Glucocorticoid receptor and Klf4 co-regulate anti-inflammatory genes in keratinocytes

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Abstract

The glucocorticoid (GC) receptor (GR) and Kruppel-like factor Klf4 are transcription factors that play major roles in the skin homeostasis by regulating the expression of overlapping gene subsets. However, whether these transcription factors cooperate in binding genomic regulatory regions in epidermal keratinocytes was not known. Here, we show that in dexamethasone-treated keratinocytes GR and Klf4 are recruited to genomic regions containing adjacent GR and KLF binding motifs to control transcription of the anti-inflammatory genes *Tsc22d3* and *Zfp36*. GR- and Klf4 loss of function experiments showed total GR but partial Klf4 requirement for full gene induction in response to dexamethasone. In wild type keratinocytes induced to differentiate, GR and Klf4 protein expression increased concomitant with *Tsc22d3* and *Zfp36* up-regulation. In contrast, GR-deficient cells failed to differentiate or up-regulate *Klf4* and *Zfp36* correlating with increased expression of the epithelium-specific *Trp63*, a known transcriptional repressor of Klf4. The identified transcriptional cooperation between GR and Klf4 may determine cell-type specific regulation and have implications for developing therapies for skin diseases.

Key words: glucocorticoid receptor, Kruppel-like factor 4, keratinocyte, transcription, inflammation.

Introduction

Glucocorticoid (GC) hormones act through the GC receptor (GR), a ligand-dependent transcription factor (TF) that regulates gene expression in virtually all cell types. The mechanisms of GR action include transactivation, direct binding to GC responsive element (GRE)-containing genes; and transrepression, interference with other transcription factors through protein-protein interactions (1, 2). GR can also repress gene expression by directly binding to recently identified simple negative GREs and recruiting transcriptional corepressors (3). GR controls a vast array of physiological processes including modulation of the inflammatory response, as well as epidermal differentiation and homeostasis (2, 4). Identifying mechanisms for the tissue-specific actions of GR is of great interest due to the widespread use of GCs as therapeutic agents, such as in inflammatory skin diseases, where prolonged usage results in undesirable side effects, including skin thinning and delayed wound healing (1, 4).

Combinatorial action of GR with cell type-specific TFs is a way to attain diverse physiological outcomes (5). While the majority of ligand-bound GR associates with pre-existing sites of open chromatin (6), it can also bind to transcriptionally silent chromatin acting as a “pioneer factor” (7). GR binding has been shown to facilitate the binding of other TFs via “assisted loading”, a process whereby GR-dependent recruitment of chromatin remodeling proteins change local nucleosome structure creating a more accessible state (8).

The Krüppel-like family of transcription factors (KLF) is formed by a class of DNA-binding proteins with a key role in development and physiology. KLF family members are known to functionally interact with other members of the nuclear hormone receptor superfamily, in a tissue specific fashion (9). Klf4 is critical for function of differentiated epithelial cells consistent with its high expression in the epidermal suprabasal layers (10). Klf4 is also one of the four factors that reprogram differentiated cells to pluripotency (11). How Klf4 is capable of regulating such diverse processes is not completely understood. Along these lines, a recent study in human keratinocytes demonstrated that Klf4 forms a complex with the epithelial-specific TF ZNF750 and chromatin regulators RCOR1 and CTBP1/2 to promote transcription of genes associated with terminal differentiation (12).

Genetic experiments established that both GR and Klf4 are critical for establishing the skin's barrier function. For instance, mice deficient in the expression of either Klf4 or GR have defective epidermal differentiation and permeability barrier acquisition (13, 14). Antenatal GC injections accelerate *in utero* barrier formation as does epidermal (keratin 5 (K5) promoter) overexpression of Klf4 (15, 16). The observation that GC treatment of developing K5-Klf4 mice further accelerated formation of the epidermal barrier suggested synergistic actions of GR and Klf4 (17). Further evidence for synergism was the identification of a gene subset similarly regulated by GC treatment or Klf4 overexpression (17). However, direct co-regulation of keratinocyte gene expression has not been demonstrated, in part due to the absence of a suitable model to evaluate GR-mediated gene regulation (14, 17). This has been recently overcome by the generation of GR epidermal knock-out (GR^{EKO}) mice in our group (18). Newborn GR^{EKO} epidermis showed aberrant expression of genes involved in epidermal barrier function, innate immunity and inflammation, many of which are also deregulated in inflammatory skin diseases, such as atopic dermatitis and psoriasis (18). These data suggest that GR may be a link between defective epidermal differentiation and inflammation during development.

In postnatal skin, the role of GR and Klf4 has been evaluated using gain or loss of function models restricted to K5 or K14 expressing tissues. In the case of GR, overexpression results in resistance to epidermal inflammation and tumorigenesis (4, 19), while loss provokes an increased sensitivity to inflammatory stressors and skin carcinogenesis (18, 20). Likewise, inducible deletion of Klf4 in the epidermis results in increased DMBA/TPA induced tumorigenesis (21). Inducible activation of Klf4 in the epidermal basal layer unexpectedly resulted in epithelial dysplasia (22), though this discrepancy may be due to the fact that depending on cellular context Klf4 can act as a tumor suppressor or oncogene (23).

In this study, we present evidence demonstrating that GR and Klf4 are recruited to GC- and KLF-responsive elements on a subset of GC-regulated genes to modulate transcription in keratinocytes. Both TFs bind to regions near the anti-inflammatory genes *Tsc22d3* and *Zfp36*, and cooperate in their regulation. Furthermore, we found that *Tsc22d3* and *Zfp36* are up-regulated during keratinocyte terminal differentiation, suggestive of a link between regulation of

inflammatory processes and terminal differentiation, or of novel roles for these genes in keratinocytes. As Klf4 is enriched in epithelial cells, it is conceivable that GR preferentially uses the GR binding sites (GBSs) adjacent to KLF sites in keratinocytes as a mechanism determining cell-type specific regulation.

