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Dual role of Act1 in keratinocyte differentiation and host defense: *TRAF3IP2* silencing alters keratinocyte differentiation while inhibiting IL-17 responses

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Abstract

TRAF3IP2 is a candidate psoriasis susceptibility gene encoding Act1, an adaptor protein with ubiquitin ligase activity that couples the IL-17 receptor to downstream signaling pathways. We investigated the role of Act1 in keratinocyte responses to IL-17 using a tetracycline inducible shRNA targeting *TRAF3IP2*. Tet exposure for seven days effectively silenced *TRAF3IP2* mRNA and Act1 protein, resulting in 761 genes with significant changes in expression (495 down, 266 up, >1.5-fold, $p<0.05$). Gene Ontology analysis revealed that genes affected by *TRAF3IP2* silencing are involved in epidermal differentiation, with early differentiation genes (*KRT1*, *KRT10*, *DSC1*, *DSG1*) being downregulated and late differentiation genes (*SPRR2*, *SPRR3*, *LCE3*) being upregulated. AP1 binding sites were enriched upstream of genes up-regulated by *TRAF3IP2* silencing. Correspondingly, nuclear expression of FosB and Fra1 was increased in *TRAF3IP2*-silenced cells. Many genes involved in host defense were induced by IL-17 in a *TRAF3IP2*-dependent fashion. Inflammatory differentiation conditions (serum addition for 4 days postconfluence) markedly amplified these IL-17 responses, while increasing basal levels and *TRAF3IP2* silencing-dependent upregulation of multiple late differentiation genes. These findings suggest that *TRAF3IP2* may alter both epidermal homeostasis and keratinocyte defense responses to influence psoriasis risk.

Keywords

keratinocytes; psoriasis; interleukin-17; differentiation; inflammation

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Introduction

As a physical barrier that protects us against exterior threats, our skin has developed a defensive system composed of both innate and acquired constituents (Gallo and Hooper, 2012, Pasparakis et al., 2014). Keratinocytes are the major cellular constituent of the epidermis and react to environmental and internal stimuli, making them major players in the regulation of skin inflammation through secretion of cytokines, chemokines, and antimicrobial peptides.

Several laboratories have implicated *TRAF3IP2* (TNF Receptor Associated Factor 3 Interacting Protein 2) as a strong candidate gene for both psoriasis and psoriasis arthritis (Ellinghaus et al., 2010, Genetic Analysis of Psoriasis et al., 2010, Huffmeier et al., 2010, Stuart et al., 2015), with the single nucleotide polymorphism (SNP) rs33980500 changing aspartic acid to asparagine in the N-terminal region of the protein (D10N), possibly by altering its interactions with the molecular chaperone HSP90 (Wang et al., 2013). *TRAF3IP2* encodes Act1 (transcription factor NF-κB activator 1), also known as CIKS (Connection to IKK and SAPK/JNK) (Leonardi et al., 2000, Li et al., 2000, Morelli et al., 2000). Act1 is recruited to the IL-17 receptor through a SEFIR domain-mediated oligomerization and is essential for appropriate regulation of IL-17-mediated signaling (Chang et al., 2006, Qian et al., 2007).

TRAF proteins (TNF receptor associated factors) bind Act1, and TRAF6 is ubiquitinated by Act1 to mediate JNK and NF-κB activation (Leonardi et al., 2000, Liu et al., 2009, Mauro et al., 2003, Morelli et al., 2000) whereas TRAF2 and TRAF5 regulate mRNA stability (Bulek et al., 2011, Sun et al., 2011). TRAF3 and TRAF4 negatively regulate IL-17 signaling by competing for Act1 binding with TRAF6 and IL17RA, respectively (Wu et al., 2015, Zepp et al., 2012, Zhu et al., 2010). Although IL-17 alone regulates the expression of a relatively small subset of approximately 40 genes in cultured keratinocytes (Chiricozzi et al., 2011, Nograles et al., 2008), biologics targeting IL-17 and its receptors are very effective treatments for psoriasis (Elyoussfi et al., 2016, Lowes et al., 2014).

