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E4F1-mediated control of pyruvate dehydrogenase activity is essential for skin homeostasis

Perrine Goguet-Rubio1,2,a,b,c,d,e, Berfin Seyran1,2,a,b,c,d,e,1, Laurie Gayte1,2,a,b,c,d,e, Florence Bernez1,a,c,f, Anne Sutter1,a,b,c,d,e, Hélène Delpech1,a,b,c,d,e,g, Laetitia Karine Linares1,a,b,c,d,e, Romain Riscal1,a,b,c,d,e, Cendrine Repond1,g, Geneviève Rodier1,a,b,c,d,e,g, Yann Herault1, Marc Sitbon1,a,b,c,d,e, Luc Pellier1,g, Claude Sardet1,a,b,c,d,e, Matthieu Lacroix2,3,a,b,c,d,e, Laurent Le Cam1,a,b,c,d,e,2,3 and Olivier Kirshg,i, Jawida Touhami1,g, Jean Noel1,a,b,c,d,1, Charles Vincent1,a,b,c,d, Nelly Pirota1,a,b,c,d,f, Guillaume Pavlovicj, A.S., H.D., L.K.L., R.R., C.R., G.R., O.K., J.T., J.N., C.V., N.P., and M.L. performed research; M.S., L.P., C.S., M.L., and L.L.C. designed research; P.G.-R., B.S., L.G., A.S., H.D., L.K.L., R.R., C.R., G.R., O.K., J.T., J.N., C.V., N.P., and M.L. performed research; G.P., Y.H., and M.S. contributed new reagents/analytic tools; F.B., M.L., and L.L.C. analyzed data; and M.L. and L.L.C. wrote the paper.

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The multifunctional protein E4 transcription factor 1 (E4F1) is an essential regulator of epidermal stem cell (ESC) maintenance. Here, we found that E4F1 transcriptionally regulates a metabolic program involved in pyruvate metabolism that is required to maintain skin homeostasis. E4F1 deficiency in basal keratinocytes resulted in deregulated expression of dihydrolipoamide acetyltransferase (Dlat), a gene encoding the E2 subunit of the mitochondrial pyruvate dehydrogenase (PDH) complex. Accordingly, E4f1 knockout (KO) keratinocytes exhibited impaired PDH activity and a redirection of the glycolytic flux toward lactate production. The metabolic reprogramming of E4f1 KO keratinocytes associated with remodeling of their microenvironment and alterations of the basement membrane, led to ESC mislocalization and exhaustion of the ESC pool. shRNA-mediated depletion of Dlat in primary keratinocytes recapitulated defects observed upon E4f1 inactivation, including increased lactate secretion, enhanced activity of extracellular matrix remodeling enzymes, and impaired clonogenic potential. Altogether, our data reveal a central role for Dlat in the metabolic program regulated by E4f1 in basal keratinocytes and illustrate the importance of PDH activity in skin homeostasis.

Significance

We found that the multifunctional protein E4 transcription factor 1 (E4F1) transcriptionally regulates a metabolic program involved in pyruvate metabolism that is required to maintain skin homeostasis. E4F1 deficiency in skin resulted in deregulated expression of dihydrolipoamide acetyltransferase (Dlat), a gene encoding the E2 subunit of the mitochondrial pyruvate dehydrogenase (PDH) complex. Accordingly, E4f1 knockout (KO) keratinocytes exhibited impaired PDH activity and a metabolic reprogramming associated with remodeling of their microenvironment and alterations of the basement membrane, leading to epidermal stem cell mislocalization and exhaustion of the epidermal stem cell pool. Our data reveal a central role for Dlat in the metabolic program regulated by E4f1 in skin and illustrate the importance of PDH activity in skin homeostasis.
inactivation in the epidermis results in ESC defects through a mechanism that involves, at least partly, the deregulation of the Bmi1–Arf–p53 pathway (7). Here, we show evidence supporting a major role for E4F1 in pyruvate metabolism that governs ESC maintenance and skin homeostasis.