Materials and Methods

Reagents and antibodies

Unless otherwise specified all chemicals are from Sigma-Aldrich. Primary antibodies used were specific for GR (sc-1004), Klf4 (sc-20691), p63 (sc-8431, Santa Cruz Biotechnology), phospho-GR Ser 211 (4161S, Cell Signaling Technology), tubulin (T6199, Sigma-Aldrich). Peroxidase-conjugated secondary antibodies were from GE HealthCare. HRP-conjugated Clean-blot IP Detection Reagent was used for immunoblotting immunoprecipitation experiments and was from Thermo Scientific. Secondary Alexa Fluor- conjugated secondary antibody was from Life Technologies.

Cell culture

Mouse primary keratinocytes (MPKs) were isolated and cultured as described (24), using complete EMEM: EMEM (Lonza) supplemented with 4% FCS (Biowest) previously treated with Chelex 100, 100 U/ml penicillin/100 µg/ml streptomycin (Biowest) and 2.5 µg/mL amphotericinB (Biowest). The skin of newborn (P0-P3) wild type mice was incubated in 0.25% trypsin at 4°C overnight. The epidermis was separated from the dermis, minced, and homogenized in complete EMEM with 0.05 mM CaCl₂. Samples were pooled and the resulting suspension was filtered through a cell strainer (BD Biosciences). Cells were counted and plated on collagen I (Gibco)-coated culture dishes in complete EMEM with 0.6 mM CaCl₂. The following day, cells were washed with DPBS (Biowest) and incubated in complete EMEM with 10 ng/mL EGF (Peprotech) and 0.05 mM CaCl₂ until nearly confluent.

GR^{EKO} and control keratinocyte cell lines were generated from 8-wk-old female mouse dorsal skin as previously described (18) and cultured on mitomycin C treated J2-3T3 feeders in collagen I-coated flasks in DMEM-Ham's F12 (3:1) medium (Gibco) supplemented with 1.8×10^{-4} mol/L adenine, 0.35 mM calcium, 7.5% FCS, 100 U/ml penicillin/100 µg/ml streptomycin (Biowest), 2 mM glutamine (Biowest), 0.25 µg/mL amphotericinB (Biowest), 5 µg/ml insulin, 10^{-10} M cholera toxin and 10 ng/ml EGF (Peprotech).

Prior to treatment with 100nM Dex, to deplete steroid hormones, cells were grown for 18-24 h in respective medium containing charcoal-stripped serum. To induce terminal differentiation, keratinocytes were incubated with medium containing 1.2 mM CaCl₂.

Chromatin Immunoprecipitation and ChIP-seq

ChIP experiments were performed as previously described (24), using MPKs or adult keratinocyte cell lines grown to confluency in 100 mm culture dishes (Thermo Scientific). Keratinocytes were incubated with freshly prepared 1% formaldehyde 8 minutes at room temperature, lysed and sonicated using the Bioruptor (Diagenode) to fragment chromatin. Immunoprecipitation was done with 3 µg of the indicated antibodies and Dynabeads Protein A (Life Technologies). After ChIP, reversion of cross-linking, and treatment with proteinase K and RNase, DNA was purified by phenol-chloroform extraction. Glycogen (Roche) was added to visualize pellet. QPCR (see below) was performed to assess the relative amplification of specified genomic sites in Dex vs mock-treated ChIPs, which were normalized to the amplification values of respective inputs. At least three biological replicates were used for each experimental group and technical triplicates were assessed to calculate the mean value ± SD. Statistical significance was calculated using the Student's t test.

GR ChIP-seq was performed as indicated by the manufacturer (Illumina-Solexa, <http://www.illumina.com>) (25). The quality of Input and ChIP DNA was first verified by Bioanalyzer (Agilent). Then, for library generation, oligonucleotide adapters were added to the DNA fragments which were then subjected to amplification and size selection. The resulting DNA quality was tested by QPCR at identical genomic regions as the original DNA. Next, DNA was sequenced by the Genome Analyzer II (Illumina) and data files processed using two different sequence alignment tools (Novoalign and MAQ) along with three different peak callers (SISSR, PeakRangers and MACS). The 123 selected peaks are those which had overlap between all algorithms and therefore are of high confidence (Table S1).

Peaks were assigned to genes using GREAT software. Gene ontology of genes with assigned peaks was carried out using DAVID (<http://david.abcc.ncifcrf.gov/>). Search of the

transcription factor binding motifs in identified GR-bound regions was done using MEME (<http://meme.nbcr.net/meme/>).

RNA isolation and Quantitative RT-PCR

Total RNA was isolated from cultured keratinocytes using Trizol (Life Technologies). RNA was treated with DNase (Thermo Scientific), for later use with primer pairs which bind within the same exon, and reverse transcribed using RevertAid H Minus Reverse Transcriptase and oligo dT primers (Thermo Scientific). QPCR using specific oligonucleotides (Table S2) and FastStart Universal SYBR Green Master ROX (Roche) was carried out in an Applied Biosystems 7500 Fast real time PCR system. At least three biological replicates were used for each experimental group and technical triplicates were assessed to calculate the mean value $\pm$ SD. Statistical significance was calculated using the Student's t test.

Klf4 knockdown

Keratinocytes were transfected with 25 nM *Klf4* specific (L04001010005) or Control (D0018101005) On-Target plus siRNA Smartpool (Thermo Scientific) using Lipofectamine 2000 (Life Technologies) and Optimem medium (Life Technologies). Transfection was carried out in DMEM-Ham's 12 medium without antibiotics or antimycotic. Medium was changed 5 hours after addition of siRNA-Lipofectamine 2000 complexes. Experiments were conducted 48 hours following transfection.

Immunoblotting

Keratinocytes were lysed in RIPA buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and samples were boiled in Laemmli buffer and separated on 8% SDS-PAGE (25 μ g/lane), then transferred to nitrocellulose membranes (Hybond ECL, GE Healthcare). Membranes were stained with Ponceau S to verify equal protein loading and transfer. Membranes were blocked with 5% nonfat dry milk in PBS-0.1% Tween 20, and incubated with primary antibodies overnight at 4°C. After washing three times

in PBS-0.1% Tween 20, membranes were incubated with peroxidase-conjugated secondary antibodies, washed again, and analyzed using ECL2 (Thermo Scientific) with the ImageQuant 4000 Biomolecular Imager (GE Healthcare). Experiments were quantitated using Image J software and were performed with a minimum of at least three biological replicates.