Keratinocyte hyperproliferation and aberrant differentiation are hallmarks of psoriasis (Elder et al., 2010, Harden et al., 2015) and skin inflammation is associated with many of the same transcription factors (TFs) required for epidermal differentiation and barrier formation (i.e., AP1, Sp1 and KLF4) (Nakamura et al., 2007, Rossi et al., 1998, Segre et al., 1999, Tsoi et al., 2012, Uluckan et al., 2015). Genes expressed in the later stages of keratinocyte differentiation are frequently also involved in host defense (Table S1). Based on these observations, we hypothesized that Act1 might play an important role in responses of differentiated keratinocytes to IL-17 relevant to the pathogenesis of psoriasis. To test this hypothesis, we assessed the effects of *TRAF3IP2* silencing under conditions promoting keratinocyte differentiation. Our results show that *TRAF3IP2* silencing increases expression of activator protein-1 (AP1) family TFs and several late keratinocyte differentiation genes in the absence of cytokine treatment, and that *TRAF3IP2* is required for IL-17 induction of keratinocyte host defense genes, particularly when differentiation occurs in an inflammatory context.

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Results

To identify IL-17-induced genes impacted by *TRAF3IP2* silencing, we engineered immortalized N/TERT-2G keratinocytes (Dickson et al., 2000) to inducibly express an shRNA targeting *TRAF3IP2* mRNA under the control of tetracycline (Tet) (Dickson et al., 2000, Stoll et al., 2010, Stoll et al., 2012). Seven days of silencing were necessary to effectively minimize Act1 protein expression (Figure S1). During this interval, keratinocytes reached confluence, which is important for optimal cytokine responsiveness (Johnston et al., 2011). In preliminary experiments, we compared high (100ng/ml) and low (10ng/ml) doses of IL-17. As assessed by qPCR, we found that genes responding rapidly to IL-17 (e.g., *TNF*, *CCL20*, *CXCL1, IL1B*, *IL8*) were more strongly induced at the higher IL-17 concentration (Figure S2a). However, we did not observe any significant expression differences in genes that respond more slowly to IL-17 (i.e., after 24h stimulation) (e.g., *DEFB4*, *LCN2*, *S100A7-9*, *IL36G*) (Figure S2b). Based on these results, we selected a concentration of 10ng/ml and a time point of 24 hours for RNA-seq experiments.

TRAF3IP2 silencing affects keratinocyte differentiation

Using RNA-seq, we surveyed the effects of *TRAF3IP2* silencing on keratinocytes, either alone or in conjunction with IL-17 (10ng/ml) and/or tumor necrosis factor-α (TNF, 10ng/ml). Four independent RNA-seq experiments were performed on N/TERT-TR-shTRAF3IP2 cells. Unexpectedly, *TRAF3IP2* silencing on its own revealed numerous changes in gene expression, with 266 genes upregulated and 495 genes downregulated (FC>1.5 or <0.67 and FDR<0.1, Tables S2 and S3, respectively). We found the Gene Ontology Biological Process (GO BP) category "epidermis development" (GO:0008544) to be prominent in both the decreased and increased gene lists (p=1.58×10^{-06} and 7.05×10^{-07}, respectively) (Figure 1a, Table S4). Early epidermal differentiation genes whose expression was decreased in *TRAF3IP2*-silenced keratinocytes included *KRT1* and *KRT10* (Fuchs and Green, 1980). Additional down-regulated genes of known relevance to keratinocyte differentiation were annotated for cell adhesion and included *DSC1* and *DSG1* (Table S4b). Validation of the same samples by qPCR confirmed their significantly reduced expression after *TRAF3IP2* silencing (Figure 1b). As shown by immunoblotting, Dsg1 and Krt10 proteins were also reduced by *TRAF3IP2* silencing in postconfluent keratinocytes (Figure 1c). These changes were reflected in the morphology of postconfluent keratinocytes, which displayed a more "cobblestone" appearance with fewer large stratifying cells after *TRAF3IP2* silencing (Figure 1d). Conversely, genes whose expression was upregulated by *TRAF3IP2* silencing were involved in late epidermal differentiation (Table S1) and included *SPRR2A-E-G*, *SPRR3*, *LCE3D-E*, and *CNFN* (Table S4a). Confirmatory assessment of late differentiation gene expression in confluent *TRAF3IP2*-silenced keratinocytes showed a trend towards increased expression of *SPRR2A-C-D-E*, and *SPRR3*, which reached significance when the cells were cultured to 4 days postconfluence to promote expression of late differentiation markers (Figure 1e). Culture to postconfluence also markedly increased expression of *LCE3D* and *LCE3E*, which showed a nonsignificant trend towards higher expression after *TRAF3IP2* silencing (Figure 1e).

