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ACTL6a Enforces the Epidermal Progenitor State by Suppressing SWI/SNF-Dependent Induction of KLF4

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SUMMARY

Somatic progenitors suppress differentiation to maintain tissue self-renewal. The mammalian SWI/SNF chromatin-remodeling complex regulates nucleosome packaging to control differentiation in embryonic and adult stem cells. Catalytic Brg1 and Brm subunits are required for these processes; however, the roles of SWI/SNF regulatory subunits are not fully understood. Here, we show that ACTL6a/BAF53A modulates the SWI/SNF complex to suppress differentiation in epidermis. Conditional loss of ACTL6a resulted in terminal differentiation, cell-cycle exit, and hypoplasia, whereas ectopic expression of ACTL6a promoted the progenitor state. A significant portion of genes regulated by ACTL6a were found to also be targets of KLF4, a known activator of epidermal differentiation. Mechanistically, we show that ACTL6a prevents SWI/SNF complex binding to promoters of KLF4 and other differentiation genes and that SWI/SNF catalytic subunits are required for full induction of KLF4 targets. Thus, ACTL6a controls the epidermal progenitor state by sequestering SWI/SNF to prevent activation of differentiation programs.

INTRODUCTION

Homeostasis of somatic tissues requires a sustainable pool of progenitor cells that can both proliferate to replenish themselves and enter a differentiation pathway to enable their progeny to perform specified tissue functions, such as barrier formation in epidermis. Maintenance of this progenitor state requires tight suppression of differentiation genes, because their premature expression can abolish proliferative capacity and trigger cell death (Melino et al., 1994). In the case of the epidermis, a stratified epithelial tissue in which basement-membrane-adherent progenitors migrate outward to undergo terminal differentiation, several epigenetic regulators have recently been found to contribute to differentiation gene repression in progenitors. Among these are DNA methyltransferase 1 (DNMT1) (Sen et al., 2010); histone methylation regulators that include JMJD3, Setd8, CBX4, Jarid2, and Polycomb proteins (Driskell et al., 2012; Ezhkova et al., 2009; Luis et al., 2011; Mejetta et al., 2011; Sen et al., 2008); and regulators of histone acetylation, HDAC1, HDAC2, and Sin3a (LeBoeuf et al., 2010; Nascimento et al., 2011; Reyes et al., 1998). These studies point to a role for epigenetic regulators from multiple classes in the repression of differentiation gene induction within somatic progenitor cells.

Among classes of epigenetic regulators, chromatin remodeling complexes are commonly composed of a catalytic adenosine triphosphatase (ATPase) subunit, which utilizes the energy from ATP to move or eject DNA-bound nucleosomes, along with regulatory subunits that modulate the conformation and activity of the entire complex. The mammalian SWI/SNF chromatin-remodeling complex is composed of 11 subunits encoded by 20 genes (Wu et al., 2009). Its catalytic ATPase subunit is either Brg1 or Brm. Brg1 is required during embryonic development, whereas Brm seems to be dispensable (Bultman et al., 2000; Reyes et al., 1998). Compared to Brg1 and Brm, there are fewer data on the function of the 18 genes that encode the regulatory subunits of the SWI/SNF complex. It is notable, however, that mice with loss of either BAF250a or BAF155 display an even more severe phenotype than that of Brg1 knockout (KO) mice (Gao et al., 2008; Kim et al., 2001), indicating that these regulatory elements may mediate critical biological functions.

In addition to classical epigenetic regulators, recent studies reveal that many proteins long considered components of the cytoskeleton can actually impact transcription (Grummt, 2006). Various types of lamins directly interact with chromatin and organize the nuclear landscape (Dechat et al., 2008). Nuclear myosin associates with RNA polymerases I and II (Vreugde et al., 2006; Ye et al., 2008), whereas nuclear actin copurifies with all three known RNA polymerases and multiple epigenetic regulating complexes (Visa and Percipalle, 2010). In addition, multiple actin-like genes, conserved from yeast to human, associate with different epigenetic regulators. It remains unclear whether these actin-like genes are required in the gene-regulatory control of progenitor differentiation.

In a search for epigenetic repressors of differentiation that are required for epidermal progenitor maintenance, we identified ACTL6a (actin-like 6a), a protein also known as BAF53a/INO80K/Arp4. Here, we show that ACTL6a expression is significantly downregulated during epidermal differentiation. Conditional deletion of *ACTL6A* in mouse epidermis abolished epidermal progenitor function, leading to cell-cycle exit, terminal differentiation, and ultimately hypoplasia, followed by tissue loss. In the human context, ACTL6a depletion exerts similar effects, decreasing progenitor clonogenicity and inducing

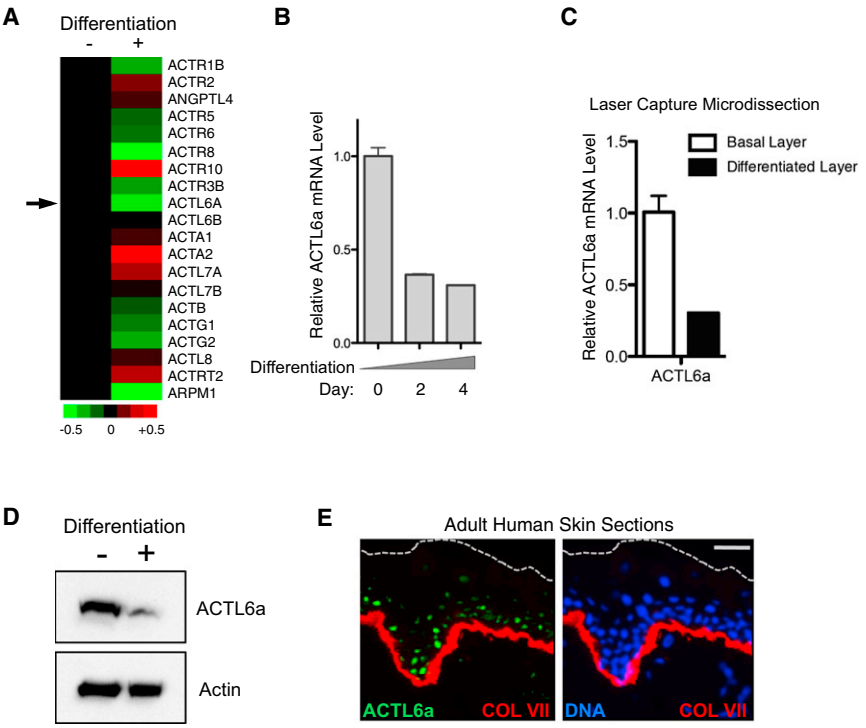

Figure 1. ACTL6a Is Downregulated during Epidermal Differentiation

(A) Heat map representing mRNA profiling analysis of all known actin-like genes comparing their expression between undifferentiated (−) and calcium-differentiated (+) human keratinocytes in vitro.

(B) ACTL6a mRNA downregulation during keratinocyte differentiation in vitro ($p < 0.001$, ANOVA). Bars represent mean ± SD.