Results

E4f1 Inactivation in Basal but Not Suprabasal Adult Keratinocytes Leads to Epidermal Defects and Exhaustion of the ESC Pool. Using E4f1–/– whole-body conditional KO mice (E4f1(K10)KO; RERT), we previously identified an essential role for E4f1 in adult skin homeostasis (7). In this genetically engineered mouse model, E4f1 inactivation was achieved in the entire skin of adult animals, including the dermal compartment. To assess the cell of origin of these skin defects, we generated new mouse strains by crossing E4f1 conditional KO mice to transgenic animals expressing the tamoxifen (tam)-inducible CreER recombinase under the control of the keratin 14 (K14) or keratin 10 (K10) promoters [hereafter referred to as E4f1(K14)KO and E4f1(K10)KO strains], allowing acute inactivation of E4f1 in adult keratinocytes of the basal or spinous layers, respectively (20).

Molecular and histological analyses of adult back skin of 8- to 12-wk-old E4f1(K10)KO animals confirmed that topical skin applications of tam activated the Cre recombinase in suprabasal 12-wk-old spinous layers, respectively (20).

Results A

E4F1 Deficiency in Basal Keratinocytes Leads to Skin Defects and Exhaustion of the ESC Pool. (A) Microphotographs of (H&E)-stained skin sections prepared from E4f1(K14)KO mice or E4f1(K14)CTR littermates, 1, 2, or 5 wk after tam administration. Dashed lines indicate the separation between the epidermis and the dermis. (Scale bars, 100 μm.) (B) Whole mounts of tail epidermis prepared from adult E4f1(K14)KO and E4f1(K14)CTR mice, 5 wk after tam application, stained with K15 antibody and DAPI. Brackets: bulge area (BG) of the HF. (Scale bar, 100 μm.) (C) Number of follicular stem cells (FSC) in back skin epidermis prepared from the same mice as in B. FACS analysis of (EdU)-CD34 double-positive FSC in back skin epidermis prepared from the same mice as in B (mean ± SEM, n = 10). (D) Number of label-retaining (EdU+) interfollicular stem cells (LRCs) detected by immunofluorescence (IF) on back skin sections prepared from adult E4f1(K14)KO mice or E4f1(K14)CTR littermates, 5 wk after tam application. Histobars represent the mean ± SEM of EdU+ cells per millimeter of epidermis (n = 5 animals per group). (E) Clonogenic assays performed with E4f1KO and CTRL primary murine keratinocytes cultured in presence or absence of 4OHT, as indicated (n = 5). Histobars represent the total number of clones per well relative to control cells (expressed as percentages) determined after rhodamine B staining. ***P < 0.001; **P < 0.01; ns, not significant.

