RACK1 Prevents the Premature Differentiation of Epidermal Progenitor Cells by Inhibiting IRF6 Expression

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TO THE EDITOR

To properly maintain barrier function, epidermal stem and progenitor cells, which reside in the basal layer of the skin, continually proliferate and differentiate to contribute to the outermost layers. Perturbations in the balance between stem cell self-renewal and differentiation leads to a variety of skin disorders (Blanpain and Fuchs, 2009). Thus, it is critical to understand the factors that govern basal layer cell fate decisions. We and others have identified IRF6, ZNF750, KLF4, and GRHL3 as transcription factors necessary to transition basal layer cells to a differentiated one (Oberbeck et al., 2019, Segre et al., 1999, Sen et al., 2012, Ting et al., 2005). However, less is known about how the levels of these transcription factors are regulated to prevent premature differentiation of stem and progenitor cells. To identify putative factors enriched in epidermal progenitor cells, we mined our previous gene expression profiling data comparing undifferentiated and differentiated primary human keratinocytes (Sen et al., 2010). Interestingly, the receptor for activated C kinase 1 also known as RACK1 (GNB2L1) was highly downregulated upon epidermal differentiation.

RACK1 is a WD40 containing scaffolding protein that was originally identified as an anchoring protein for activated protein kinase C (Ron et al., 1994). As a scaffolding protein, it has been found to be associated with a large variety of proteins to affect a wide range of cellular processes (Li and Xie, 2015). RACK1 knockout mice are embryonic lethal at the gastrulation stage, which has precluded further examination of RACK1 in normal tissue homeostasis (Volta et al., 2013). Currently, it is unclear whether RACK1 has any role in stem cell fate decisions.

To answer this question, we use human skin as a model system. Staining of adult human skin showed that RACK1 is expressed primarily in the basal layer (Figure 1a). Notably, the vast majority of RACK1 did not co-localize with keratin 1 (K1) which is expressed in the differentiated layers of the skin (Figure 1a). In primary human keratinocytes, RACK1 mRNA and protein levels are downregulated upon induction of differentiation with calcium (Figure 1b). To test the function of RACK1, we knocked down RACK1 using two distinct shRNAs (RACK1i-A and RACK1i-B). Significant depletion of RACK1 as compared to scrambled shRNA controls (CTRi) was achieved on both the mRNA and protein levels (Figure 1c). Loss of RACK1 expression resulted in the spontaneous upregulation of epidermal differentiation genes and inhibited cell proliferation without impacting apoptosis (Figure 1d-f). Together, these data suggest that the loss of RACK1 led the cells to prematurely differentiate and exit the cell cycle.

Next, we analyzed the gene expression profile of RACKi in comparison to CTRi keratinocytes grown in proliferation conditions. The loss of RACK1 resulted in the differential
expression of 1,221 genes (573 upregulated and 648 downregulated) (Supplementary Figure S1a and Supplementary Table S1). Genes with decreased expression were enriched in gene ontology (GO) terms such as DNA replication and cellular macromolecule biosynthesis (Supplementary Figure S1b). Upregulated genes were enriched in GO terms such as epidermis development and establishment of skin barrier (Supplementary Figure S1b). Premature expression of these differentiation genes may be due to the increased expression of transcription factors such as IRF6, KLF4, GRHL3, and ZNF750 (Supplementary Figure S1c, Supplementary Table S1). ARCHS4 analysis of the 573 genes with increased expression upon RACK1 depletion identified IRF6, GRHL3 and ZNF750 as the top 3 transcription factors to be co-expressed with this expression profile (Supplementary Figure S1d) (Lachmann et al., 2018). To confirm that RACK1 is regulating the transcriptional levels of these proteins (as opposed to post-transcriptional regulation), we quantified the formation of newly synthesized IRF6, GRHL3, KLF4, and ZNF750 RNA in CTRi and RACKi cells by 5-ethynyluridine (EU) labeling. The loss of RACK1 resulted in a significant increase in nascent IRF6, GRHL3, and ZNF750 mRNA (Supplementary Figure S1e). Thus, RACK1 acts as a transcriptional repressor of transcription factors required for epithelial cell differentiation.