Immunoprecipitation

Keratinocytes were lysed in buffer containing 50 mM Tris pH 7.6, 150 mM NaCl, 5 mM EDTA, 0.5% Igepal CA-630 with protease and phosphatase inhibitors (Roche). Proteins were immunoprecipitated using 2 μ g of Klf4 specific antibody or rabbit IgG as a negative control and Dynabeads Protein A (Life Technologies). Following washing with buffer containing 0.2% Igepal CA-630, proteins were eluted from beads by boiling in Laemmli buffer for analysis by immunoblotting.

Results and Discussion

To understand how GR transcriptionally regulates keratinocyte homeostasis and differentiation, we performed ChIP-sequencing (ChIP-seq) analysis in mouse primary keratinocytes (MPKs) that were treated either with vehicle or Dex for 2h. Following peak calling, we identified GR bound to 123 regulatory sequences upon Dex treatment (Table S1).

Our data identified a limited number of GBSs relative to other ChIP-seq approaches that reported GBSs were within the range of thousands (26-28), however, subsequent validation confirmed the accuracy of the data (see below). The identified GR ChIP-seq peaks corresponded to both known and novel GR-target genes in keratinocytes. The anti-inflammatory genes *Tscd22d3/Gilz* and *Zfp36*, the circadian rhythm gene *Per1*, and the receptor tyrosine kinase-like orphan receptor *Ror1*, among others, have been previously reported as GR-targets in other cell types (29). In keratinocytes, these genes are known to be regulated by Dex, though the precise binding sites of GR and the mechanism of regulation in this cell type were unknown (30). Importantly, the relatively small number of GBSs identified in our ChIP-seq is in agreement with the finding that few genes were detected as having changes in expression in human keratinocytes treated with Dex for 1 or 4 h, as compared to later time-points where the expression of hundreds of genes was altered (30).

We first confirmed the regulation of identified genes by hormone-activated GR in an independent experiment in MPKs. ChIP of Dex or mock-treated (2h) cells using an anti-GR antibody followed by quantitative (Q)-PCR demonstrated direct, ligand dependent recruitment of GR to putative regulatory sequences of *Per1*, *Tscd22d3* and *Zfp36* (Fig. 1A). Differences were statistically significant, as assessed by Student's t test. Consistently, *Tscd22d3* and *Per1* expression was induced by 6-fold or more and *Zfp36* by almost 2-fold after hormone treatment (Fig. 1B). These results validated our ChIP-Seq data and confirmed that the identified high confidence peaks were “true” GR primary targets.

To overcome the inherent difficulties in working with primary keratinocytes (17), we established spontaneously immortalized adult keratinocyte cell lines (20). There are differences in the basic biology of keratinocytes depending on the developmental stage and whether they

are primary or immortal (31). Therefore, we evaluated whether Dex treatment induced similar GR recruitment and gene expression changes in our control adult cell line (CO) relative to primary newborn keratinocytes. We focused on *Tsc22d3* and *Zfp36* because they are known anti-inflammatory genes whose regulation and function in the epidermis are not well understood. Indeed, both GR recruitment (Fig. 1C) and gene induction of *Tsc22d3* and *Zfp36* (Fig. 1D, CO) occurred to a similar extent in the adult keratinocyte cell line as observed in newborn primary cells (Fig. 1A,B), indicating that in the case of these genes developmental stage (or primary or immortal status) does not affect regulation by GCs. We used a GR deficient cell line derived from adult GR^{EKO} epidermis to unequivocally determine the dependence of Dex-induced changes in gene expression on this TF (Fig. 1E; 20). In GR^{EKO} cells, neither *Tsc22d3* nor *Zfp36* were induced by Dex, showing that the presence of GR is necessary for this process (Fig. 1D, GR^{EKO}).

We searched the identified GR ChIP-seq peaks for the presence of consensus GRE (AGAACAAnnTGTTCT) using MEME (<http://meme.nbcr.net/meme/>) and found GRE motifs in 64% of putative regulatory regions (Table S1). Considering that GR binds to composite elements, we searched for co-occurrence of binding motifs of other TFs in the sequences of the GR bound peaks. In the 123 sequences analysed, several over-represented TF motifs, including KLF (43%) and AP-1 (28%) were identified (Table S1).

GR transrepression of AP-1 dependent transcription is well known, especially in the regulation of genes associated with inflammation (29). However, AP-1 can also facilitate the accessibility of GR to genomic binding sites by maintaining the chromatin in a more accessible conformation (32). In contrast, there are fewer examples for functional interactions between GR and KLFs, which occur in a tissue specific fashion, due to differing expression patterns of individual KLF members (9, 33). For instance, Klf11, in a neuronal cell line and Klf15 in a lung cell line were found to co-bind with GR to regulatory regions of target genes (34, 35). A very recent study in macrophages identified GR and KLF binding sites in genes encoding *Klf2* and *Klf9* and in the GR targets *Cited* and *Mt2* (33). Our ChIP-Seq data also suggest that GR and KLFs co-regulate a KLF member, as putative sites for both TFs were identified in *Klf13* (Table

S1). Aside from these specific examples, the significance of the cooperation between KLFs and GR is still limited.

We next focused on Klf4 as a possible candidate for binding KLF motifs in the identified regions bound by GR given the known involvement of both TFs in epidermal development and homeostasis. Comparison of previous gene expression studies has shown an extensive overlap between genes regulated by GR and Klf4, mostly related to epidermal differentiation (17, 18, 24, 36). Additionally, there is an overlap between direct GR targets identified here and Klf4 regulated genes in human keratinocytes (Table S1; 37).