E4F1 Controls Pyruvate Metabolism in Keratinocytes Through Transcriptional Regulation of the E2 Subunit of the Pyruvate Dehydrogenase Complex Dlat. Using a pan-genome ChIP approach combined with next-generation sequencing (ChIP-seq), we identified E4f1 binding sites at the whole-genome level in primary mouse embryonic fibroblasts and embryonic stem cells (18, 22). Functional annotation of E4f1 direct target genes indicated a significant enrichment in genes implicated in metabolism, including a set of five genes encoding core components or regulators of the mitochondrial pyruvate dehydrogenase (PDH) complex (PDC), a multimeric complex that converts pyruvate into Acetyl-CoA (AcCoA). In embryonic stem cells, this set of E4f1-controlled genes includes the E2 and E3 subunits of the PDC, dihydrolipoamide acetyltransferase (Dlat) and dihydrolipoyl dehydrogenase (Dld), the regulatory subunit of the PDH phosphatase complex (Pdp), the pyruvate transporter of the inner mitochondrial membrane Bp44l/Mpc1, (23, 24), and the mitochondrial transporter Slc25a19 that transports the PDH cofactor thiamine pyrophosphate (25). These results prompted us to evaluate whether E4F1 also controlled this set of PDH-related genes in primary keratinocytes. First, we confirmed by quantitative ChIP that endogenous E4F1 was recruited to the promoter of Dlat, Dld, Slc25a19, and Bp44l in cultured primary murine keratinocytes (Fig. 2D). Similarly to E4f1KO fibroblasts and muscle cells, E4f1-deficient keratinocytes displayed a marked decrease of Dlat mRNA level. However, the expression of Dld, Pdp, Slc25a19, and Bp44l/Mpc1 remained unchanged upon E4f1 inactivation, suggesting that other E4F1-independent mechanisms contribute to their expression in keratinocytes (Fig. 2B). The mRNA levels of other PDH-related genes, including Pdp1, which encodes the E1 complex D1 component, were also confirmed to change significantly upon inactivation of E4f1 (Fig. 2C). Next, we sought to assess whether E4f1 inactivation impacted the expression of the other PDH components, Slc25a19, Bp44l, and Dld. Therefore, we compared the expression profiles of these components at the protein level in E4f1-deficient and control keratinocytes cultured in the presence and absence of 4OHT. Western blot analysis confirmed that E4f1-deficient keratinocytes displayed a marked decrease of Dld expression (Fig. 2D). Conversely, the expression of Slc25a19 and Bp44l was not affected by E4f1 inactivation, indicating that other E4F1-independent mechanisms contribute to their expression in keratinocytes (Fig. 2B). The mRNA levels of other PDH-related genes, including Pdp1, which encodes the E1 complex D1 component, were also confirmed to change significantly upon inactivation of E4f1 (Fig. 2C).
subunit of the PDC, the PDH -kinases 1/4 (Pdk1, Pdk4) and - phosphatases 1/2 (Pdp1, Pdp2) remained unchanged in E4f1-deficient keratinocytes (Fig. S5). Decreased Dlat expression was confirmed at the protein level, as shown by immunostaining of skin samples prepared from E4f1(K14)KO animals, 1 wk after tam-administration. This decrease was further confirmed by immunoblotting both in tam-treated E4f1(K14)KO mice and control (CTR) primary keratinocytes in basal conditions or after addition of glucose (mean ± SEM; n = 5). (A) Immunohistochemistry analysis of MCT4 expression in skin sections prepared from E4f1(K14)KO mice and control littermates, 1 wk after tam administration. (Scale bar, 50 μm.) (B) Protein levels of acetylated-lysine histone H4 and β-actin (loading control) determined by immunoblotting in total protein extracts prepared from epidermis of E4f1(K14)KO mice or E4f1(K14)CTR littermates, 1 wk after tam administration. Immunoblotting in (C) total protein extracts prepared from epithelium of E4f1(K14)KO mice and E4f1(K14)CTR littermates, 1 wk after tam administration, or (D) E4f1KO or CTR cultured primary keratinocytes. *Non-specific band. (E) Immunostaining of DLAT in skin sections prepared from the same mice as in C. Sections were counterstained with DAPI. (Scale bar, 50 μm.) (F) Metabolic reprogramming of E4f1-deficient keratinocytes. Next, we characterized the metabolic consequences of impaired PDH activity in E4f1-deficient keratinocytes. We postulated that decreased PDH activity in E4f1-deficient keratinocytes triggered a decrease of glucose-derived AcCoA production and the redirection of the glycolytic flux toward lactate production (Fig. 3A). Consistent with this hypothesis and the role of AcCoA as a donor substrate for acetylation reactions, E4f1KO keratinocytes exhibited decreased histone H4 acetylation, as shown by immunoblotting using an anti-pan-acetyl lysine histone H4 antibody (Fig. 3B). Moreover, in line with increased pyruvate metabolism by the NADH-dependent lactate dehydrogenase (LDH), E4f1KO keratinocytes displayed an increased NAD+/NADH ratio (Fig. 3C). Increased expression of the glucose transporter GLUT1 suggested that glucose uptake increased upon E4f1 inactivation in keratinocytes (Fig. S6F). These cells also exhibited increased expression of the monocarboxylate transporter MCT4 that favors the efflux of lactate outside the cell (Fig. 3 D and E). Accordingly, increased lactate secretion by E4f1KO keratinocytes was evidenced by a change in their extracellular acidification rate (ECAR) (Fig. 3F). Other metabolic changes were observed in E4f1-deficient keratinocytes, as illustrated by increased fatty acid oxidation (FAO) (Fig. 3G). This adaptive metabolic response was likely sufficient to sustain mitochondrial respiration because no significant difference was observed in oxygen consumption upon E4f1 inactivation in keratinocytes cultured in complete medium (Fig. S6D). Analyses of tam-treated E4f1(K14)KO mice and control littermates confirmed that E4f1-deficient keratinocytes underwent the same metabolic
reprogramming in vivo. Thus, immunohistochemistry (IHC) analyses of skin samples prepared from these animals indicated that E4f1 inactivation in basal keratinocytes resulted in increased expression of GLUT1, MCT4, and of CD147/BASIGIN, a chaperone required for MCT4 relocalization at the cytoplasmic membrane (Fig. 3H and Fig. S6C). Strikingly, E4f1(K14)KO mice exhibited lactic acidemia and increased level of circulating ketone bodies, a by-product of FAO (Fig. 3I and J). Moreover, the clonogenic potential of E4f1/ko keratinocytes was partly rescued by addition of exogenous acetate that can replenish AcCoA pools (Fig. 3K), confirming that the profound metabolic reprogramming of E4f1-deficient keratinocytes impinged on their biological functions.