We performed double knockdown of RACK1 and IRF6 to determine if premature differentiation due to loss of RACK1 can be rescued. IRF6 was chosen because it is an early epidermal differentiation transcription factor directly downstream of P63 (Moretti et al., 2010). Depletion of RACK1 and IRF6 prevented the increase in IRF6 mRNA and protein levels observed with just RACK1 knockdown (Figure 2a-b). Preventing the increase in IRF6 levels in RACK1i keratinocytes also blocked the expression of differentiation genes such as TGM1, DSG1, SPPR1A, LCE3D, FLG, GRHL3, KLF4, and ZNF750 (Figure 2c). To assess the extent of rescue as a result of IRF6 knockdown in RACK1i cells, we assayed 60 additional genes that were differentially expressed upon RACK1 depletion (Supplementary Figure S2 and Supplementary Table S1). Of the 36 genes increased, 92% (33/36) were restored similar to control (CTRi) levels upon RACK1 and IRF6 double knockdown (Supplementary Figure S2a-b). The 3 genes that were still increased in expression included MTRNR2L2, CALB1, and PAK3 (Supplementary Figure S2a-b). Of the 24 genes downregulated upon RACK1 loss, 67% (16/24) were restored to control levels upon double knockdown (Supplementary Figure S2c-d). The genes that were not rescued include MCM7, ASF1B, ETV1, PLLP2, PEG10, PLLP, APLN, and CPVL (Supplementary Figure S2c-d). Overall, 82% (49/60) of the differentially expressed genes were restored similar to control levels upon double IRF6 and RACK1 knockdown. To explore if knockdown of IRF6 and RACK1 will rescue the RACK1i phenotype in a tissue setting, we
regenerated human skin for all three groups. We chose an early timepoint (Day 3) of skin regeneration where terminal differentiation proteins filaggrin (FLG) and loricrin (LOR) are not yet expressed in control tissue (Figure 2d). In the absence of RACK1, the skin was prematurely differentiated as FLG and LOR could be readily detected. Depletion of IRF6 and RACK1 simultaneously prevented the expression of the terminal differentiation proteins, suggesting that RACK1 prevents premature differentiation in epidermal progenitor cells by repressing IRF6 expression (Figure 2d).

IRF6 is upregulated during epidermal differentiation by P63 however it is unclear how IRF6 levels are repressed in undifferentiated keratinocytes (Moretti et al., 2010). Here we show that RACK1 suppresses IRF6 expression in epidermal progenitor cells where RACK1 protein levels are the highest. Upon knockdown of RACK1, levels of newly synthesized IRF6 mRNA is significantly increased. Furthermore, RACK1 prevents premature differentiation of epidermal stem and progenitor cells partly by repressing IRF6 expression since loss of IRF6 and RACK1 rescues ~82% of the RACK1i differentially expressed genes that were tested. Since double knockdown of IRF6 and RACK1 doesn't completely rescue all the genes affected by RACK1 depletion, it is likely that RACK1’s phenotype is due to the premature expression of a combination of transcription factors. These other factors could include GRHL1, OVOL1, KLF4, GRHL3, ZNF750, and DSP which all increase in mRNA expression and are co-expressed with the genes differentially expressed upon RACK1 loss (Supplementary Figure S1c-d, Supplementary Table S1).

In summary, we have found a basal layer enriched protein that is critical for preventing premature differentiation by inhibiting the expression of differentiation promoting transcription factors.
DATA AVAILABILITY
Datasets related to this article can be found at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE181265, hosted at the Gene Expression Omnibus (GEO): GSE181265.