(C) ACTL6a mRNA levels from human epidermal laser-capture microdissection of the undifferentiated progenitor-containing basal layer and differentiating suprabasal layers ($p < 0.05$, t test). Bars represent mean ± SD.

(D) ACTL6a protein downregulation during keratinocyte differentiation in vitro.

(E) ACTL6a protein localizes primarily to less differentiated layers in human epidermal tissue in vivo (ACTL6a, green; collagen (COL) VII basement-membrane marker, red; nuclear stain with Hoechst 33342, blue; scale bar represents 50 μm; dotted line indicates epidermal tissue upper boundary). See also Table S2.

ectopic expression of differentiation genes, whereas enforced ACTL6a expression suppressed differentiation. ACTL6a target-gene characterization identified KLF4 (Krüppel-like factor 4) as a key target of ACTL6a repression. KLF4 loss significantly compensated for the defects caused by ACTL6a depletion in progenitors. Of the three epigenetic regulatory complexes well characterized as containing ACTL6a, namely the TIP60 and KAT2a histone acetyltransferase (HAT) complexes and the SWI/SNF chromatin-remodeling complex, only depletion of the ARID1A/BAF250a member of the Brg1- or Brm-containing SWI/SNF complex recapitulated ACTL6a effects. Consistent with a role for ACTL6a in regulating SWI/SNF impacts on progenitor gene regulation, ACTL6a was necessary for impairment of Brg1 or Brm binding to differentiation-gene promoters. These data suggest that ACTL6a maintains the undifferentiated progenitor state by opposing SWI/SNF-enabled activation of KLF4 and other epidermal differentiation genes.

RESULTS

ACTL6a Is Downregulated during Epidermal Tissue Differentiation

Analysis of actin-like gene expression during calcium-induced epidermal keratinocyte differentiation in vitro identified ACTL6a as one of the most downregulated actin-like genes (Figures 1A–1C; Table S2 available online). For examining ACTL6a messenger RNA (mRNA) expression within intact epidermal tissue, laser-capture microdissection was used for the separation of the undifferentiated progenitor basal epidermal layer from the suprabasal differentiating layers. In agreement with in vitro findings, ACTL6a mRNA levels were decreased in differentiated layers relative to the basal layer (Figure 1C). This mRNA downregulation was reflected in ACTL6a protein levels as well, which also decreased during differentiation (Figure 1D). Within intact tissue, ACTL6a protein was likewise most strongly expressed in less differentiated cells adjacent to the epidermal basement membrane (Figure 1E). ACTL6a expression is thus downregulated in epidermal differentiation.

Conditional Epidermal ACTL6a Deletion Ablates Progenitor Function

The downregulation of ACTL6a during epidermal differentiation suggested the possibility that ACTL6a may impact this process. To explore this, we undertook targeted *ACTL6A* deletion in mouse epidermis using Cre recombinase driven by the epidermal basal layer keratin 14 (K14) promoter (Huelsken et al., 2001). We first undertook conditional *ACTL6A* deletion in adult mouse epidermis using a K14-driven tamoxifen-inducible Cre-estrogen receptor (Cre-ER) ligand domain fusion (Vasioukhin et al., 1999) (Figure S1A). Cre activation via topical tamoxifen application to a small region of mouse back skin was followed clinically by scaling, then thinning, ultimately leading to erosion and loss of epidermis by day 16 (Figure 2A); this loss of surface epithelium was also visible in the perioral area, where mice lick the topical agent (Figure S1B). Over this time period, ACTL6a-deleted tissue underwent progressive epidermal hypoplasia (Figures 2B and 2C; Figure S1C) prior to a complete tissue failure. In concert with this, loss of ACTL6a was associated with induction of both early (keratin 1) and late (loricrin) differentiation-protein expression in the basement-membrane proximal basal progenitor layer, where such proteins are normally never expressed (Figure 2D). A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay for apoptosis showed no significant difference in the interfollicular epidermal tissue between KO and control animals (Figure S1D); however, there was a profound loss of proliferation (Figures 2D and 2E; Figures S1F and S1H). We have further investigated the function of