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We considered the possibility that *TRAF3IP2* silencing might impact proliferation, apoptosis or cell attachment, thereby altering gene expression by affecting the degree of confluence. To this end, we investigated apoptosis (Figure 2a), cell counts (Figure 2b), and cell viability (Figure 2c) at different time points under standard attachment conditions and found them to be unaffected by *TRAF3IP2* silencing. *TRAF3IP2*-silenced keratinocytes also retained the same ability as control cells to express differentiation markers when subjected to non-adherent conditions known to induce differentiation (Watt, 1989, Watt et al., 1988) (Figure 2d). Although culture under non-adherent conditions decreased keratinocyte viability by approximately 20% and abrogated Ki-67 staining, there were no differences in these parameters comparing control and *TRAF3IP2*-silenced cells (Figure 2e-f). Indicative of a lack of non-specific effects of Tet (Ahler et al., 2013), we observed no effects on expression of differentiation genes (Figure S3a) or on cell growth (Figure S3b) in an N/TERT-derived line expressing an irrelevant shRNA targeting enhanced green fluorescent protein. Thus, we could not account for the observed *TRAF3IP2*-dependent differences in keratinocyte differentiation by confounding factors related to culture conditions.

We compared our RNA-seq results to inhibitory RNA (RNAi)-based transcriptome studies deposited in the Gene Expression Omnibus (GEO) database (Table S5) and found that genes modulated by *TRAF3IP2* silencing overlapped significantly with genes whose expression is altered by silencing of Kruppel-like factor-4 (*KLF4*), a key TF regulating keratinocyte differentiation and barrier function during mouse skin development (Segre et al., 1999). Moreover, expression of *KLF4* was downregulated by *TRAF3IP2* silencing, both in the RNA-seq data (FC=0.65, p=0.0085), and by qPCR (FC=0.71, p=0.0007). Other keratinocyte RNAi studies targeting *TP63*, *ZNF750, ANCR*, *ACTL6A*, and *EXOSC9* also manifested significant overlap with genes whose expression is modulated by *TRAF3IP2* silencing (Table S5). Notably, each of these genes is involved in the control of epidermal differentiation (Bao et al., 2013, Kretz et al., 2012, Mistry et al., 2012, Sen et al., 2012, Truong et al., 2006). Additionally, genes affected by *TRAF3IP2* silencing significantly overlapped with genes regulated in suprabasal vs. basal epidermis (Table S5) (Gulati et al., 2013).

We used semiparametric generalized additive logistic models (Swindell et al., 2013) to ask whether TF binding motifs within promoter regions were enriched as a function of *TRAF3IP2* silencing. This analysis revealed significant enrichment for AP1 motifs within 2 kb upstream of the transcription start sites of genes upregulated by *TRAF3IP2* silencing (Figure 3a, Table S6). In pursuit of this observation, we measured the expression of various AP1 family members in *TRAF3IP2*-silenced cells by qPCR and Western blotting. We found that *FOSB* and *FOSL1* mRNA levels were elevated (FC=1.87 and 2.20, p=0.0484 and 0.0030, Figure 3b), as were the corresponding FosB and Fra1 protein levels in nuclear extracts (Figures 3c and 3d).

TRAF3IP2 silencing has prominent effects on the induction of host defense and *SPRR2* family genes by IL-17

Our RNA-seq experiment included stimulation by IL-17, TNF or both. A Venn diagram summarizing the overall results of this experiment is presented in Figure S4. Consistent with

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prior results of others (Chiricozzi et al., 2011), many more genes were modulated by TNF alone or IL-17 in conjunction with TNF than by IL-17 alone. Herein, we focus on the effects of *TRAF3IP2* silencing on IL-17 stimulation, for which Act1 is known to be a direct downstream mediator (Chang et al., 2006, Sonder et al., 2011).

RNA-seq identified 38 upregulated and 45 downregulated genes in response to IL-17 in control cells (Figure S4). Three microarray-based publications investigating the effects of IL-17 in monolayer normal human keratinocytes were identified in the GEO database, and each of them manifested highly significant overlap with our results (Chiricozzi et al., 2011, Nograles et al., 2008, Swindell et al., 2012) (Table S7). Table S8 presents fold-change expression values for all genes in the RNA-seq experiment displaying significantly altered expression in response to IL-17 in control cells, compared to their expression in *TRAF3IP2*-silenced KC.