CONFLICTS OF INTEREST
The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTIONS
Conceptualization: JL, GLS; Data curation: JL, MT, YC; Formal Analysis: JL; Investigation: JL, MT, YC; Funding acquisition: GLS; Writing-Original Draft Preparation: JL, GLS; Writing-Review or Editing: JL, GLS.

Patient consent for experiments was not required because primary human keratinocytes were purchased from Life Technologies (C0015C).
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FIGURE LEGENDS

**Figure 1.** RACK1 is required for maintaining epidermal progenitor cell function.
(a) Immunofluorescent staining of human skin with antibodies against RACK1 (red) and Keratin 1 (K1: green), a marker of the suprabasal layer. Merged image includes Hoechst 33342 staining of nuclei. n=3. Scale bar=10um. (b) RACK1 mRNA and protein expression in proliferating (-Ca^{2+}) and differentiated (+Ca^{2+} day 3) primary human keratinocytes maintained in 2D culture. For RT-qPCR analysis of RACK1 mRNA levels, expression was normalized to L32. n=3. Data represented as mean ± SD. ** p<0.01 vs proliferating (-Ca^{2+}) cells (unpaired t-test). For Western blot of RACK1 protein levels, β-actin is shown as loading control. Representative image is shown and n=3. (c) Primary human keratinocytes were knocked down with control (CTRi) or RACK1 (RACK1i) shRNAs and the remaining RACK1 mRNA and protein levels were measured by RT-qPCR and Western blotting respectively. Two separate shRNAs (RACKi-A, RACK1i-B) targeting different regions of RACK1 were used. RACK1 mRNA expression was normalized to L32 levels. n=3. Data represented as mean ± SD. *** p<0.001 vs CTRi (unpaired t-test). For Western blot of RACK1 protein, β-actin is shown as loading control. Representative image is shown and n=3. (d) RT-qPCR analysis of the relative mRNA expression of epidermal differentiation genes in CTRi and RACK1i cells. Cells were cultured in proliferation conditions. Expression of differentiation genes were normalized to L32 levels. n=3. Data represented as mean ± SD. * p<0.05 vs CTRi (unpaired t-test). (e) Proliferation assay of CTRi vs RACK1i cells. Plotted values represent the relative increase in fluorescence at each timepoint relative to starting point (day 0). n=3. Data represented as mean± SD. ** p<0.01 vs CTRi (1-way ANOVA). (f) Percent of apoptotic cells, comparing CTRi and RACK1i cells, were analyzed by flow cytometry. Only cells positive for Annexin-V and negative for Propidium Iodide was considered as apoptotic. 6000 cells per measurement. n=3. Data represented as mean ± SD. ns= not significant.

**Figure 2.** RACK1 inhibits IRF6-mediated differentiation of epidermal progenitor cells.
(a) RACK1 and IRF6 mRNA expression in primary human keratinocytes transfected with control (CTRi), RACK1 (RACK1i), or RACK1(RACK1i) plus IRF6 (IRF6i) siRNA in 2D culture. Cells were cultured in proliferation conditions. For RT-qPCR analysis, L32 was used for internal normalization. n=3. Data represented as mean ± SD. * p<0.05, ** p<0.01 (unpaired t-test). (b) Western blot of samples described in (a) for RACK1 and IRF6 protein levels. β-actin is shown as loading control. Representative image is shown and n=3. (c) RT-qPCR analysis of the relative mRNA expression of epidermal differentiation genes for samples described in (a). L32 was used as an internal normalization control. n=3. Data represented as mean ± SD. * p<0.05, ** p<0.01, *** p<0.001 vs CTRi (1-way ANOVA). (d) Immunofluorescent staining of late differentiation marker filaggrin (FLG: green) and loricrin (LOR: red) in regenerated human skin (day 3). Nuclei was stained with Hoechst (blue). Scale bar =20um. Quantification of FLG and LOR signal intensity is shown to the right of their respective images. n=3. Data represented as mean ± SD. * p<0.05 vs CTRi (1-way ANOVA).
**Supplementary Figure S1.** RACK1 represses differentiation gene transcription in epidermal progenitor cells.