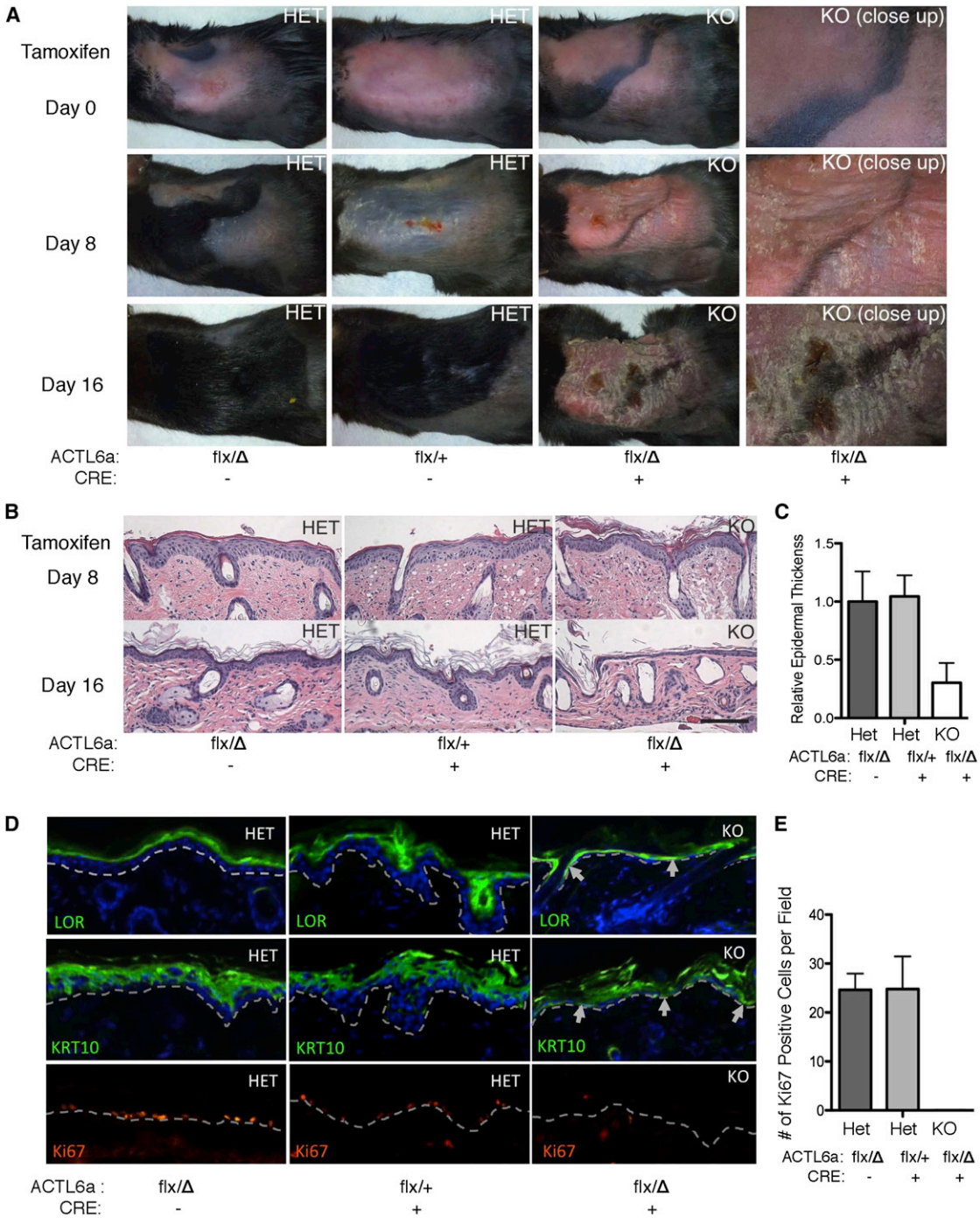

Figure 2. Conditional Epidermal *ACTL6a* Deletion Ablates Progenitor Function

(A) Time-course images of *ACTL6a* conditional KO and heterozygous littermate control (HET) skin before (day 0) and after (day 8, day 16) tamoxifen induction of Cre-ER-mediated *ACTL6a* deletion in mouse epidermis.

(B) Histology of mouse back skin sections from ACTL6a conditional KO and littermates over the same time period (scale bar represents 50 μm); note the hypoplastic tissue collapse in KO epidermal tissue by day 16, after which point no epidermis could be detected.

(C) Quantification of average epidermal tissue thickness (n = 5). $p < 0.0001$, ANOVA. Bars represent mean ± SD.

(D) Differentiation-protein expression (Loricrin [LOR], green; Keratin 1 [KRT1], green) and Ki67 expression (orange); the dotted line denotes the basement membrane. The arrowheads point out cells adjacent to the basement membrane expressing differentiation proteins in KO tissue.

(E) Mitotic indices calculated by the number of Ki67-positive cells per field in the indicated genotypes. $p < 0.0001$, ANOVA. Bars represent mean ± SD.

See also Figures S1 and S2.

ACTL6a in embryonic epidermal tissue development using constitutive K14-Cre. Similar to its effect in adults, loss of ACTL6a during embryogenesis led to epidermal hypoplasia (Figures S2B and S2D). Newborn ACTL6a KO animals die within only a few hours after birth and are characterized by thin-appearing skin with epidermal erosions and the induction of differentiation proteins in the basal layer (Figures S2A and S2B), without a significant increase in apoptosis (Figure S2E). Consistent with areas of epidermal erosion, the capacity of the epidermis to exclude dye is also significantly impaired in newborn KO animals compared to their littermates (Figure S2A). *ACTL6a* deletion therefore leads to proliferative failure and premature terminal differentiation, which in turn leads to epidermal tissue loss in both embryonic development and adult tissue homeostasis.

ACTL6a Is Required for Repression of Premature Progenitor Differentiation

To further characterize the function of ACTL6a in epidermal homeostasis, we performed mRNA expression profiling on human epidermal keratinocytes treated with two independent ACTL6a small hairpin RNAs (shRNAs) (Figure 3). In undifferentiated keratinocytes, ACTL6a depletion altered a total of 467 differentiation genes (327 [70%] induced, 120 [30%] repressed; Tables S3 and S4). ACTL6a-regulated genes displayed a significant ($p < 2 \times 10^{-59}$, Fisher's exact test) overlap of 267 genes with the published calcium-induced epidermal keratinocyte differentiation profile (Sen et al., 2010), with most genes changing in the same direction (Figure 3A). Genes that were upregulated in undifferentiated keratinocytes by ACTL6a loss were significantly enriched with gene oncology (GO) terms relevant to epidermal differentiation (Figure 3B); no specific GO terms were highly enriched in downregulated genes (Figure S3B). Dramatic derepression of specific well-characterized differentiation genes was confirmed with ACTL6a loss, at mRNA and protein levels (Figures 3C–3E). These data confirm that, in addition to the murine tissue context, ACTL6a is required for repression of differentiation-gene expression in the human setting as well.

Consistent with a function for ACTL6a in maintaining the undifferentiated progenitor state, ACTL6a loss impaired clonogenic growth, with ACTL6a-depleted cells producing only an average of 14% of the colonies (>1 mm^2) seen with the control (Figures 3E–3G; Figure S3F). We next tested the effects of altering ACTL6a function in organotypic human epidermal tissue, a setting that accurately recapitulates spatial patterns of epidermal gene expression (Truong et al., 2006). With striking similarity to conditional KO mouse epidermis, organotypic human epidermal tissue displayed hypoplasia and ectopic differentiation occurring at the normally undifferentiated basal layer (Figures 3H and 3I). Further supporting the notion that ACTL6a represses differentiation to maintain epidermal progenitor function, enforced expression of ACTL6a in all layers of regenerating human epidermal tissue impaired differentiation-gene expression (Figure 4; Figure S4A). ACTL6a is thus required for sustaining epidermal self-renewal and for preventing premature progenitor differentiation.

ACTL6a Represses Differentiation in Part through KLF4

To search for downstream targets through which ACTL6A represses differentiation, we performed gene set enrichment analysis (GSEA) using the ACTL6a gene set. A number of previously characterized transcriptional activators of epidermal differentiation were included among ACTL6a target genes, including KLF4, GRHL3, PRDM1, and HOPX, all of which were significantly upregulated by ACTL6A knockdown. When comparing the genes regulated by these transcription factors, we found that 227 of the 467 genes within the ACTL6A-regulated gene set (48.6%) are also regulated by KLF4 with a significant p value of 3.5×10^{-69}. In contrast, only 8% of ACTL6a gene set is regulated by GRHL3, PRDM1, and HOPX combined (Figures 5A–5C). This suggests that a significant portion of the genes regulated by ACTL6a are also KLF4 targets.

In addition to its well known role in the embryonic stem cell setting, the transcription factor KLF4 is also highly expressed in differentiated layers of epidermal tissue, where it has been characterized by targeted gene disruption as a critical and nonredundant activator of differentiation (Jaubert et al., 2003; Segre et al., 1999). Loss of ACTL6a in KO mouse epidermal tissue causes derepression of KLF4 and dysregulation of KLF4 targets such as CDSN (Figures 5D and 5E; Figures S2F and S5D). To validate the genetic interaction between ACTL6A and KLF4, we performed double RNAi studies to investigate the impact of KLF4 depletion in the context of ACTL6a loss. KLF4 depletion effectively suppressed the induction of differentiation genes caused by ACTL6a loss, indicating that KLF4 is required for the derepression of differentiation observed with ACTL6a loss (Figures 5F–5I; Figures S5B and S5C). Therefore, both bioinformatic and genetic studies identify KLF4 as a downstream target through which ACTL6a exerts a portion of its repression actions on differentiation.