**Metabolic Reprogramming of E4f1-Deficient Keratinocytes Associates with Remodeling of the Microenvironment and Loss of Adhesion of the ESC with the Basement Membrane.** In many tumors, increased lactate secretion has been linked to the remodeling of the extracellular matrix (ECM) and degradation of the basement membrane (BM) by ECM-remodeling enzymes (26). To further characterize the consequences of the metabolic reprogramming of E4f1-deficient keratinocytes, we performed histological analyses of E4f1(K14)KO skin. Electron microscopy analyses indicated that E4f1 inactivation in basal keratinocytes resulted in disorganization of the BM, which appeared either diffused with thinner lamina densa or focally disrupted (Fig. S7A). Alterations of the BM in E4f1KO skin was confirmed upon staining of skin sections by the Comori reticulin method, which stains the argyrophilic (silver staining) fibrous structures present in the BM (Fig. S7B). Immunostaining of skin samples prepared from E4f1(K14)KO mice with anti-laminin V antibody showed that the expression pattern of this essential component of the BM was diffused and focally discontinuous in E4f1KO skin sections compared with its defined and continuous pattern in control samples (Fig. 4A). This defect also correlated with an abnormal expression pattern of integrin β4 (Itgβ4). In areas showing broad disruption of the BM, Itgβ4 expression was not restricted to the basal pole of keratinocytes but was also detected at the apical or lateral sides of both basal and suprabasal keratinocytes (Fig. 4A). Remodeling of the ECM within the dermal compartment was also evidenced by picro-Sirius red staining of collagen fibers on skin sections prepared from tam-treated E4f1(K14)KO mice (Fig. S7C). These results led us to investigate whether the massive remodeling of the ECM and alterations of the BM observed upon E4f1 inactivation resulted from increased activity of ECM-remodeling enzymes. Increased matrix metalloproteinase 9 (MMP9) and cathepsin activities were detected by gelatin-zymography in protein extracts prepared from total skin samples of tam-treated E4f1(K14)KO mice (Fig. 4B and Fig. S7D). Moreover, increased MMP2, MMP9, and cathepsin activities were also evidenced in the culture medium of E4f1KO primary keratinocytes (Fig. 4 C and D). Addition of the LDH-inhibitor oxamate in the culture medium decreased cathepsin activities, confirming that their induction resulted from the metabolic reprogramming of these cells (Fig. 4D). Moreover, stable expression of ectopic TIMP1, a broad MMP inhibitor, in feeder cells partly rescued the clonogenic potential of E4f1KO ESC (Fig. 4E). Improved clonogenicity of E4f1-deficient ESC was also observed upon incubation with GM6001, a pharmacological MMP inhibitor with broad spectrum (Fig. 4F). Taken together, these data indicate that the induction of ECM remodeling enzymes in E4f1-deficient keratinocytes is a consequence of their metabolic reprogramming and impinges on their clonogenic potential. Based on these results, we hypothesized that the observed disruption of the BM impacted on the maintenance of ESC within their normal microenvironment, leading to the definitive exhaustion of the ESC pool. To test this hypothesis, we analyzed EdU* LRCs on skin sections prepared from E4f1(K14)KO mice or control littermates 2 wk after tam administration and evaluated their localization within the epidermis. The same skin sections were also processed to assess MCT4 expression as a surrogate marker of the metabolic reprogramming of E4f1KO ESC. LRCs were identified by IF on skin sections prepared from E4f1(K14)KO or control littermates 2 wk after tam administration and evaluated their localization within the epidermis. The same skin sections were also processed to assess MCT4 expression as a surrogate marker of the metabolic reprogramming of E4f1KO ESC. LRCs were identified by IF on skin sections prepared from E4f1(K14)KO or control littermates 2 wk after tam administration and evaluated their localization within the epidermis. The same skin sections were also processed to assess MCT4 expression as a surrogate marker of the metabolic reprogramming of E4f1KO ESC. LRCs were identified by IF on skin sections prepared from E4f1(K14)KO or control littermates 2 wk after tam administration and evaluated their localization within the epidermis.