(a) RNA-Seq analysis of primary human keratinocytes infected with control (CTRi) and RACK1 (RACK1i-A, RACK1i-B) shRNAs. Samples were performed in biological duplicates. Cells were grown in proliferation conditions. Significantly changed genes were identified with ≥1.5 fold change and p≤0.05 (DESeq2). Heatmap of genes that are significantly increased (red) or decreased (blue) are displayed. (b) Top gene ontology (GO) terms for the genes significantly upregulated (573 genes) and downregulated (648 genes) upon RACK1 knockdown. (c) RT-qPCR analysis of the relative expression of epidermal differentiation genes. RT-qPCR was performed with epidermal progenitor cells transfected with siRNAs targeting control (CTRi) or RACK1 (RACKi) sequences. n=3. Data are graphed as mean ± SD. **p < 0.01, ***p < 0.001 vs CTRi (unpaired t-test) (d) The top 10 transcription factors co-expressed with the 573 genes upregulated upon RACK1 knockdown. The analysis was performed using ARCHS4. (e) Nascent RNA expression analysis of genes coding for differentiation promoting transcription factors analyzed in (c) was measured in CTRi and RACKi cells using in vivo EU labeling and qPCR of newly synthesized RNA. n=3. Data are graphed as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 vs CTRi (unpaired t-test).
Supplementary Figure S2. RACK1 and IRF6 double knockdown restores most of the gene expression of RACK1i phenotype to control levels. (a-b) Primary human keratinocytes were transfected with control (CTRi), RACK1 (RACK1i) or RACK1 (RACK1i) plus IRF6 (IRF6i) siRNA in 2D culture. Cells were cultured in proliferation conditions. RT-qPCR analysis of the relative mRNA expression of genes upregulated in RACK1i cells. (c-d) RT-qPCR analysis of the relative mRNA expression of genes downregulated in RACK1i cells. Differentially expressed genes chosen in Supplementary Figure 2 were obtained from the RNA-Seq on RACK1 knockdown cells (Supplementary Figure 1 and Supplementary Table 1). n=3. Data represented as mean ± SD. * p<0.05, ** p<0.01, *** p<0.001 vs CTRi (1-way ANOVA) for the entire figure.
Figure 1

(a) Images showing RACK1 expression and K1 expression with a merged image.

(b) Graph showing RACK1 mRNA expression levels with fold change compared to -Ca^{2+}.

(c) Graph showing RACK1 mRNA expression levels with fold change compared to CTRi.

(d) Bar chart showing mRNA expression levels for various proteins with fold change compared to CTRi.

(e) Line graph showing proliferation fold change over days.

(f) Bar chart showing apoptotic cells percentage over days.
Figure 2

(a) mRNA expression

(b) Protein expression

(c) mRNA Expression

(d) Immunofluorescence
METHODS

Cell culture
Primary human epidermal keratinocytes derived from neonatal foreskin (Thermo Fisher Scientific; C0015C) were cultured in EpiLife medium (Thermo Fisher: MEPI500CA) supplemented with human keratinocyte growth supplement (HKGS, Thermo Fisher: S0015). Keratinocytes were differentiated by seeding in full confluence with 1.2 mM calcium for 3 days. Amphotropic phoenix cells (ATCC CRL-3214) were cultured in DMEM with 10% fetal bovine serum. All cells were maintained in a 37°C and 5% CO₂ incubator.