ACTL6a Regulates Epidermal Homeostasis through the SWI/SNF Complex

Recent studies suggest that ACTL6a can associate with different epigenetic regulators, including the Tip60 HAT complexes, the KAT2a HAT complexes, and the SWI/SNF chromatin-remodeling complex (Park et al., 2002; Tea and Luo, 2011; Zhao et al., 1998). To examine which complex might be relevant to ACTL6a function in this setting, we performed a loss-of-function analysis for key functional components of each of these complexes. Depletion of either KAT2a or Tip60 failed to significantly alter differentiation-gene expression (Figures S6I and S6J and data not shown). In contrast, depletion of the largest component of the SWI/SNF complex, BAF250a/ARID1A, but not BAF250b/ARID1B, produced impacts similar to ACTL6a loss: decreased clonogenic growth and premature induction of differentiation (Figures S6A–S6H). ACTL6a shares a very similar distribution pattern in the fast protein liquid chromatography (FPLC) fractions of undifferentiated human keratinocyte cellular extracts with the catalytic subunit (Brg1 or Brm) of the SWI/SNF complex (Figure S7A). In addition, overexpression of ACTL6a mutant M1 with K226/E227A mutations that impair binding with Brg1 (Nishimoto et al., 2012) also impaired the suppression of differentiation compared to wild-type ACTL6a (Figures S4B–S4D). Taken together, these results suggest that the function of ACTL6a in repressing progenitor differentiation may involve the SWI/SNF complex.

ACTL6a Loss Permits SWI/SNF Complex Binding to Differentiation Genes

To explore the basis for the functional relationship between ACTL6a and the SWI/SNF complex, we performed chromatin

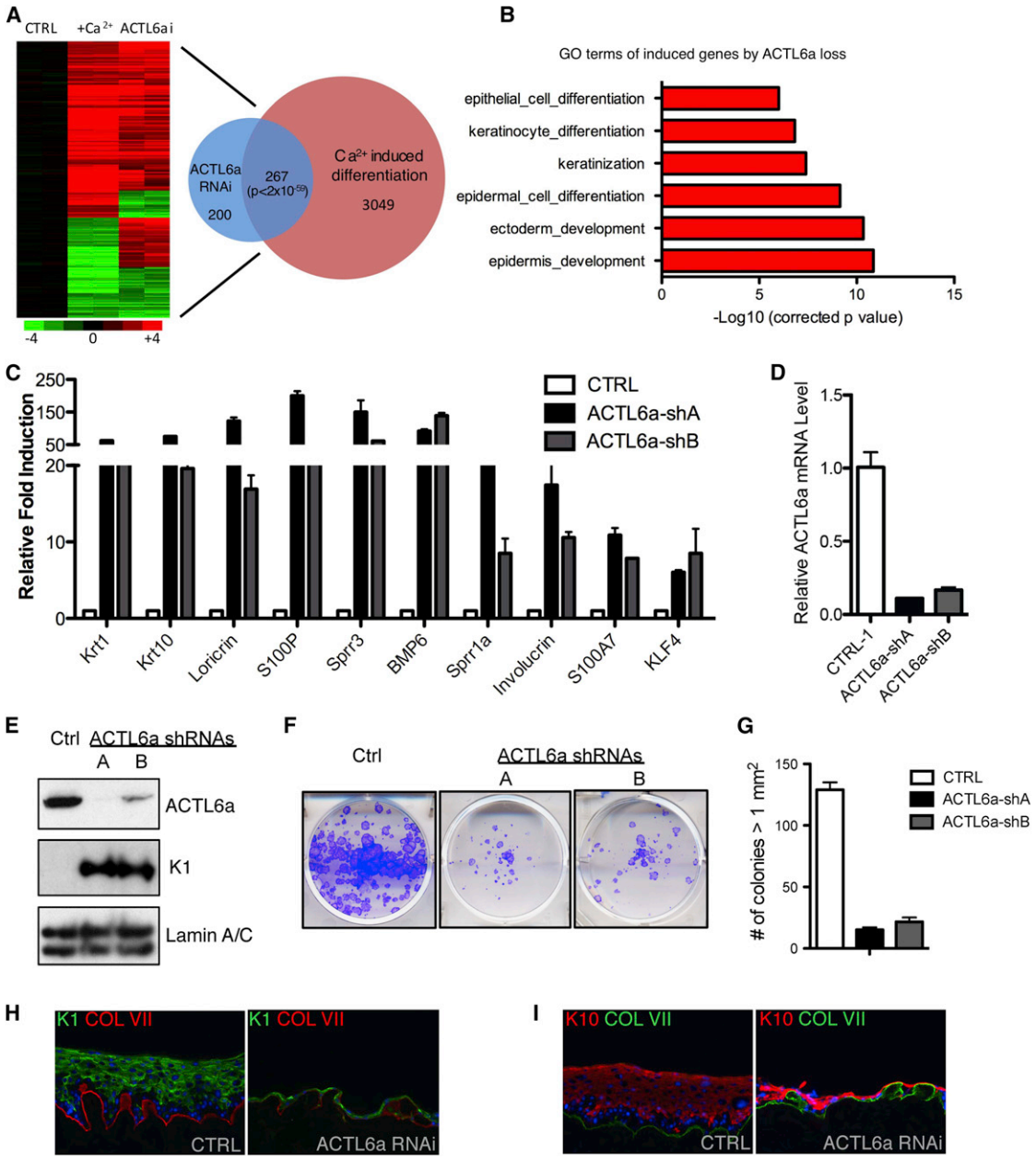

Figure 3. ACTL6a Is Required for Repression of Differentiation

(A) Heat map (left) and Venn diagram (Rezai-Zadeh et al., 2003) illustrating the overlap between expression changes identified with ACTL6a loss and calcium-induced differentiation ($p < 2 \times 10^{-59}$, Fisher's exact test). Genes induced are colored in red, and repressed genes are colored in green.

(B) GO analysis demonstrating that ACTL6a loss induces differentiation-associated genes.

(C) qRT-PCR verification of array data showing mRNA levels of differentiation-associated genes during ACTL6a loss. Bars represent mean ± SD.

(D) Verification of ACTL6a knockdown by duplicate independent shRNAs. Bars represent mean ± SD.

(E) Immunoblots of ACTL6a-depleted keratinocytes demonstrating loss of ACTL6a protein as compared to empty vector controls.

(F) Clonogenic assays of human keratinocytes with ACTL6a RNAi or empty vector controls. $p < 0.005$, ANOVA.

(G) Colonies >1 mm^2 in clonogenic assays are quantified (n = 2 per group). $p < 0.001$, ANOVA. Bars represent mean ± SD.

(H and I) Organotypic human epidermal tissue comparing ACTL6a loss with empty vector control for the K1 differentiation marker (H; green) and comparing ACLT6a loss versus empty vector control for the K10 differentiation marker (I; red).

See also Figure S3 and Tables S3 and S4.

immunoprecipitation (ChIP) analysis to examine Brg1 and Brm binding to differentiation promoters as a function of ACTL6a. Because ACTL6a expression is significantly downregulated during human keratinocyte differentiation, we first compared the localization of the SWI/SNF complex in undifferentiated human keratinocytes with calcium-induced differentiated keratinocytes.