GR-bound sequences near *Tscd22d3* and *Zfp36* contained consensus GREs as well as adjacent KLF motifs (Table S1 and Fig. 2A). We assessed Klf4 recruitment to putative *Tsc22d3* and *Zfp36* regulatory regions by ChIP-QPCR and found statistically significant increases in Klf4 binding to both (4.7-fold and 2.9-fold, respectively) upon Dex treatment of control cells (Fig. 2B). In GR-deficient keratinocytes, in the case of *Tsc22d3*, Dex-dependent recruitment of Klf4 was decreased by almost 3-fold relative to that observed in control cells. The slight induction (1.7 fold) of Klf4 binding to *Tsc22d3* in Dex-treated GR^{EKO} cells, was not statistically significant but may reflect a role of the mineralocorticoid receptor, closely related to GR and able to bind GCs. Importantly, the binding of Klf4 to *Zfp36* was greatly reduced both in the absence and presence of Dex (0.22 fold and 0.39 fold, respectively) in GR^{EKO} relative to control cells (Fig. 2B). The Dex- and GR-dependent recruitment of Klf4 to both genomic regions suggests cooperation between both TFs in regulating these anti-inflammatory genes. Given that several KLFs promote the anti-inflammatory program in macrophages, functional cooperation between GR and this family of TFs was postulated as a mechanism to counteract inflammation in these cells (33).

To further analyze the contribution of Klf4 to the regulation of *Tsc22d3* and *Zfp36*, we transfected the control cell line with siRNA specific for *Klf4* and achieved approximately 60% knockdown (Fig. 3). Dex-induction of *Tsc22d3* was decreased by approximately 2-fold in cells expressing the *Klf4* siRNA relative to control siRNA. Our results demonstrate that not only GR but also Klf4 is required for induction of *Tsc22d3* in the presence of Dex. While Klf4 was

required for *Tsc22d3* induction, decreased Klf4 levels did not affect that of *Zfp36*. This may be due to incomplete knockdown or to a different role of Klf4 in the regulation of this gene.

There is controversy regarding whether GCs regulate *Klf4* gene expression in epidermal keratinocytes. While *Klf4* mRNA was not up-regulated by antenatal GC treatments in developing mouse skin (17), it was induced by Dex treatment of cultured human keratinocytes (30). We assessed *Klf4* basal gene expression in adult cell lines and found decreased mRNA levels in GR^{EKO} vs control cells (Fig. S1); however, no *Klf4* induction was found in control keratinocytes treated with Dex for 3 to 24h (Fig. 3 and data not shown). In contrast, in other cell types such as macrophages, Klf4 is induced by Dex treatment possibly through GR recruitment to two putative GREs located at -3830 bp and +5896 bp relative to the Klf4 transcription start site (33). These differences may be due to cell-type specific expression of co-regulators.

We also evaluated whether GR and Klf4 associated upon treatment with Dex (Fig. S2). While a small amount of GR was detected in Klf4 immunoprecipitates in the absence of hormone, it did not increase upon Dex treatment, indicating a mechanism other than direct protein-protein interaction for GC-induced recruitment of Klf4 (Fig. S2). We speculate that the mechanism of Klf4 recruitment could be similar to the Dex-induced *de novo* remodeling of chromatin near GR binding sites (enriched for canonical GREs) and assisted loading of the TF C/EBP β observed in liver (28).

It is well known that treatment of cultured keratinocytes with high calcium concentrations (1.2 mM) induces cell differentiation. Consistent with its role in terminal differentiation, *Klf4* is induced in human primary keratinocytes grown in high calcium medium for 72h (37). GR protein has also recently been described to be up-regulated in keratinocytes cultured in high calcium conditions (38). To evaluate whether Klf4 induction can compensate for the loss of GR in keratinocytes, confluent control and GR^{EKO} cells were grown in high calcium (1.2 mM) containing medium for 0 or 72h (Fig. 4). Control keratinocytes underwent terminal differentiation in the presence of high calcium, featuring cell flattening, extensive cell-cell contacts, stratification and formation of cornified envelopes (Fig. 4A; asterisks). The expression of the differentiation marker *Sprr2d* was strongly induced in calcium-treated control

cells, as shown by RT-QPCR (Fig. 4B). In contrast, GR^{EKO} cells showed an aberrant morphology featuring spindle-like phenotype with decreased cell-cell contacts even in low calcium conditions (Fig. 4A). In high calcium medium, GR^{EKO} cells failed to properly differentiate, as seen by the lack of cornified envelopes (Fig. 4A) which was further confirmed by the lack of *Spr2d* induction (Fig. 4B).

Importantly, neither *Klf4* mRNA nor protein was induced in calcium treated GR^{EKO} cells, whereas both were induced in the control (Figs. 4B and 5C,D). We did not observe *Nr3c1* up-regulation in terminally differentiated control cells; however GR protein levels did show a modest increase in agreement with Yoon *et al.* ((38); Figs. 4B and 5C,D). Notably, we observed an increase of *Tsc22d3* and *Zfp36* expression in differentiating control cells (Fig. 4B). In contrast, in GR^{EKO} cells, calcium treatment did not result in up-regulation of *Zfp36*; and *Tsc22d3* showed a 3-fold decrease in induction relative to the control (Fig. 4B). We postulate that the decreased induction of these genes, in addition to that of *Klf4*, in GR^{EKO} cells may interfere with keratinocyte differentiation. For instance, the negative signaling of TSC22D3 on the RAS/RAF/ERK pathway (1, 2) could be important in halting proliferation during differentiation. While ZFP36 is best known for destabilization of *Tnf* and *Csf2*, currently there are more than 40 known targets including a wide range of cytokines, which can affect keratinocytes, and factors involved in cell cycle control such as Cyclin D1 (39).

As *Klf4* expression was not affected by short treatments with Dex (Fig. 3), we considered other mechanisms for its misregulation in GR^{EKO} cells. We previously observed that Δ Np63 expression, the predominant isoform of *Trp63* in epidermis (40), was reduced in oral epithelia of K5-GR transgenic mice, suggesting negative regulation by GR (41). Importantly, Δ Np63 represses *Klf4* expression by directly binding its promoter (42), and the expression of both TFs is mutually exclusive in the epidermis, with Δ Np63 expressed basally and *Klf4* suprabasally. We evaluated whether GR could regulate *Klf4* indirectly by modulating p63 expression, first by testing whether p63 isoform levels were affected by Dex in control and GR^{EKO} cell lines (Fig. 5A). The levels of both Δ Np63 and TAp63 were diminished by Dex in

control keratinocytes while no effect was observed in GR^{EKO} cells (Fig. 5A). These data show that activated GR is capable of repressing the expression of *Trp63* isoforms. Strikingly, TAp63 levels were elevated by 2-fold in GR^{EKO} relative to control cells (Fig. 5A). While TAp63 has tumor suppressor properties (43), inducible epidermal overexpression causes hyperproliferation and a failure to commit to terminal differentiation (44), indicating functions dependent on cellular context and on the ratio to the Δ Np63 isoform.