GO BP term enrichment analysis of transcripts upregulated by IL-17 in control keratinocytes implicated genes annotated for host defense responses (Table S1), including *CAMP, CCL20, ELF3, NFKBIZ, SAA1, S100A7-9-12,* and *VNN1*) (Figure 4a, Table S9). The induction of host defense-related genes by IL-17 was markedly blunted in *TRAF3IP2*-silenced cells, with the result that no host defense annotations remained significant after silencing (Figure 4b, Table S9). Genes involved in late keratinocyte differentiation were also induced by IL-17 in control cells, including multiple *SPRR2* family members, *LCE3D*, *LCE3E*, and *SPRR3.* We confirmed by qPCR that multiple host defense genes, including *CXCL2, DEFB4, IL36G, LCN2, NFKBIZ, S100A7-8-9-12, SAA1, SAA2,* and *ZC3H12A*, were significantly induced by IL-17 in a *TRAF3IP2*-dependent fashion (Figure 4c; all genes tested by qPCR are shown in Figure S5). Transient transfection of N/TERT KC with 2 different shRNAs (both different from that used in the lentiviral construct) confirmed the specificity of the effect of *TRAF3IP2* silencing on IL-17 responses (Figure S3c). Protein secretion analysis showed significant reduction of human β-defensin-2 (encoded by *DEFB4*), Lipocalin-2, IL-8 and CXCL1 protein levels in cells silenced for *TRAF3IP2* (Figure 4f). We also confirmed that multiple *SPRR2* genes were induced by IL-17, and for all of them except *SPRR2D*, induction was significantly blunted by *TRAF3IP2* silencing (Figure 4d). However, we were unable to confirm IL-17 induction of *LCE3D*, *LCE3E*, or *SPRR3* in either control or *TRAF3IP2*-silenced cells (Figure S5a). Finally, the early differentiation genes *KRT1*, *KRT10*, *DSG1*, and *DSC1* were not significantly altered by IL-17 treatment (Figure 4e).

Psoriatic epidermis *in vivo* manifests a distinctive differentiation program known as “regenerative maturation”, which is also seen in wound healing (Gottlieb et al., 1992, Mansbridge et al., 1984). We approximated a similar state of inflammatory differentiation *in vitro* by growing keratinocytes to four days postconfluence in the presence of serum, as previously described (Van Ruissen et al., 1996). As shown in Figure 5a, expression of the early differentiation markers *KRT1*, *KRT10*, *DSC1*, and *DSG1* was markedly reduced under inflammatory differentiation conditions, relative to confluent cells. As already noted for IL-17 stimulation experiments performed at confluence (Figure 4), these genes were not induced in response to IL-17 under inflammatory differentiation conditions, and with the exception of *KRT10*, were further reduced in *TRAF3IP2*-silenced cells. In contrast, the late differentiation genes *LCE3D*, *LCE3E*, *SPRR2A-C-D-E*, and *SPRR3* were expressed at

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much higher levels in keratinocytes exposed to serum for four days postconfluence, relative to confluent controls without serum (Figure 5b). Notably, several late differentiation genes were further induced in response to *TRAF3IP2* silencing under these conditions, including *LCE3D*, *LCE3E*, *SPRR2A* and *SPRR2E* (Figure 5b).

We then examined the effects of *TRAF3IP2* silencing on transcripts induced by IL-17 in the context of inflammatory differentiation. Multiple host defense genes (*CCL20, CXCL1*, *DEFB4*, *IL36G, NFKBIZ*, *LCN2*, *SAA1*, SAA2, *S100A7-8-9-12*, and *ZC3H12A*) were much more strongly induced by IL-17 under inflammatory differentiation conditions compared to confluent cells, and all of these inductions were blocked by *TRAF3IP2* silencing (Figure 5c; all genes tested are shown in Figure S6). While *SPRR2A, 2B, 2E, and 2F* were also induced by IL-17 under inflammatory differentiation conditions, only the inductions of *SPRR2A* and *SPRR2F* were significantly inhibited by *TRAF3IP2* silencing (Figure 5b).

Discussion

The physiological importance of Act1 is reflected in its ability to interact with multiple signaling pathways downstream of the IL-17 receptor (Gaffen, 2009, Gu et al., 2013, Linden, 2007, Wu et al., 2012) and its role in a variety of inflammatory pathologies (Claudio et al., 2009, Kang et al., 2010, Pisitkun et al., 2010, Pisitkun et al., 2012, Qian et al., 2008, Swaidani et al., 2011, Weaver et al., 2013, Wuet al., 2012, Zepp et al., 2011). *TRAF3IP2* resides in a known susceptibility locus for inflammatory diseases such as psoriasis and psoriasis arthritis (Ellinghaus et al., 2010, Genetic Analysis of Psoriasiset al., 2010, Huffmeier et al., 2010), Crohn's disease (Ciccacci et al., 2013) and Type 1 diabetes (Bergholdt et al., 2012). Moreover, IL-17 and its receptor are specific therapeutic targets in psoriasis (Lowes et al., 2014, Martin et al., 2013).