**Fig. 4.** E4f1 inactivation in basal keratinocytes results in alterations of the BM, remodeling of the ECM and ESC mislocalization. (A) IF analysis of Laminin V (LamV, red) and Itgβ4 (green) expression in skin sections prepared from E4f1(K14)KO and CTR mice, 2 wk after tam administration. Sections were counterstained with DAPI (blue). (Scale bars, 50 μm.) (B, Left) Representative zymogel analysis of MMP activities in protein extracts prepared from skin samples of E4f1(K14)KO and CTR mice, 2 wk after tam administration. Normalization, β-actin immunoblotting was performed on the same protein extracts. (Right) Quantification of MMP2 and MMP9 activities (mean ± SEM, n = 5 animals per group). (C, Left) MMP activities measured by zymography in conditioned media (CM) from E4f1KO or CTR cultured primary keratinocytes. For normalization, β-actin expression was determined by immunoblotting in protein extracts prepared from the same cells. (Right) Quantification of MMP2 and MMP9 activities (mean ± SEM, n = 4). (D, Left) Cathepsin activities measured by zymogel in CM from E4f1KO or CTR primary keratinocytes cultured in the presence or absence of oxamate. (Right) Quantification of cathepsin activities (mean ± SEM, n = 3). (E) Clonogenic assays performed with E4f1KO and CTR primary keratinocytes in presence of feeders transduced with empty or TIMP1-encoding retrovirus (mean ± SEM, n = 9). (F) Clonogenic assays performed with E4f1KO and CTR primary keratinocytes in presence or absence of the MMP-inhibitor GM6001 (mean ± SEM, n = 6). (G) Mislocalization of E4f1KO ESC. LRCs were identified by IF on skin sections prepared from E4f1(K14)KO and CTR mice, 2 wk after tam administration. MCT4 staining was performed to identify metabolically reprogrammed cells. Sections were counterstained with DAPI. (Scale bar, 30 μm.) Arrows indicate EdU* LRCs. Histograms represent the total number of EdU* LRCs per millimeter of epidermis and their respective localization in the basal or suprabasal (SB) layers (mean ± SEM, n = 4 animals per group). ***P < 0.001; **P < 0.01; *P < 0.05; ns, not significant.
keratinocytes. Two weeks after tam administration, E4f1 KO epidermis displayed approximately the same number of EdU^+ LRCs than control epidermis (0.25 ± 0.05 vs. 0.23 ± 0.02 per millimeter, respectively). However, the number of E4f1 KO LRCs in a suprabasal position was significantly increased compared with LRCs of control epidermis that remained, as expected, in the basal layer (suprabasal: 0.1 ± 0.04 vs. 0.01 ± 0.01; basal: 0.14 ± 0.03 vs. 0.21 ± 0.003 per millimeter, respectively) (Fig. 4G).