We next examined the expression of p63 isoforms in control and GR^{EKO} keratinocytes grown in high calcium medium for 72 h (Fig. 5B). Here, we observed an increased expression of both Δ Np63 (1.8-fold) and TAp63 (6.6-fold) isoforms in GR^{EKO} cells prior to incubation with calcium. As the calcium experiments are done in the presence of complete serum and the Dex treatments with charcoal-stripped serum, it is likely that other lipophilic compounds may participate in the basal regulation of Δ Np63. In the presence of calcium, Δ Np63 was up-regulated in GR^{EKO} keratinocytes, while TAp63 expression was not affected (Fig. 5B). Importantly, immunoblotting experiments confirmed the results of the RT-QPCR experiments (Fig. 5C, D). Since *Zfp36* is a p63-target gene (45), it is feasible that the reduced *Zfp36* mRNA levels detected in GR^{EKO} cells upon high calcium are not only due to the lack of GR but also to the augmented p63.

A major aim in the field is to understand how individual or combined TFs modulate transcriptional programs in epidermal development and disease. Until now, two genome wide chromatin binding studies have been done for Klf4 in keratinocytes but none for GR (12, 46). This study provides the first direct evidence that GR and Klf4 bind at genomic regulatory regions near the anti-inflammatory genes *Tsc22d3* and *Zfp36*. Our observation that these genes were up-regulated during keratinocyte terminal differentiation in a GR-dependent manner is suggestive of roles beyond modulating inflammation. GC treatment is a standard therapy for countering skin inflammation, with GR activation suppressing inflammatory pathways in both keratinocytes and skin immune cells (4). In immune cells, Klf4 can mediate inflammatory signaling (47-49), however, whether it does so in keratinocytes was unknown. Taken together,

our data shows direct cooperation between Klf4 and GR in keratinocyte gene regulation and uncovers a role for Klf4 in regulating anti-inflammatory genes in these cells.

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Figure legends

Figure 1. *Tsc22d3* and *Zfp36* are direct GR transcriptional targets in keratinocytes

Experiments were performed in cultured mouse primary keratinocytes MPKs (A, B) or immortal adult keratinocyte cell lines (C, D).

A GR ChIP-QPCR demonstrates recruitment to putative regulatory sequences of *Per1*, *Tsc22d3*, and *Zfp36* upon Dex treatment (100nM, 2h). Fold GR recruitment (mean values $\pm$ SD) in Dex- vs mock- treated MPKs is shown, with asterisks denoting statistically significant differences between groups (Student's t-test, n=3; *p<0.05, **p<0.01).

B Dex treatment (100 nM, 3h) induces gene expression of *Per1*, *Tsc22d3*, and *Zfp36* in MPKs as measured by RT-QPCR. Relative gene expression (mean values $\pm$ SD) in Dex- versus mock-treated samples is shown, with asterisks denoting statistically significant differences between groups (Student's t-test; n=3; **p<0.01).

C GR ChIP-QPCR demonstrates recruitment to putative regulatory sequences of *Tsc22d3* and *Zfp36* upon Dex treatment (100 nM, 2h) in control cell line. Fold GR recruitment (mean values $\pm$ SD) in Dex- vs mock- treated cells is shown, with asterisks denoting statistically significant differences between groups (Student's t-test, n=4; *p<0.05, **p<0.01).

D Gene expression of *Tsc22d3* and *Zfp36* in Dex- (100 nM, 3h) or mock-treated GR^{EKO} and control (CO) cell lines was assessed by RT-QPCR. Relative gene expression (mean values $\pm$ SD) in Dex- versus mock-treated samples is shown, with asterisks denoting statistically significant differences between treatment groups (Student's t-test; n=3; *, p<0.05; **, p<0.01).

E GR deficiency in the GR^{EKO} relative to the control cell line (CO) is shown by immunoblotting. Tubulin was used as a loading control.

Figure 2. *Klf4* is recruited to *Tsc22d3* and *Zfp36* in Dex-treated cells in a GR dependent manner.

A Genomic sequences near *Tsc22d3* and *Zfp36* to which ligand-bound GR was recruited in ChIP-seq. GREs and adjacent KLF and/or AP-1 sites are underlined.

B *Klf4* recruitment to regions near *Tsc22d3* and *Zfp36* increases upon Dex treatment (100 nM, 2h) in control but not in GR^{EKO} cells. Fold *Klf4* recruitment (mean values $\pm$ SD) in Dex- relative to mock-treated CO and GR^{EKO} cells is shown, with asterisks denoting statistically significant differences between treatment groups (Student's t-test; n=4; *, p<0.05; **, p<0.01).

Figure 3. Knockdown of *Klf4* interferes with Dex-induction of *Tsc22d3* but not *Zfp36*.

Tsc22d3 and *Zfp36* showed statistically significant increases in expression following treatment with Dex (100 nM, 3h) in cells transfected with control but not *Klf4* siRNAs as shown by RT-QPCR. Note the reduction of *Klf4* levels in samples transfected with gene specific siRNA. Fold change (mean values $\pm$ SD) in expression relative to mock-treated control siRNA transfectants is shown, with asterisks denoting statistically significant differences between treatment groups (Student's t test; n=4; *, p<0.05; **, p<0.01).

Figure 4. Defective calcium-induced terminal differentiation in GR^{EKO} cell line.

A Phase contrast images show morphological characteristics of calcium-induced differentiation in control (CO) but not GR^{EKO} cells after 72h in the presence of 1.2 mM calcium. Note the presence of cornified envelopes in control cells (asterisks) in contrast to striking defective differentiation of the GR^{EKO} cells. Bar: 50 μ m.