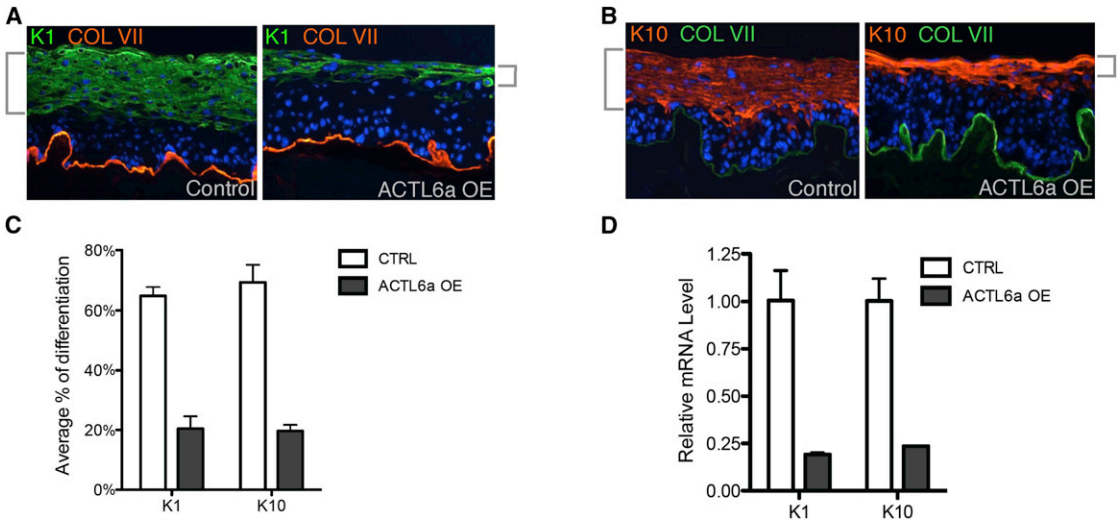

Figure 4. ACTL6a Overexpression Suppresses Differentiation

(A) K1 differentiation marker (green) with enforced ACTL6a expression throughout the epidermis versus control. Note the decreased expanse of expression of K1 in less differentiated regenerating epidermal layers (brackets) in ACTL6a-overexpressing tissue compared to control.

(B) K10 expression comparing ACTL6a overexpression versus control.

(C) Quantification of differentiation markers K1 and K10 by percentage of relative thickness of K1- and K10-expressing cells in tissue sections (n = 5). $p < 0.005$, t test. Bars represent mean ± SD.

(D) Quantification of K1 and K10 mRNA levels in tissue. $p < 0.005$, t test. Bars represent mean ± SD.

See also Figure S4.

Compared to undifferentiated cells, differentiated keratinocytes displayed enhanced binding by SWI/SNF, as well as by RNA polymerase II, at the promoters of differentiation genes, including *KLF4* as well as *KRT10*, *S100A9*, *SPRR3*, and *BMP6* (Figures 6A and 6B). Moreover, depletion of ACTL6a in undifferentiated progenitor populations enhanced the binding of both Brg1 and/or Brm as well as RNA polymerase II to differentiation-gene promoters but failed to alter binding to other gene promoters (Figures 6C and 6D; Figures S7C and S7D). This suggests that ACTL6a loss permits SWI/SNF to bind and activate differentiation genes, a model that would predict that loss of Brg1 and Brm SWI/SNF complex catalytic subunits would impair differentiation.

Consistent with this model, previously reported Brg1 and Brm double KO mice displayed impaired epidermal differentiation-gene induction and deficient tissue-barrier formation (Indra et al., 2005). In agreement with these prior data, simultaneous loss of both Brm and Brg1 by two independent sets of RNAi sequences suppressed the expression of differentiation genes in organotypic human epidermal tissue (Figures 6E and 6F). This suppression of differentiation mediated by Brg1 and Brm loss was also accompanied by suppression of the transcription factor KLF4 (Figure 6G), indicating that intact SWI/SNF complex function is required for full induction of KLF4 and epidermal differentiation. Taken together, our data suggest a model in which ACTL6a helps to maintain the undifferentiated progenitor state by inhibiting SWI/SNF complex binding to and activation of *KLF4* and other differentiation-gene promoters (Figure 7).

DISCUSSION

Here, we present data indicating that ACTL6a is required for the repression of epidermal progenitor differentiation, in part via suppression of KLF4, and that ACTL6a prevents SWI/SNF complex binding to the promoters of *KLF4* and other differentiation genes. The necessity of SWI/SNF complex function in induction of epidermal differentiation was confirmed by double depletion of the Brg1 and Brm catalytic subunits, a loss-of-function experiment that confirmed that full KLF4 induction also requires SWI/SNF action. Taken together, these data support a hypothetical model in which ACTL6a sustains the epidermal progenitor phenotype, at least in part, by preventing the SWI/SNF complex from binding to and activating the expression of differentiation genes.

Although recent studies demonstrate that ACTL6a can associate with multiple epigenetic regulatory complexes, our data indicate that the SWI/SNF complex may be most relevant to ACTL6a action in this setting. Previous studies in various systems suggest that, in addition to the SWI/SNF chromatin-remodeling complex, ACTL6a can associate with HAT complexes, including those containing Tip60 and KAT2a (Lee et al., 2003; Tea and Luo, 2011). The majority of the previous functional analysis on ACTL6a's role during development has been in the nervous system and only recently in the hemopoietic system (Krasteva et al., 2012; Lessard et al., 2007; Yoo et al., 2009). For *Drosophila* olfactory projection neuron dendrite targeting, the ACTL6a homolog Bap55 functions through Tip60 histone acetyltransferase (Tea and Luo, 2011). However, in vertebrates, the switch of ACTL6a by its homolog ACTL6b in the SWI/SNF complex is critical for the differentiation process of neural stem cells (Lessard et al., 2007). The present studies in the mammalian epidermal setting indicate that the Tip60 histone acetyltransferase is not required for ACTL6a effects, whereas multiple subunits associated with the SWI/SNF complex, including BAF250a, Brg1, and Brm, are actively involved in repressing differentiation