Nearly all studies aiming to elucidate the function of Act1, including the report of a spontaneous mutation leading to an atopic dermatitis-like phenotype, and the effects of the psoriasis-related Act1(D10N) on Th17 cell hyperactivity, have been performed in mouse models (Matsushima et al., 2010, Wang et al., 2013). Little is thus known about the role of Act1 in humans, with the exception of one report on the loss of IL-17 responses in human chronic mucocutaneous candidiasis resulting from a different Act1 mutation (T536I) (Boisson et al., 2013). To address this gap, we used RNA-seq to identify genes whose expression was impacted by *TRAF3IP2* silencing in human keratinocytes, then followed up on those studies using qPCR.

TRAF3IP2 silencing differentially impacts early and late keratinocyte differentiation genes

Even though monolayer keratinocyte cultures do not faithfully replicate the physiology of intact skin (Klingenberg et al., 2010), confluent keratinocytes manifest many aspects of the terminal differentiation program (Kolly et al., 2005, Poumay et al., 1999, Poumay and Pittelkow, 1995). Utilizing this model system (Figure 1), we found that *TRAF3IP2* silencing reshapes differentiation-related gene expression by markedly reducing the expression of the early differentiation genes *KRT1*, *KRT10*, *DSC1*, and *DSG1* (Figure 1b), yielding a more “cobblestone” and less stratified phenotype (Figure 1d). In contrast, late differentiation

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genes of the *SPRR2, SPRR3,* and *LCE3* families were upregulated after *TRAF3IP2* silencing, depending on the differentiation context (Figures 1e and 5b). This biphasic phenotype is reminiscent of Krt1/Krt10 knockout mice, which manifest markedly reduced Dsg1 and Dsc1 protein levels but retain effective epidermal barrier function (Wallace et al., 2012). Further suggestive of a role in keratinocyte differentiation, we found that *TRAF3IP2*-responsive genes overlapped significantly with genes regulated in suprabasal epidermis versus normal skin (Gulati et al., 2013) and genes regulated by *KLF4*, *ZNF750*, *ANCR*, *ACTL6A* and *EXOSC9*, which have all been implicated in epidermal differentiation (Bao et al., 2013, Kretz et al., 2012, Mistry et al., 2012, Sen et al., 2012, Truong et al., 2006) (Table S5).

Analysis of TF binding sites among genes upregulated by *TRAF3IP2* silencing revealed significant enrichment of motifs for members of the AP1 family (Figure 2a), and correspondingly, we found AP1 family members FosB and Fra1 to be more highly expressed in *TRAF3IP2*-silenced keratinocytes (Figure 2c-d). It is difficult to extrapolate these globally-derived results to individual genes, due to varying promoter structures, trans-acting factors, and chromatin configurations. Nevertheless, given that AP1 sites are present in most *SPRR2* genes (Cabral et al., 2001) and that AP1 suppresses expression of *KRT1* and *KRT10* in keratinocytes (Rutberg et al., 2000), it is tempting to speculate that both increased expression of late differentiation genes and decreased expression of early differentiation genes might be mediated by increased AP1 activity in the context of *TRAF3IP2* silencing. In the *in vivo* context of psoriasis, we have shown that non-coding SNPs residing near psoriasis GWAS signals frequently disrupt AP1 binding sites (Swindell et al., 2015). Given that genes whose expression was altered by *TRAF3IP2* silencing were also enriched for TF binding sites other than those belonging to the AP1 family (Figure 3a) that the profile of genes affected by *TRAF3IP2* silencing overlap with responses to silencing of other transcription factors in keratinocytes (Table S5), it is likely that TFs outside the AP1 family may also be involved in mediating the effects of *TRAF3IP2* silencing.

Because gene expression patterns change substantially as keratinocytes pass through confluence (Kolly et al., 2005, Paragh et al., 2010, Radoja et al., 2006, Tran et al., 2012) or are separated from their substratum (Banno and Blumenberg, 2014), it was important to rule out *TRAF3IP2*-related differences in response to cell proliferation, death, or detachment, which might affect the confluence of the cultures. Keratinocyte apoptosis was not detectably influenced by *TRAF3IP2* silencing (Figure 2a), nor was cell growth (Figure 2b-c). *TRAF3IP2* silencing also did not affect the differentiation process in cells subjected to suspension culture (Figure 2d), suggesting that the *TRAF3IP2*-dependent effects we have observed require cell-substratum contact.