B Gene expression of *Spr2d*, *Nr3c1*, *Klf4*, *Tsc22d3*, and *Zfp36* in CO or GR^{EKO} cells treated for 0 or 72h with 1.2 mM calcium was assessed by RT-QPCR. Changes in gene expression (mean values $\pm$ SD) relative to t0 are shown, with asterisks denoting statistically significant differences relative to untreated controls (Student's t-test; n=4; *, p<0.05; **, p<0.01). The graph has a logarithmic scale.

Figure 5. GR negatively regulates *Trp63* isoforms in keratinocytes.

A RT-QPCR shows that Dex-treatment (100nM, 3h) repressed expression of *Trp63* isoforms Δ Np63 and TAp63 in CO but not in GR^{EKO} cells. Note the elevated levels of TAp63 in GR^{EKO} relative to CO cells. Mean values $\pm$ SD are shown, with asterisks denoting statistically significant differences relative to mock-treated (V) samples (Student's t-test; n=3; *, p<0.05; **, p<0.01).

B Δ Np63 and TAp63 isoforms show elevated expression during calcium-induced terminal differentiation in GR^{EKO} versus CO cells as measured by RT-QPCR. Mean values $\pm$ SD are shown, with asterisks denoting statistically significant differences in GR^{EKO} relative to CO cells at each time-point (Student's t-test; n=3; *, p<0.05; **, p<0.01).

C Immunoblotting shows induction of GR and Klf4 protein during terminal differentiation in CO but not in GR^{EKO} cells. Note the increase in p63 in GR^{EKO} cell extracts. Tubulin was used as a loading control.

D. Quantification of immunoblotting experiments. Mean values $\pm$ SD are shown, with asterisks denoting statistically significant differences between 0 and 72 h of calcium treatment within a genotype (Student's t-test; n=3; *, p<0.05; **, p<0.01), and hashes denoting statistically significant differences in GR^{EKO} relative to CO cells at each time-point (Student's t-test; n=3; #, p<0.05; ##, p<0.01).

Figure 1

Figure 2

A

Tsc22d3, chrX:137063804-137064012

GGGCAAAAAACAGAATGTT**CAG**CATTCAAGGGAGGGGGCAGGAGTTGGGTTCTGCCT**GAGTAATTCTTG**
GRE
KLF
AP-1

Zfp36, chr7:29161362-29161638

TCCCGCACATTCCGT**CCTCGCCGCCCGCCCCGCCCCACCCCG**CCCTCCTTCCTTGGCCCTGTGGGGACGGAA
GRE
KLF

ACATCCCGTTCCTGCCCGAGCTGGGTCAAGAGCCGGAGGGACAGGACCAGAG**CACCCCTTAC**
KLF

B

Figure 3

Figure 4

A

B

Figure 5

Figure S1- Reduced *Klf4* expression in GR^{EKO} versus CO cells.

Klf4 expression was assessed in GR^{EKO} versus CO cells by RT-QPCR.

Mean values $\pm$ SD are shown, with asterisks denoting statistically significant differences in GR^{EKO} relative to CO (Student's t-test; $n=3$; *, $p<0.05$).

Figure S2- Evaluation of GR-Klf4 interaction via co-immunoprecipitation.

Lysates of cells treated with Dex for 0, 30 or 60 min were subjected to immunoprecipitation with antibody specific for Klf4 or a nonspecific rabbit IgG (t0). Input controls and immunoprecipitates were analysed by immunoblotting with GR, p-GR and Klf4 antibodies.