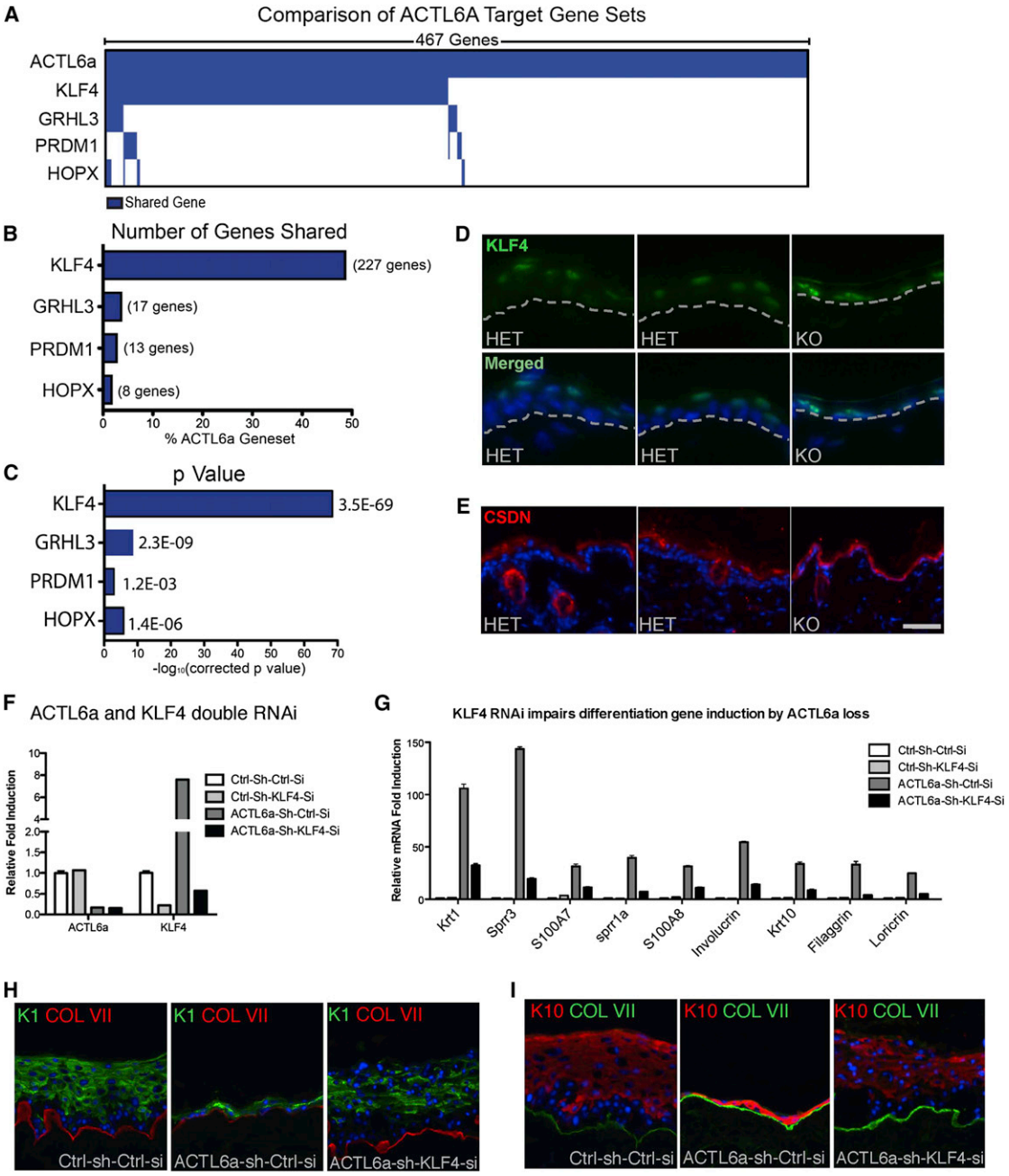

Figure 5. ACTL6a Suppresses Differentiation Partially by Repressing KLF4

(A) GSEA comparing the ACTL6a-controlled gene set (fold change >2) with the gene sets controlled by the known epidermal-differentiation-mediating transcription factors whose expression levels changed with ACTL6a loss, including KLF4, GRHL3, PRDM1, and HOPX. Shared genes are indicated in dark blue.

(B) Bar graph comparing the percentage of shared gene numbers with the ACTL6a data set. KLF4 controls 48.6% of the ACTL6a gene set.

(C) Bar graph comparing the p values (−log-corrected p value) in the GSEA analysis.

(D) Staining of KLF4 in vivo in adult mice epidermis, comparing KO versus littermate HET controls on day 16 after initial tamoxifen treatment. KLF4 is induced on the basal layer in KO mouse tissue.

(E) Staining of the KLF4 target, CDSN, comparing KO mouse tissue versus HET controls.

(F) Relative mRNA expression levels of KLF4 and ACTL6a in a double RNAi experiment using ACTL6a shRNA and KLF4 siRNA. $p < 0.0001$, ANOVA. Bars represent mean ± SD.

(G) Relative mRNA expression of differentiation-associated genes with ACTL6a and KLF4 double RNAi. $p < 0.0001$, ANOVA. Bars represent mean ± SD.

(H) K1 staining (green) of human epidermal tissue sections from tissues with ACTL6a and KLF4 double RNAi compared to controls. Note the rescue of ACTL6a tissue collapse by KLF4 depletion.

(I) K10 staining (red) of human epidermal tissue sections from tissues with ACTL6a and KLF4 double RNAi compared to controls.

See also Figure S5.