Interestingly, this atypical feature of E4f1 KO LRCs was particularly evident within focal epidermal lesions exhibiting MCT4 positivity, whereas LRCs remained in contact with the BM in the adjacent MCT4+ areas of the same epidermis (Fig. 4G). Five weeks after tam administration, the number of LRC diminished in E4f1 KO epidermis, confirming that E4f1 inactivation finally ended in the exhaustion of the ESC pool (Fig. 1D). Thus, these data show that the metabolic reprogramming triggered by E4f1 inactivation in basal keratinocytes associates with the remodeling of their microenvironment and alterations of the BM, leading to the loss of attachment of the ESC within their normal niche and their definitive loss.

**Discussion**

Our analyses performed in different mouse models where E4f1 was genetically inactivated in the basal or the spinous layers of the epidermis show that the complex skin phenotypes observed upon E4f1 inactivation originate from defects in basal keratinocytes. Our results indicate that E4f1 deficiency in these cells leads to a metabolic reprogramming of keratinocytes that affects skin homeostasis and ended in the definitive exhaustion of the ESC pool. We found that this metabolic shift, which includes the redirection of the glycolytic flux toward lactate production, is a direct consequence of PDH deficiency. Moreover, our data identify DLAT, the E2 subunit of the PDC, as an essential component of this metabolic program regulated by E4f1 in keratinocytes.

Whether E4f1-mediated control of the PDC in keratinocytes is clinically relevant remains to be determined. It is worth noting, however, that a homozygous nonsynonymous mutation in the coding region of the E4f1 gene has been recently identified in a patient presenting clinical symptoms resembling those of Leigh syndrome patients (27). Although skin abnormalities have been reported only in some Leigh syndrome patients (28), they are part of the broad spectrum of clinical manifestations that are commonly observed in several mitochondrial disorders (29). Further investigations are necessary to evaluate whether E4f1-mediated control of mitochondrial activities, which likely extend beyond the control of the PDC, contribute to the skin manifestations observed in these patients.

Another pathological situation that has been associated with changes in PDH activity is cancer. Interestingly, the metabolic rewiring of E4f1 KO keratinocytes is reminiscent of the one observed in many cancer cells that display increased aerobic glycolysis, even in high oxygen conditions, an effect known as the Warburg effect. It is well established that PDH deregulation in cancer cells can result from posttranslational modifications of PDC subunits by inhibitory kinases (PDKs), activating phosphatases (PDPs), or the lipooxidase SIRT4 (30, 31). We failed to detect deregulation of Pdks and Pdps mRNA levels in E4f1 KO keratinocytes, and our data rather support the notion that transcriptional control of Dlat is the main mechanism by which E4f1 controls PDH activity in normal epidermal cells. It remains to be seen whether E4f1-mediated control of Dlat is an alternative regulatory mechanism of the PDC in skin cancer cells. Nevertheless, our data clearly show that the control of PDH activity by E4f1 in basal keratinocytes is essential for normal skin homeostasis.