Gene symbol	Chrom	Chrom_start	Chrom_end	pvalue (-10*log10)	GRE MOTIF	KLF MOTIF	AP1 MOTIF
Per2	chr1	93332598	93333431	227,42	GTGTACAGAATGTTCTCGGC	ACCCTCCCAACACA	
Ralb	chr1	121405904	121406699	375,29	AGGGACAGAGAGTCCAGGAC		CTGACTCA
Nuak2	chr1	134231906	134232468	335,65	GGCGGGATCATGATGTTCTT		TGAGGCAC
Gpr161	chr1	167221433	167222346	521,77		GGCCTGGGGCAGAG	
AK017125	chr1	174479598	174480468	408,64	AGGGACAGAGAGTTCCTCAC	TTCGACCACACCCC	
Mthfd1l	chr10	6240400	6241159	375,8	ACACAGCACATAGTGTTCT	AGTGTCTGGGAAGG	
Moxd1	chr10	24115658	24116564	437,16	TCAGAGGACAGTATGTACTG		
Anapc16	chr10	59436557	59437575	968,69	AGCCAGAACACAATGTTCTC	TGAGAGGGGAGCAG	
Unc5b	chr10	60267884	60268713	291,1			
Lrrc20	chr10	60927495	60928398	601,34	GCAGGGCACTCTGTGTACCC	TCGGGCCACACCCA	
Eif4ebp2							
Mknk2	chr10	80126961	80127794	557,47	AGGAACAGCTCGTCCTAGGA		
Spred2	chr11	19749542	19750339	338,36			
Phf15	chr11	51729371	51730267	1887,93	CAGGACACCCAGTCCTTGGC	ATGGGCCTCACACC	
Cdkn2aipnl							
Per1	chr11	68910113	68911085	852,61	AGAACACGATGTTCC	CCCTGCCCCACATT	
Krt42	chr11	100148051	100148802	306,78		CCGCACCCACCCA	
Eif1							
Axin2	chr11	108872438	108873630	2554,95		ACGTGCCCCCTCTCT	
Rgs9							
Sphk1	chr11	116394671	116395715	334,06			
Bahcc1	chr11	120113752	120114488	432,05		GGGGTGGGGCAGAA	
Id2	chr12	25854455	25855011	582,92	CCTGAGCACTCGGTGTTCTT		GTGACTCA
Tmx1	chr12	71725183	71726107	265,87		AGGGTGTGGCCAGG	
Frmd6							
Fntb	chr12	77984680	77985374	540,5	AGGATCAGAATGTTCTGTAG	AGGGTGAGGCCCAG	
Sel1l	chr12	93117244	93117946	537,46	ACGAACATTCTGTCCCAGAA		ATGACTCA
Rin3	chr12	103566223	103566907	258,14	AAGTACATTGTGTCCTTTGA	AGGGTGGGGGTGGG	
Klf6	chr13	5840515	5841102	467,77		ACCTTCCACTCCCT	
Edn1	chr13	42469301	42470237	833,31	AGCAGGGGACACTGTGTTCCA	CACTTCCCCTCCCT	
Jarid2	chr13	44964462	44965677	610,17			
Gadd45g	chr13	52001157	52001951	346,71		CCCAGCCCTACACT	
AK014969	chr13	52344718	52345676	595,48	ATGAACACGATGTTCCCTGA		
Diras2							
AK144747	chr13	90653286	90654112	332,96	TCCCAGCACGGGGTGTTCTT		ATGACTCA
Atp6ap1l							
Hexb	chr13	97960232	97961100	581,96	TCTCAGGACCGACTGACCCA		
Grhl2	chr15	37122163	37122985	699,67	GGGAACACTGTGTCCTTGTA		ATGACTCA
Azin1	chr15	38386738	38387747	326,58			
Eif3h	chr15	51561900	51562839	1048,8			
AK163289	chr15	61439440	61440444	315,03			
Micall1	chr15	78961713	78962462	364,9	AGTCACAGAATGTCCTTGGC		GTGACTGA
Krt6a	chr15	101527667	101528519	696,28	GCACTGCTCACTTTGTTCCC		
Zc3h7a	chr16	11143747	11144537	1228,4		GGCGTCGGGACCGG	
Parn	chr16	13506704	13507717	408,05		ACTTGCCCCACCCA	
Gnb1l	chr16	18547751	18548560	687,23			TGAGTCA
AK053957	chr16	25058959	25060033	545,38		CCGGGCCCCCTCCCA	
Leprel1	chr16	26248772	26249554	329,44	TAGAACATTGTATTCTTGCC		TGAGTCAT
Cldn1							
AK029404	chr16	31598510	31599566	427,38	AGGTACATCCAGTCCTGAGC		
Sidt1	chr16	44233136	44233676	385,25	TGGTACAATTTGTGCCTGGT		TGAGTCAC
Gm608							
Cblb	chr16	52094514	52095248	326,83	AAGTACAGACTGTCCCAGGA		TGAGTCTC
Filip1l	chr16	57391129	57392285	2087,76			

N6amt1	chr16	87267971	87268910	902,63	GTCTGGCACTCACTCATCCA	TCCTGCCTGTCCCC	GTGACCCA
BC099513	chr16	95462765	95463864	659,75	TCACGGAACATATTGTACCT		
Ets2	chr16	95880846	95881801	645,89			
Hsf2bp	chr17	32048871	32049713	813,46	GGGAACATTCCGTCTCGGAA		
H2-T3	chr17	36367717	36368869	1320,18		CCCTCTCCCTCCCC	
AK138323							
Mir715	chr17	39979485	39986313	3100			
Mir715	chr17	39979485	39986313	3100			
Mir715	chr17	39979485	39986313	3100			
Mir715	chr17	39979485	39986313	3100			
Mir715	chr17	39979485	39986313	3100			
Tcf4	chr18	68851589	68851983	633,78			
Mc2r							
AK009639	chr19	5849726	5850617	1565,36	AGGTACATTCCGTTCCAGGC	TGGGCTGGGCCGGG	
Hoga1	chr19	42132697	42133507	527,69	GGCCATCACACCGTGTTCCT		GTGACTCA
Camk1d	chr2	5299883	5300572	692,92			
4921504E06Rik	chr2	19501239	19502198	240,9	AGACAGAATGCTCTGTTCAg		GTGACTCA
Pax8	chr2	24295381	24296340	475,02	GGAGGGCACATGATGTACAA		
Itgb6	chr2	60537740	60538748	1361,25	CTGTACATTCTGTGCTGCTT	TGAGTGAGGAGAGG	
Tcp1111	chr2	104545348	104546337	540,82	GGGAACAATTTGTGCTTTAG		
Bcl2l1	chr2	152645660	152646435	420,7	TGAAGGGACACTGTGTTCCC		
Pxmp3	chr3	5860456	5860990	2237,96			
Zbtb7b	chr3	89182266	89183149	544,95	AGGAACAAGACATTCCCTGC		TGAGTCA
S100a10	chr3	93359704	93360284	314,37	TAGCACAAAGGGTTCCTGGA	TGGGTGTGGCCTAG	GTGACTCA
Wars2	chr3	98953718	98954556	403,97			
Pdlim5	chr3	142035231	142036203	586,07	AGGAACAGAATGTCCCAGAC		
4930503B20Rik	chr3	146320253	146321062	605,28	AGGTACACAATGTTCCAGCA		ATGACTGA
Ror1	chr4	100073937	100075431	416,9			
BC051525	chr4	120289222	120289771	765,77	GGGAACACAGAGTCCTGGGC	GGGGTGGGGACCTG	
Trnp1	chr4	133047438	133048255	385,8	AGCAGGAATGGAATGTTCT	CCCTCCCCACCCC	
Clic4	chr4	134842633	134843533	553,41	CCGCAGAACGCCCTGTACCG	CTCTGCCCTACCCA	
Srrm1							
Kif17	chr4	137834057	137834838	852,11	GGCAGGGACATCATGTTCTT		GTGACTCA
Fblim1	chr4	141125723	141126527	262,92	GCCCAGCACCTTCTGTTCT	ACCAACCCCACTCC	
H6pd	chr4	149353543	149354307	402,8		ATTCCCCCACACT	
Errfi1	chr4	150155907	150156755	334,9		TCCAGCCCCTTCCA	
MRPL33	chr5	31936324	31937148	376,22	TAGAACAGAACATCCCTGGG		
Dok7	chr5	35480489	35481131	387,2	CAGAGGCACGGAATGTTCCA		TGAGTCTC
Tgfb3	chr5	107552818	107553616	351,82	TAAGAGCACTGGCTGTTCTA	TAGGAGGGGCTGGA	ATGACTCA
Myo1h	chr5	114806421	114807295	303,27	TGCAGGAATGGTATGTACCC		
Cdk8	chr5	147072041	147073399	1708,11			
Hipk2	chr6	38785066	38785917	297			ATTACTCA
Tra2a	chr6	49186023	49186710	636,67			
D6Mm5e	chr6	82977753	82978472	589,14	GCTGGGAACCTCCTGTACTT		
Tead4	chr6	128231964	128232801	315,09	GCAAAGAAGTGTCTGTTCT		
Hif3a	chr7	17643227	17644190	353,76	GAGGACATTCCGTCCTGTAC	AGGGTGGGGGTAGG	
Zfp36	chr7	29160976	29161895	509,74	CCGCACATTCCGTCCTCGCC	CCCCGCCCCACCCC	
Klf13	chr7	71041604	71042464	1831,31	GGCGGGAACAGCATGTTCTC	TCTTGCCCAAGCCA	
Nr2f2	chr7	77181488	77182319	277,29	CAAAAGAACAAGCTCTACTT		CTGACTCA
Abhd2	chr7	86481426	86482048	713,42	AGGAACACCATGTCTTGAG		AGTGACTCA
AK005418	chr7	117260116	117261077	278,32	TAGTACATTTGTACTGTAC	TGGGTGGGGAGAGA	
Copb1	chr7	121346072	121346998	377,11	AGGGAGCACAGTATGTTCCA		TGAGTAAC
Adam12	chr7	141428774	141429766	412,02			
Hpgd	chr8	58899121	58900287	352,74	ATGTACATTCTGTTCTATCC		TGACTCA
Fbxo8							

