation⁴⁶. Also, among the genes that were differentially expressed is *Slurp1*, encoding a secreted member of the LY6/PLAUR family of proteins⁴⁷, which was decreased between 7- to 9-fold in *ED-L2/Cre;Klf4^{loxP/loxP}* mice. SLURP1 is a marker of late keratinocyte differentiation expressed in the granular layer of the skin⁴⁷ and, as a key ligand for the $\alpha 7$ nicotinic acetylcholine receptor⁴⁸, is important for terminal differentiation of epidermal keratinocytes, for homeostasis, and for the formation of the skin barrier⁴⁹. Moreover, mutations in *SLURP1* are the cause of Mal de Meleda⁵⁰, a rare autosomal recessive disease characterized by transgressive palmoplantar keratoderma or hyperkeratosis of the palms and soles⁵¹. Of note, tylosis, or focal non-epidermolytic palmoplantar keratoderma, is strongly associated with esophageal squamous cell cancer⁵². Interestingly, *envoplakin*, has been mapped to the tylosis esophageal cancer (TOC) locus, a region on chromosome 17q25 commonly deleted in sporadic esophageal cancers⁵³. While these implications are intriguing, the roles of these genes in esophageal epithelial homeostasis, hyperproliferation, and carcinogenesis remain to be elucidated, and investigations of expression and function of these factors in esophageal epithelial cells are currently underway.

In conclusion, using a novel conditional knockout mouse model for *Klf4*, we demonstrate that *Klf4* is required for normal homeostasis in tongue, esophagus, and skin. In the esophagus specifically, loss of *Klf4* produces epithelial hypertrophy, increased proliferation, altered cell morphology with evidence of delayed cellular maturation, and eventually esophageal epithelial dysplasia by six months of age. Thus, we show here that *Klf4* is necessary for differentiation and maintenance of normal homeostasis in esophageal epithelia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BrdU	5-bromo-2-deoxyuridine
Klf4	<i>Krüppel</i> -like factor 4
Klf5	<i>Krüppel</i> -like factor 5
qPCR	quantitative real-time PCR
TBP	TATA box binding protein gene
TOC	tylosis esophageal cancer

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Fig. 1. *Klf4* was deleted successfully in esophageal epithelia of *ED-L2/Cre;Klf4^{loxP/loxP}* mice. (A–B) Esophageal sections from *Gt(ROSA)26^{tm1Sor}* control mice (A) showed no epithelial staining for β -galactosidase, while *ED-L2/Cre;Gt(ROSA)26^{tm1Sor}* mice (B) had staining throughout the epithelia. (C–D) Both control (C) and *ED-L2/Cre;Klf4^{loxP/loxP}* mice (D) demonstrated nuclear staining for *Klf4* in the skin. (E–F) In esophageal epithelia, control mice (E) had nuclear *Klf4* expression in cells of the suprabasal layer while *Klf4* expression was lost in *ED-L2/Cre;Klf4^{loxP/loxP}* mice (F). Scale bars (A–F), 10 μ m.

Fig. 2. *ED-L2/Cre;Klf4^{loxP/loxP}* mutant mice had altered esophageal epithelial homeostasis. Compared to 1 month-old (A–B) and 3 month-old littermate controls (E–F), H&E stained esophageal epithelia from *ED-L2/Cre;Klf4^{loxP/loxP}* mice were hypertrophic and keratinocytes showed altered morphology (C–D, G–H). The basal layers at these time points also appeared thickened and irregular. By 6 months of age, compared to littermate controls (I–J), *ED-L2/Cre;Klf4^{loxP/loxP}* mice had evidence of delayed keratinocyte maturation and showed moderate dysplasia (K–L). Note the budding of epithelia into the lamina propria and the lack of surface keratinization in *ED-L2/Cre;Klf4^{loxP/loxP}* mice. Scale bars (A, C, E, G, I, K), 50 μ m; (B, D, F, H, J, L), 10 μ m.

Fig. 3.

Ultrastructural abnormalities in *Klf4*-deficient esophageal epithelia from 3 month-old mice. Assessment of ultrastructure of littermate controls (A, C) and *ED-L2/Cre;Klf4^{loxP/loxP}* mutant mice (B, D). (A–B) Compared to controls (A), esophageal epithelia of *ED-L2/Cre;Klf4^{loxP/loxP}* mice (B) demonstrated changes in the orientation of cells as they progressed from the basal to suprabasal layers. (C–D) In the superficial layer, compared to controls (C), cells from *ED-L2/Cre;Klf4^{loxP/loxP}* mice (D) had increased nuclear to cytoplasmic ratios. Typically, cells in this layer have small compacted nuclei. Scale bars (A–D), 2 μ m.