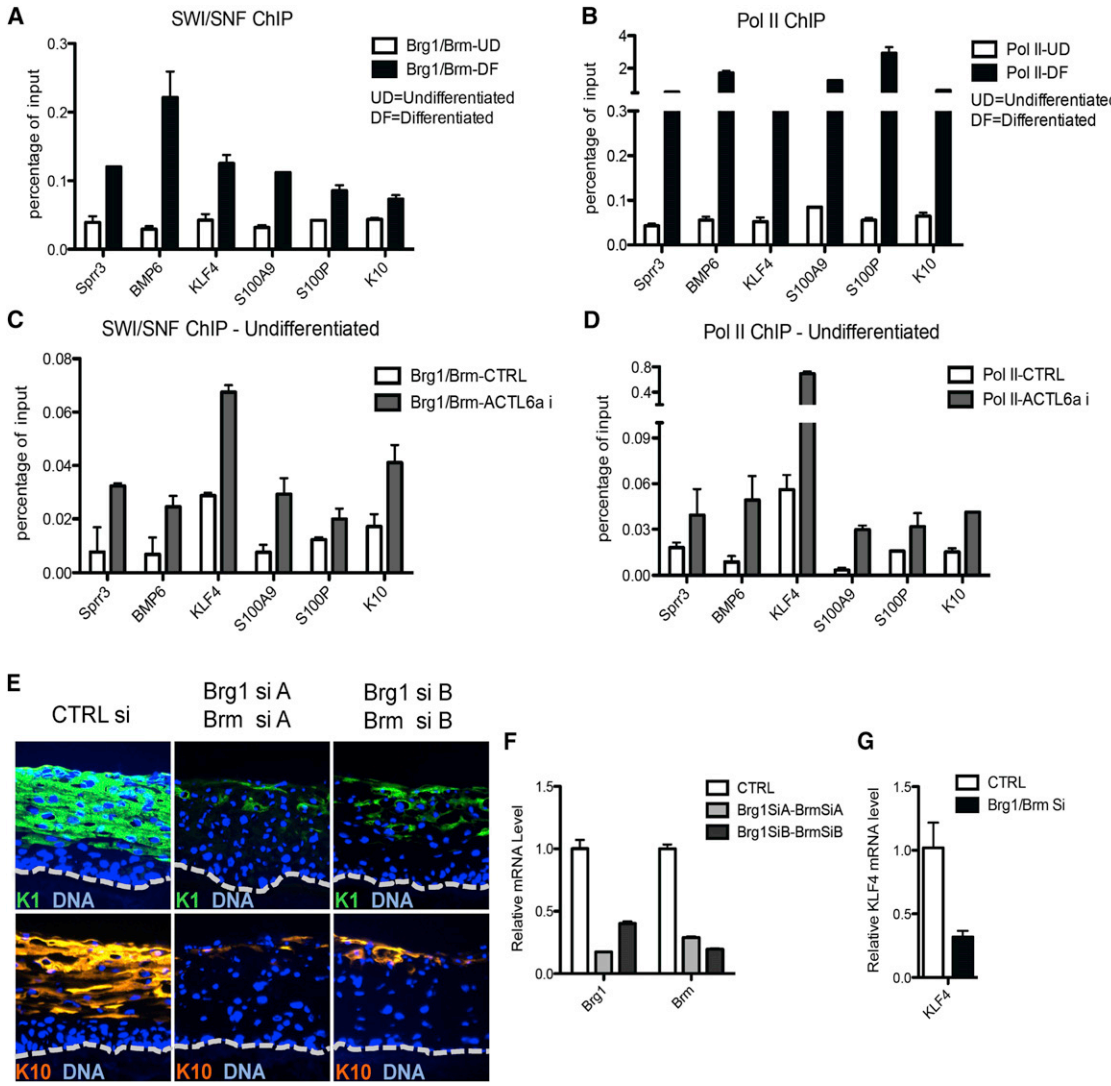

Figure 6. ACTL6a Loss Facilitates SWI/SNF Complex Targeting to Differentiation Genes

(A) ChIP analysis of the Brg1 and Brm SWI/SNF complex components at differentiation-gene promoters, comparing undifferentiated and differentiated human keratinocytes. $p < 0.0001$, ANOVA. Bars represent mean ± SD.

(B) ChIP analysis of RNA polymerase II at differentiation-gene promoters, comparing undifferentiated and differentiated human keratinocytes. $p < 0.0001$, ANOVA. Bars represent mean ± SD.

(C and D) ChIP analysis of the SWI/SNF complex (C) and RNA polymerase II (D) enrichment at promoter regions of representative differentiation-associated genes using undifferentiated human keratinocytes treated with shRNA against ACTL6a or control shRNA. $p < 0.05$, ANOVA. Bars represent mean ± SD.

(E) Brg1 and Brm double depletion using two independent sets of siRNAs inhibits differentiation in regenerating organotypic human epidermal tissue. K1, green; K10, orange; dotted line, basement membrane.

(F) Quantification of the knockdown efficiency of siRNAs targeting Brg1 or Brm. Bars represent mean ± SD.

(G) Loss of Brg1 and Brm also inhibits the expression of KLF4 in differentiated human keratinocytes. Bars represent mean ± SD.

See also Figures S6 and S7.

in the progenitor state and in activating normal differentiation-gene expression.

The presence of ACTL6a in progenitor cell-containing populations is associated with suppression of the targeting of the SWI/SNF complex to differentiation genes, and ACTL6a depletion relieves this suppression in these undifferentiated cells. Previous studies in lymphocytes indicated that >80% of total SWI/SNF complexes in resting lymphocytes do not associate tightly with chromatin; however, a rapid and tight association of the complex can be induced by cell stimulation (Zhao et al., 1998). A similar mechanism may operate during epidermal differentiation in that ACTL6a loss may stimulate the relocation of a portion of SWI/SNF complexes to chromatin at differentiation-gene promoters. This would also be in agreement with recent studies in T helper cells, in which Brg1 binding to differentiation genes was associated with gene induction (De et al., 2011).

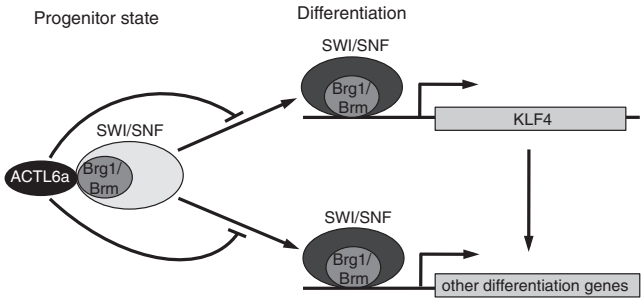

Figure 7. Hypothetical Model of ACTL6a Action in Regulating Epidermal Tissue Differentiation

In undifferentiated conditions, ACTL6a prevents Brg1- or Brm-containing SWI/SNF complex binding to and activation of KLF4 and other differentiation gene promoters. During differentiation, ACTL6a downregulation facilitates SWI/SNF binding to and activation of KLF4 and other differentiation genes. In addition, the composition of the SWI/SNF complex also changes during differentiation (reflected by the different shades of gray).

The targeting of the SWI/SNF complex during differentiation may result from a variety of inputs. The combination of different regulatory subunits and the resulting overall conformation are hypothesized to directly affect the targeting and activity of the SWI/SNF complex (Wu et al., 2009). Therefore, the physical association between ACTL6a and the SWI/SNF complex is likely to form a specific conformation that may have less affinity to differentiation genes. On the other hand, multiple transcription factors and histone modifications can also play important roles in recruiting the SWI/SNF complex (Debril et al., 2004; Kowenz-Leutz and Leutz, 1999; Salma et al., 2004). Therefore, it is formally possible that the recruitment of the SWI/SNF complex to differentiation genes upon ACTL6a loss is indirectly mediated by the transcription factors derepressed by ACTL6a loss, such as KLF4. It is noteworthy, however, that KLF4 depletion did not totally account for the full spectrum of differentiation-gene derepression caused by ACTL6a loss. This partial rescue of the ACTL6a loss defect could be due to a number of factors, including action by transcription factors other than KLF4, such as GRHL3, PRDM1, HOPX, and others.

KLF4 is an essential transcription regulator directly dictating multiple biological processes including cell proliferation, differentiation, tumorigenesis, and pluripotency (Rowland and Peeper, 2006; Vangapandu and Ai, 2009). However, very little is known about how the expression levels of KLF4 are precisely controlled to enable its multiple roles in different settings. During epidermal differentiation, KLF4 expression increases substantially, and both KLF4 loss of function and ectopic expression significantly disturb epidermal growth and differentiation (Jaubert et al., 2003; Segre et al., 1999). Our findings have identified ACTL6a as a negative regulator of KLF4 in the undifferentiated progenitor state, and the downregulation of ACTL6a during epidermal tissue differentiation thus appears to enable the upregulation of KLF4 to help drive differentiation.

In addition to ACTL6a, we have observed that several other actin-like proteins also change their expression during differentiation, including ACTR8, which is functionally related to the Ino80 chromatin-remodeling complex. It is interesting to note that the expression of ACTL6b is below the level of detection by multiple methods in our lab, including quantitative RT-PCR and RNA sequencing. ACTL6b was previously shown to replace ACTL6a in associating with the SWI/SNF complex and dictating the neuronal differentiation processes (Lessard et al., 2007). The lack of detectable epidermal ACTL6b expression indicates that the regulation of differentiation at the level of nuclear actin-like proteins is significantly different in epidermis versus the neuronal tissue. Consistent with our findings in the epidermal tissue, ACTL6a is essential for the progenitor function in the hemopoietic system, where little ACTL6b is detected (Krasteva et al., 2012; Kuroda et al., 2002; Olave et al., 2002). Future studies on the functions of other actin-like proteins in multiple tissues will further enhance our knowledge of developmental regulation by these regulators and the epigenetic regulatory complexes with which they associate.