Gene silencing
Knockdown of RACK1 in primary human keratinocytes was achieved with short hairpin RNA (shRNA) or small interfering RNA (siRNA). For shRNA dependent knockdown, shRNAs targeting RACK1 were first cloned into the pSuper retroviral vector (Sen et al., 2004). The resulting retroviral construct (3 μg) were then transfected into amphotropic phoenix cells using Lipofectamine 2000 (Life Technologies: 11668027). Supernatant containing the retrovirus were collected at 48 h post transfection and used to infect keratinocytes. Infection of the keratinocytes occurred over 2 consecutive days. Each day, the viral supernatant, plus 5 μg/ml polybrene (SigmaAldrich; H9268), were placed on the cells and centrifuged at 1700 g for 1 h. Selection of infected cells occurred in medium supplemented with 1 μg/ml puromycin. The sequence for shRACK1-A and shRACK1-B is 5’-GGATGAGACCAACTATGGA-3′ and 5’-GAGATAAGACCATCATGGA-3′ respectively. The control shRNA construct was generated as previously described (Sen et al., 2004, Sen et al., 2010).

For siRNA mediated knockdown, lipofectamine RNAiMAX (Thermo Fisher 13778) was used. 25μl of RNAiMAX and 10nM siRNA (final concentration) were used for each 10cm plate according to manufacturer’s instructions. The siRNAs used are as follows: Control siRNA (Ambion Silencer Select negative control 4390844), RACK1 siRNA (Dharmacon M006876-01), and IRF6 siRNA (Dharmacon M012227-01).

Regenerated human skin
Human skin equivalents were generated by seeding 10⁶ genetically modified primary human keratinocytes onto devitalized human dermis as previously described (Li and Sen, 2015, Li et al., 2021). The tissue was cultured at the air–liquid interface for 3 days and then harvested for RNA or placed in OCT for cryo-sectioning.

Gene expression analysis
Total RNA was extracted from cells and regenerated human skin using GeneJET RNA purification kit (Thermo Scientific K0732) according to manufacturer’s instructions. Nanodrop was used to measure RNA concentration. A total of 1μg of RNA was reverse transcribed using Maxima cDNA synthesis kit (Thermo Fisher: K1642) and quantitative PCR was performed using the Bio-Rad CFX96 machine. L32 was used as internal control for normalization. Sequence of primers can be found in the primer sequences excel file.

Nascent RNA capture and expression analysis
Newly synthesized RNA was captured using Click-iT Nascent RNA Capture kit (Thermo Fisher C10365) according to manufacturers instructions and expression was measured by qPCR. In brief, cells were labelled with EU (0.2mM) 4 days after transfection. After 4 hrs of incubation with EU, total RNA was isolated from cells using TRIzol (Invitrogen 15596026) and Zymo Directzol RNA Miniprep kit (Zymo Research R2052). 1ug of total RNA was biotinylated with biotin azide and 500ng of biotinylated total RNA was incubated with streptavidin magnetic beads to
isolate newly synthesized RNA. Bead captured RNA was immediately used as template for cDNA synthesis and analyzed by qPCR as stated above.

**Western blot**

Cells were homogenized in RIPA buffer (Thermo Fisher, 89900) containing phosphatase inhibitor (Thermo Fisher, A32957) and protease inhibitor (Sigma-Aldrich, 11836170001). Protein concentration of the lysate was determined using BCA protein assay kit (Thermo Fisher, 23225). 30μg of protein per sample was resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 3% BSA plus 0.5% Tween 20 in PBS and incubated in primary antibody overnight at 4°C. The primary antibodies used were against the following proteins: RACK1 (Santa Cruz, sc-17754) at 1:1000, IRF6 (Cell Signaling, 6948), and b-actin (Santa Cruz Biotechnology, SC-47778) at 1:5000. After washing, the membranes were incubated with secondary antibody, and visualized with LICOR Odyssey Imager and Image Studio Lite software. The secondary antibodies used were IRDye 680RD Donkey anti-rabbit IgG (LICOR: 926-68073) at 1:10,000 and IRDye 800CW donkey anti-mouse IgG (LICOR: 926-32212) at 1:10,000.