Banp	chr8	124551887	124552816	494,46	AAGAACAGGCTGTGCTTGGT		TGAGTCAG
Cadm1	chr9	47170289	47170911	709,96	GGCCAGTACAATTTGTTTCCT	TGGGAGGGGTAAA	TGAGGCAG
Zbtb16	chr9	48531655	48532316	626,54	GGCCAGCACTGCGTGTTCCCT		
Zbtb16	chr9	48545244	48546123	357,38	GGGTACAGTGTGTTCTTGGA		
Zc3h12c	chr9	51913134	51914248	461,61	TGCAAGAACCAAATGTTCTC		CTGACTCA
Rdx							
Gcnt3	chr9	69895308	69896173	418,72	TTGTACATGCAGTCCCTGCA	CCCTGCCCAGCCCT	
Rpl29	chr9	106314210	106315267	273,93			
Cspg5	chr9	110183619	110184270	474,14		AGCGTGGGGACCAT	
2610002l17Rik							
Lars2	chr9	123370513	123371653	1022,6			
Rap2c	chrX	48321683	48322064	489,34	GACAAGCACAGGCTGTTCTT		
Frmd7							
Il1rapl1	chrX	84483527	84484212	539,48			
Tsc22d3	chrX	137063416	137064179	433,76	GCAAAAAACAGAATGTTTCAG	AGGGAGGGGCAGGA	TGAGTAAT

Sevilla et al., Table S2.

ChIP-qPCR

Gene Symbol	Description	Gene Bank	Product (bp)	Forward primer	Reverse primer
Per1	Period circadian clock 1	NM_001159367.1	200	TACAGGACCGCTGTCGTTGGG	CGTCAAAGCGGAGGCAGGAGG
Tsc22d3	TSC22 domain family, member 3	NM_001077364.1	91	GGAGGGAATGCAACTGGGAG	CCCCTCCCTTGAATGCTGAA
Zfp36	Zinc finger protein 36	NM_011756.4	180	TCCTCTATCAAGTCCGCCCA	AGGAACGGGATGTTTCCGTC

RT-qPCR

Gene Symbol	Description	Gene Bank	Product (bp)	Forward primer	Reverse primer
Hprt1 (housekeeping)	Hypoxanthine phosphoribosyltransferase 1	NM_013556	142	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG
Klf4	Kruppel-like factor 4	NM_010637.3	185	GTGCCCCGACTAACCGTTG	GTCGTTGAACTCCTCGGTCT
Nr3c1	GR alpha	NM_008173.3	182	AAAGAGCTAGGAAAAGCCATTCT	TCAGCTAACATCTCTGGGAATTCA
Per1	Period circadian clock 1	NM_001159367.1	298	GGGAGACAAGAAGCCCCCGGA	GTGTCGGCGACCAGGGGGAA
Sprr2d	Small proline-rich protein 2d	NM_011470.1	336	TGGTACTCAAGGCCGAGA	TTTGTCTGATGACTGCTGAAGAC
Tsc22d3 (Gilz)	TSC22 domain family, member 3	NM_001077364.1	111	CTGTTGGCCTCGACTGCTG	GCCGAAAGTTGCTCACGAAG
Zfp36	Zinc finger protein 36	NM_011756.4	112	CCACCTCCTCTCGATAACAAGA	GCTTGGCGAAGTTCACCCA

Supplemental Data Legends.

Figure S1. Reduced Klf4 expression in GR^{EKO} versus CO cells. Klf4 expression was assessed in GR^{EKO} versus CO cells by RT-QPCR. Mean values $\pm$ SD are shown, with asterisks denoting statistically significant differences in GR^{EKO} relative to CO (Student's t-test; n=3; *, p<0.05).

Figure S2. Evaluation of GR-Klf4 interaction via co-immunoprecipitation. Lysates of cells treated with Dex for 0, 30 or 60 min were subjected to immunoprecipitation with antibody specific for Klf4 or a nonspecific rabbit IgG (t0). Input controls and immunoprecipitates were analysed by immunoblotting with GR, p-GR and Klf4 antibodies.

Table S1. Annotated genes identified by GR ChIP-seq in MPKs after 2h of Dex treatment. Gene symbol, chromosomal location (start and end coordinates), p-value, and over-represented GRE, KLF and AP-1 motifs in the genomic sequences are shown.

Table S2. List of primers used for validating the identified GR ChIP-seq targets.