TRAF3IP2-dependent effects of IL-17 on host defense genes are potentiated by keratinocyte differentiation in an inflammatory environment

Consistent with our observation that confluent keratinocyte monolayers display increased responsiveness to cytokine stimulation compared to subconfluent keratinocytes (Johnston et al., 2011), stratified keratinocytes in a reconstituted human epidermis model have been shown to be substantially more sensitive to IL-17, compared to monolayer keratinocytes

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(Chiricozzi et al., 2014). Our use of confluent cultures may help to explain why we identified a similar number of genes influenced by IL-17 treatment as previous studies, despite using a lower IL-17 concentration (10ng/ml vs. 100-200ng/ml in previous studies, Table S7).

Given that an important function of differentiated keratinocytes is to provide a physical barrier to external threats while acting as the earliest sentinel for innate immunity, it is not surprising that IL-17-inducible genes were involved in both host defense as well as some late keratinocyte differentiation responses (Figures 4, 5, and Table S9) (Li et al., 2014). Multiple host defense genes were strongly induced by IL-17 in a *TRAF3IP2*-dependent fashion under confluent conditions (Figure 4), even more so in the context of inflammatory differentiation (Figure 5). We also confirmed IL-17 inducibility of several late differentiation genes belonging to the *SPRR2* family (Figures 4 and 5). In contrast, the early differentiation genes *KRT1*, *KRT10*, *DSG1* and *DSC1* were unresponsive to IL-17 (Figure 4) and markedly down-regulated under inflammatory differentiation conditions, with additional declines after *TRAF3IP2* silencing (Figure 5a).

Overall, then, the effect of *TRAF3IP2* silencing is to decrease expression of early differentiation genes and increase expression of late differentiation genes under basal conditions, while inhibiting host defense responses to IL-17. We do not believe that the loss of IL-17 responses after *TRAF3IP2* silencing is secondary to a differentiation block, because many late differentiation genes are actually expressed at higher levels after *TRAF3IP2* silencing, rather than lower levels as would be predicted for a differentiation block. Rather, we would suggest that because (a) keratinocytes are increasingly exposed to the microbial environment as they mature, (b) IL-17 is implicated in host responses to a variety of pathogens [reviewed in (Huppler and Gaffen, 2016)], (c) differentiated keratinocytes manifest enhanced responsiveness to IL-17 (Chiricozzi et al., 2014), and (d) Act1 is a well-established downstream mediator of IL-17 action (Chang et al., 2006, Qian et al., 2007), that Act1 plays an increasingly important role in mediating IL-17 responses to the microbial environment as keratinocytes become more terminally differentiated.

The *SPRR2* genes were distinctive among the late differentiation genes, in that they were inducible by both IL-17 and *TRAF3IP2* silencing. Correspondingly, they also appear to function in both host defense and late differentiation, as they are involved in both cornified envelope formation and antioxidant defense (Vermeij et al., 2011, Vermeij and Backendorf, 2010). Despite their similarity in structure, the *SPRR2* genes manifest different patterns of tissue-specific and stimulus-specific expression involving variation in promoter elements (Cabral et al., 2001). The fact that these genes display different patterns of responsiveness to IL-17 under inflammatory differentiation conditions (Figure 5) suggests that they have evolved different regulatory signals to adapt to various stimuli.

Act1 and psoriasis pathogenesis: Possible dual roles in epidermal homeostasis and inflammation

It remains mysterious how a putative loss-of-function variant of Act1 (the D10N variation) is associated with increased risk of psoriasis and psoriatic arthritis (Ellinghaus et al., 2010, Huffmeier et al., 2010). Act1 is a multidomain protein with multiple downstream effectors