**Apoptosis assay**

Cells were trypsinized and stained with Annexin V Alexa Fluor 488 (Life Technologies: A13201) and Propidium Iodine (BD Biosciences 550825) for 15 minutes at room temperature. Stained cells were analyzed by flow cytometry using Guava Easycyte 8HT (Millipore). Apoptotic cells were defined as Annexin V positive and PI negative cells. At least 6000 cells were counted for each sample in triplicate.

**Cell proliferation assay**

5,000 cells were plated on a 24-well plate in triplicate for each condition and each time point. Media was changed every 48 h. Cell abundance at each time point was assessed using AlamarBlue (Thermo DAL1025) according to manufacturer’s instructions. Fluorescence was measured following 2 h incubation at 37°C using SpectraMax iD3 microplate reader (Molecular Devices). To generate the proliferation curve, fluorescent signal at day 1 was set to 1 and subsequent measurements were calculated relative to day 1 fluorescence signal.

**Immunostaining**

For immunofluorescence staining of cryosectioned tissue, cryostections (6μm) were fixed in 10% buffered formalin, blocked in 2.5% goat serum, 0.3% Triton X-100 and 3% bovine serum in PBS, and incubated with primary antibody (diluted in blocking buffer) overnight at 4°C. The primary antibodies used were against: RACK1 (Santa Cruz, sc-17754) at 1:100, Keratin1 (Biolegend: PRB-149p) at 1:500, Filaggrin (Abcam, ab218397) at 1:500, and Loricrin (Abcam 198994) at 1:500. After 3 washes with PBS, bound primary antibody was detected by fluorescent-dye-conjugated secondary antibodies. These secondary antibodies include: Alexa Fluor 555-conjugated goat anti-mouse IgG (Thermo Fisher: A11029) at 1:500, Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Thermo Fisher, A21206) at 1:500, Alexa Fluor 555-conjugated goat anti-rabbit IgG (Thermo Fisher: A21429) at 1:500, Alexa Fluor 488-conjugated goat anti-mouse IgG (Thermo Fisher, A11001) at 1:500. The nuclei were counterstained with Hoechst 33342 in PBS (1:1000, Thermo Fisher, H3570). Slides were then washed 3 times with PBS and mounted in Fluoromount (Thermo Fisher, 00-4958-02). Images were taken with an Olympus Dx71 camera mounted to a fluorescence microscope.
RNA sequencing and analysis

Control (CTRi) and RACK1 (RACK1i) knockdown samples (transduction with retroviruses expressing shRNAs) were cultured in proliferation conditions for 7 days and harvested for RNA-Seq. Sequencing of extracted RNA was performed on the Illumina Hi Seq 2000, carried out by the Scripps Next Generation Sequencing Core Facility. Reads were aligned to the GENCODE v19 transcriptome hg19 using TopHat2 (Kim et al., 2013) with default settings. Normalization of reads and differential gene expression between samples was calculated by DESeq2 (Love et al., 2014) using Partek Flow (Partek Incorporated, http://www.partek.com/partek-genomics-suite). Enrichr (Kuleshov et al., 2016) was used to generate gene ontology (GO) terms for lists of differentially expressed genes. The statistical threshold of $p \leq 0.05$ and ≥1.5 fold change was used to create a gene list of differentially upregulated and downregulated genes in comparison to control samples. Partek Flow was used to generate heatmaps representing relative expression of genes. ARCHS4 (Lachmann et al., 2018) was used to identify co-expressing transcription factors from the list of differentially upregulated genes identified in RACK1i keratinocytes (Lachmann et al., 2018).

Statistical analysis

All data are presented as mean±s.d. and analyzed using Graphpad Prism. Statistical significance was assessed using two-way ANOVA when comparing multiple treatments between two or more groups, one-way ANOVA when comparing multiple treatments within a single group or an unpaired t-test when comparing two treatments.

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