Fig. 4. *ED-L2/Cre;Klf4^{loxP/loxP}* mice had increased proliferation and apoptosis in esophageal epithelia. (A–B) At 3 months of age, proliferating cells were confined to the basal layer in both control (A) and *ED-L2/Cre;Klf4^{loxP/loxP}* mice (B). (C) Quantitation of BrdU-labeled cells revealed a statistically significant 1.9-fold increase in cell proliferation in *ED-L2/Cre;Klf4^{loxP/loxP}* mice ($p=0.006$). (D–E) Compared to littermate controls (D), TUNEL staining of esophageal epithelia from *ED-L2/Cre;Klf4^{loxP/loxP}* mice (E) demonstrated increased numbers of apoptotic cells in the superficial layers at 3 months of age. (F) By quantitation of TUNEL positive cells, apoptosis was increased 2.7-fold in *ED-L2/Cre;Klf4^{loxP/loxP}* mice ($p=0.001$). Scale bars (A–B, D–E), 50 μ m.

Fig. 5. *Klf4* deletion altered esophageal keratinocyte differentiation. (A–B) Staining for keratin 14 (red), which is normally confined to cells in the basal layer, as in controls (A), revealed expansion of the expression domain to include cells within the first few layers of suprabasal cells in *ED-L2/Cre;Klf4^{loxP/loxP}* mice (B). (C–D) Compared to controls (C), expression of keratin 13, a marker of keratinocyte differentiation, was decreased and shifted toward the luminal surface in *ED-L2/Cre;Klf4^{loxP/loxP}* mice (D). (E–F) Keratin 4, another keratinocyte differentiation marker, was strongly expressed in the suprabasal layers of controls (E), but expression was markedly decreased in *ED-L2/Cre;Klf4^{loxP/loxP}* mice (F). DAPI (blue) was used as a nuclear counterstain for immunofluorescence (A–B, E–F). Scale bars, 10 μ m.

Fig. 6.

Klf4 inhibited expression of pro-proliferative Klf5. (A) Klf5 staining was seen in nuclei of cells only in the basal layer of the esophagus. (B) In contrast, *ED-L2/Cre;Klf4^{loxP/loxP}* mice showed expression of Klf5 in both basal and suprabasal cells. (C) By qPCR, *Klf5* expression was increased by 54% in esophageal epithelia of *ED-L2/Cre;Klf4^{loxP/loxP}* mice ($p=0.01$). (D) ChIP assays of mouse primary esophageal keratinocytes demonstrated Klf4 binding to the 5' regulatory region of *Klf5* between -601 and -419. Cells were also treated with calcium to induce differentiation. Input was DNA extracted before immunoprecipitation. Anti-IgG antibody was used as a negative control. (E) Luciferase reporter assays with the 1 kb region immediately upstream of the *Klf5* translational start site revealed a 53% decrease in activity ($p<0.05$) when cells were co-transfected with a *Klf4* expression vector, compared to control. (F) Overexpression of *Klf4* in mouse primary esophageal keratinocytes resulted in a 45% decrease in *Klf5* mRNA by qPCR ($p<1\times10^{-9}$). (G) Western blotting and subsequent quantitation revealed a 98% decrease in Klf5 protein when Klf4 was overexpressed by 7-fold in these cells. A model for Klf4 and Klf5 in the switch from proliferation to differentiation in esophageal epithelia is shown in (H).

Table 1

Differentially regulated genes on microarray confirmed by qPCR

Gene name	Fold change by microarray*	Fold change by qPCR*	p-value
<i>Msln</i>	3.2	20.4	0.0002
<i>Ctsc</i>	2.2	2.3	0.0003
<i>Evpl</i>	-2.0	-3.6	0.008
<i>Lce1d</i>	-2.0	-3.6	0.0004
<i>Timp2</i>	-2.1	-1.7	0.01
<i>Tcf23</i>	-2.2	-4.3	0.0005
<i>Lce1a2</i>	-2.2	-3.6	0.0002
<i>Lce1a1</i>	-2.2	-4.0	0.002
<i>Igfbp6</i>	-2.6	-2.4	0.0002
<i>Lce1b</i>	-2.9	-4.8	0.0003
<i>Wnt5a</i>	-3.0	-2.9	0.0001
<i>Dkk1l</i>	-5.0	-2.1	0.005
<i>Klk5</i>	-5.0	-5.6	0.0005
<i>Slurp1</i>	-9.0	-7.1	0.0004

* *ED-L2/Cre;Klf4^{loxP/loxP}* mice compared to littermate controls