EXPERIMENTAL PROCEDURES

Cells and Organotypic Culture

Primary human keratinocytes were isolated from fresh surgically discarded newborn foreskin and cultured in complete Keratinocyte-SFM (Life Technologies, #17005-142) and Medium 154 (Life Technologies, #M-154-500). Organotypic regeneration of human epidermal tissue was performed as previously described (Truong et al., 2006). Biological replicates were performed in all cases using primary cells from at least three independent unrelated donors.

Gene Transfer and Knockdown

Gene transfer by viral transduction was performed as described (Sen et al., 2010). shRNAs targeting ACTL6a were ordered from Open Biosystems. For small interfering RNA (siRNA) knockdown, 1×10^6 cells were electroporated with 1 nmol siRNA using the Amaxa Human Keratinocyte Nucleofector Kit (Lonza, #VAPD-1002). For Brg1 and Brm knockdown, either the ON-TARGETplus SMARTpool RNAi reagents (Dharmacon) were used, or single ON-TARGETplus siRNA were used, including Brg1 Si A (Dharmacon, #J-010431-12), Brg1 Si B (Dharmacon, #J-010431-09), Brm Si A (Dharmacon, #J-017253-07), and Brm Si B (Dharmacon, #J-017253-05). For KLF4 siRNA, the ON-TARGETplus siRNA (Dharmacon, #D-005089-19) were used.

KO Mice

ACTL6a-targeted mice were generated as described separately (J.T., unpublished data). Mouse toes or 3mm of tail tissue were cut with clean surgical scissors and heated in 75 μl of Reagent A (25 mM NaOH, 2 mM EDTA) at 95°C for 1 hr. After it was cooled down to room temperature, tissue was mashed with a pipette tip to aid the release of genomic DNA. Reagent B (75 μl; 40 mM of Tris-HCl; pH 7.5) was then added for neutralization. For genotyping, 1 μl of each tissue's genomic DNA extraction was used in a 20 μl PCR reaction. Tamoxifen (Sigma-Aldrich, #T5648-1G) was dissolved in 100% ethanol at a concentration of 0.5 mg per 100 μl. Mice were aged for least 40 days, and their back hair was shaved prior to tamoxifen treatment. Each mouse received 0.5 mg tamoxifen each time applied directly on its back skin every 2 days, for a total of three times. All animal studies were performed in accordance with P.A.K.'s currently approved protocol (Stanford Institutional Animal Care and Use Committee #4045).

Protein Expression and Tissue Analysis

For immunoblot analysis, 20–50 μg of cell lysates were loaded per lane for SDS-PAGE and transferred to polyvinylidene fluoride membranes. For immunofluorescence staining, tissue sections (7 μm thick) were fixed using either 50% acetone and 50% methanol or 4% formaldehyde. Primary antibodies were incubated at 4°C overnight, and secondary antibodies were incubated at room temperature for 1 hr. The affinity-purified antiserum against ACTL6a and the J1 antiserum against Brg1/Brm were raised by the Crabtree Laboratory. Other antibodies used in this study include anti-BAF53a (Novus Biologicals), anti-Krt1 (Covance), anti-Krt10 (Neomarkers), anti-Ki67 (Neomarkers), anti-Loricrin (Covance), ms-anti-CollagenVII (Millipore), pAb-anti-CollagenVII

(Calbiochem), goat-anti-CDSN (Santa Cruz Biotechnology), and rat-anti-Nidogen (Santa Cruz Biotechnology).

Quantitative RT-PCR Expression Analysis

For quantitative RT-PCR (qRT-PCR), total RNA was extracted using RNeasy Plus (QIAGEN) and subsequently subjected to reverse transcription using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). qRT-PCR analysis was performed using the Mx3000P instrument with the SYBR Green Master Mix (Fermentas). Samples were run in duplicate and normalized to levels of GAPDH mRNA or 18S ribosomal RNA for each reaction. Primer sequences are listed in Table S1.

mRNA Expression Profiling and Analysis

Amplification and labeling of complementary DNA (cDNA) probes and hybridization to the Human Genome U133 Plus 2.0 microarray chip (Affymetrix) were performed by the Stanford Protein and Nucleic Acid Facility. Data analysis was performed using R. Each data set for an experiment was filtered for probes that had an expression value $\geq$100 in at least one of the samples, along with a p value $\leq$0.05 based on significance analysis of microarrays. Pairwise comparisons between the RNAi-treated samples and the control samples were performed for finding probes that showed $\geq$2-fold expression change. Additional gene sets were acquired from the Gene Expression Omnibus (GRHL3: GSE7381, Yu et al., 2006; KLF4: GSE32685, Sen et al., 2012) or taken from the supplemental tables for HOPX (Yang et al., 2010) and PRDM1 (Magnúsdóttir et al., 2007). Significant genes were identified as having a $\geq$2-fold change with p value <0.05. GSEA was conducted using Genomica software with a p value cutoff of 0.05.

ChIP

ChIP assays were performed essentially as described previously (Euskirchen et al., 2011) with minor modifications. Human keratinocytes were crosslinked either with 1% formaldehyde alone or with dual crosslinking of both 2 mM disuccinimidyl glutarate and 1% formaldehyde. The chromatin was sonicated for the production of fragments with an average length between 200 and 500 bp. The sonicated chromatin was immunoprecipitated overnight at 4°C with J1 antibody (Khavari et al., 1993), Poll II, or the same amount of immunoglobulin G control. Following reverse crosslinking, the samples were treated with RNase and proteinase K, and the DNA was purified using the QIAGEN PCR Purification Kit. ChIP product (2 μl) was used for each qPCR reaction.

Colony-Formation Assay

Mouse fibroblast 3T3 cells were treated with 15 μg/ml mitomycin C (Sigma-Aldrich) in Dulbecco's modified Eagle's medium for 2 hr, then trypsinized and plated at 8×10^5 cells per well in a 6-well plate. The media was changed to keratinocyte growth media 24 hr after plating. A total of 300 keratinocytes were seeded onto the feeder layer 24 hr after the media change. Media was changed every 2 days for 14 days. At the end of 14 days, the cells were washed with PBS to remove the 3T3 cells, then fixed in 1:1 acetone/methanol for 5 min. The plate was allowed to air dry for 3–5 min, and then colonies were stained with crystal violet.

Skin-Barrier Analysis

The skin-barrier analysis was performed essentially as previously published (Scholl et al., 2007). In brief, live newborn mice or E17.5 embryos (dissected and washed in PBS) were incubated overnight at 37°C in a 5-bromo-4-chloro-3-indlyl-β-D-galactopyranoside (X-gal) reaction mix (100 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 3 mM $K_3Fe[CN]_6$, 3 mM $K_4Fe[CN]_6$, and 1 mg/ml X-gal [pH 4.5]). Images were taken the next morning, after incubation.

ACCESSION NUMBERS

Profiling data has been deposited with the GEO accession code GSE36222.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2012.12.014.

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