Interestingly, as with cancer cells, we show that the metabolic reprogramming of E4f1 KO basal keratinocytes results in increased activity of ECM-remodeling enzymes, including MMPs and cathepsins. The exact molecular mechanism by which increased glycolysis activates MMP activity in cancer cells remains controversial. Previous studies have suggested that the MCT-chaperone CD147/BASIGIN increases MMP activity through a yet undefined mechanism (32). However, recent data contradict this working model (33). Whatever the mechanism, the high glycolytic profile and increased activity of tissue-remodeling enzymes of fully transformed cells have been associated with their increased migratory and invasive properties that contribute to metastatic dissemination. Our data show that the metabolic reprogramming of normal E4f1 KO keratinocytes recapitulates some features of cancer cells, including their ability to induce the focal degradation of the basement membrane and to remodel their microenvironment. Here, we show that these alterations impact on ESC maintenance within their niche, leading to their mechanical elimination and ending in the complete exhaustion of the ESC pool. Interestingly, we previously reported that the ability of E4f1 to control the Bmi1–ARF–p53 pathway partly contributes to ESC self-renewal (7). These data raise interesting questions regarding the connection between the metabolic reprogramming of E4f1-deficient keratinocytes and the deregulation of the p53 pathway in these cells. The potential crosstalk between PDH activity and the control of the p53 pathway is a promising hypothesis that warrants further investigation.
It was recently proposed that basal keratinocytes rely more on glycolysis to sustain their energetic demand than their differentiated progeny in which mitochondrial-reactive oxygen species trigger epidermal differentiation through Notch and β-catenin signaling (6). Our data do not necessarily contradict this model, but provide clear evidence that when glycolysis is further increased in basal keratinocytes, such as in E4F1-deficient cells, this profoundly alters epidermal homeostasis and ESC maintenance. Our results also question the mechanisms leading to the inhibition of keratinocyte differentiation observed in E4f1KO epidermis.

Altogether our results identify E4F1 as an essential regulator of the metabolite status of basal keratinocytes and stress the importance of a tight control of the PDH activity for epidermal homeostasis.

Materials and Methods

Generation of Mouse Models and Experimental Treatment. Generation of E4f1 KO and E4f1 KO sc mice was previously described (7, 19). These mice were intercrossed with K14CreER (20) or K10CreR (21) mice to generate experimental groups (E4f1−/−, K14CreER, E4f1−/−, K14CreER, E4f1−/−, K10CreR (K14KO mice were previously described (7) were approved by the regional ethics committee for animal welfare (Comité éthique pour l’expérimentation animale de Languedoc Roussillon, protocol 12068). Oligonucleotides used for genotyping these animals are provided in SI Materials and Methods.

Histology, IHC, and Immunolabeling of Skin Sections. IHC and immunolabeling of skin sections were performed as previously described (7) using the following antibodies: anti-DLAT (sc-32925 Santa Cruz), MCT4 (sc-502329 Santa Cruz), BAG51 and (G-19 sc-9757, Santa Cruz), Laminin V (generous gift from C. Fera’s laboratory, University of Nice, Nice, France), Int1 (553745 BD Pharmingen), K14 (PRB-155P Covance), K10 (PR-BBP-159P Covance), Involucrin (sc-15230 Santa Cruz).

Culture of Primary Keratinocytes. Murine primary keratinocytes were isolated from newborn skin as previously described (7) and grown in calcium-free Eagle’s MEM (Bio-Whittaker; Lonza) supplemented with 8% (vol/vol) calcium-free FBS (Sigma). Cre-mediated recombination was achieved by adding 4-hydroxy Tamoxifen (40HT, Sigma; 1 μM final) to the culture medium.

Lactate, Ketone Bodies, and PDH Activity Measurement. Lactate and ketone bodies concentration were measured from tail blood samples using a lactometer (EKF Diagnostics) and β-ketone strips (Optimum, Abbott). PDH activity was measured with PDH Enzyme Activity Dipstick Assay Kit (Abcam) and PDH Activity Colorimetric Assay kit (Biovision) according to the manufacturers’ recommendations.

Statistical Analyses. The unpaired Student’s t test was used in all analyses. Statistical significance was expressed as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

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