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(Wu et al., 2012), and it is speculative to equate Act1 knockdown in our experiment with loss of function from the D10N mutation. However, loss of Act1 function (whether due to the D10N mutation or other, more indirect mechanisms) might explain the increased expression of late differentiation genes that is characteristic of psoriasis (Li et al., 2014). Indeed, the most highly up-regulated gene co-expression module that we identified in psoriatic lesions (module P26) (i) contained *SPRR2A*, *2B*, *2D, and 2E*, and showed strong overlap with genes expressed in differentiated vs. basal epidermis, and genes impacted by silencing of the key epidermal regulators *STAU*, *TINCR*, *ZNF750*, and *KLF4* (Li et al., 2014). However, this aspect of *TRAF3IP2* function cannot account for the increased expression of many IL-17-induced host defense genes in psoriasis, as a loss-of-function mutation would be expected to decrease, rather than increase expression of these genes. As might be expected given its key role in inflammation, IL-17 signaling is subject to several levels of feedback regulation (Garg et al., 2013), including one involving the ribonuclease MCPIP1 encoded by *ZC3H12A* (Garg et al., 2015). In this regard, it is notable that *TRAF3IP2* silencing markedly inhibits the expression of *ZC3H12A* (Figures 4 and 5), which might be expected to relax this negative feedback regulation. While further studies are needed to understand the mechanistic function of *TRAF3IP2* in keratinocyte's differentiation as well as the complex role of the Act1D10N mutation in keratinocytes with respect to the pathogenesis of psoriasis, our findings concur with others linking the processes of late keratinocyte differentiation and host defense (Akiyama et al., 2014, Gutowska-Owsiak et al., 2012), and suggest a role for *TRAF3IP2* in the integration of these processes.

Materials and Methods

Immortalized N/TERT-2G keratinocytes expressing a Tet operator were stably infected with a lentivirus coding for an shRNA directed against the 3′UTR of *TRAF3IP2* mRNA to create N/TERT-TR-sh*TRAF3IP2* cells, as previously described (Stollet al., 2010). To silence *TRAF3IP2*, these cells were treated with 1μg/ml Tet, typically for 7 days, with feeding every 2 days.

RNA-seq libraries were prepared using the Illumina mRNA-Seq kit (Illumina, San Diego, CA) and analyzed as described previously (Li et al., 2014). Gene Ontology (GO) analysis of RNA-seq data was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery, http://david.abcc.ncifcrf.gov) (Huang da et al., 2009). RNA-seq results have been submitted to GEO (GSE86451). For qPCR experiments, total RNA was isolated using RNeasy plus kit (Qiagen, Valencia, CA) followed by reverse transcription (High-Capacity cDNA Reverse Transcription kit, Applied Biosystems) and qPCR on an ABI PRISM 7900HT system (Applied Biosystems). Statistical analysis of qPCR data was performed using Prizm (GraphPad, La Jolla, CA), using two-tailed t-tests with Bonferroni correction for multiple comparison testing of different experimental conditions, as appropriate. Additional methodological details are described in the Supplementary Information.

Supplementary Material

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Abbreviations

Act1	transcription factor NF-κB activator 1
AP1	activator protein 1

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CIKS	connection to IKK and SAPK/JNK
FC	fold change
FDR	false discovery rate
GEO	Gene Expression Omnibus database
GO	Gene Ontology
qPCR	quantitative polymerase chain reaction
RNA-seq	high-throughput cDNA sequencing
SD	standard deviation
SNP	single nucleotide polymorphism
TCF	transcriptional co-factor
Tet	tetracycline
TF	transcription factor
TNF	tumor necrosis factor-α

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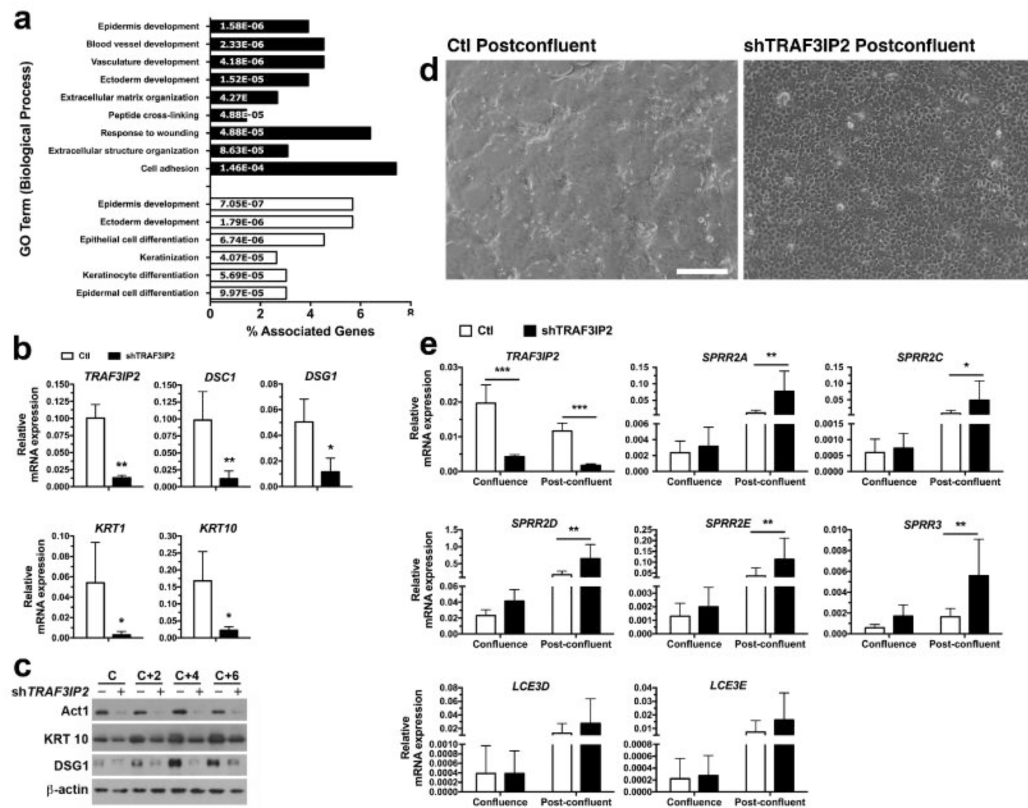

Figure 1. *TRAF3IP2*-silenced keratinocyte display changes in epidermal differentiation-related gene expression

(a): Bar plot representing the percentage of genes downregulated (black bars) or upregulated (white bars) by *TRAF3IP2* silencing classified by Biological Processes. *p*-values are indicated within the bars.

(b): Relative mRNA expression of *TRAF3IP2*, *KRT1*, *KRT10*, *DSC1* and *DSG1* in N/TERT-TR-sh*TRAF3IP2* cells cultivated with or without Tet for 7 days. Bar represent mean +SD of 4 independent experiments that were used for RNA-seq. *, $p<0.05$ and **, $p<0.001$

(c): Immunoblot showing the abundance of Act1, Dsg1 and Krt10 in N/TERT-TR-sh*TRAF3IP2* cells cultivated with or without Tet until confluent (C) and 2, 4, or 6 days after confluence. β-actin is shown to represent equal loading and immunoblot is representative of 2 independent experiments.

(d): Representative phase-contrast microphotographs of 4 days postconfluent control cells or *TRAF3IP2*-silenced cells. Scale bar is 20μm.

(e): Relative expression of late differentiation gene mRNAs in N/TERT-TR-sh*TRAF3IP2* cells cultivated with or without Tet for 7 days or brought to post-confluence for 4 days. Bar represent mean+SD of 3 independent experiments that were used for RNA-seq. *, $p<0.05$ and **, $p<0.001$

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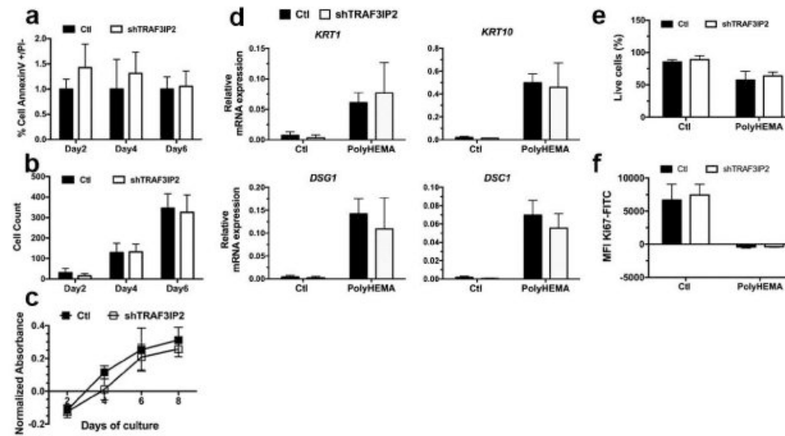

Figure 2. Inhibition of differentiation in *TRAF3IP2*-silenced keratinocyte is not due to changes in cell proliferation or viability

(a-b) Flow cytometry analysis of N/TERT-TR-sh*TRAF3IP2* cultured in the presence (black) or not (white) of Tet for the indicated time. Cells were stained for annexin V and propidium iodide, and assessed for annexin V staining (**b**) or cell counts (**c**). Result represents mean +SD of 4 independent experiments.

(c): N/TERT-TR-sh*TRAF3IP2* were cultured for the indicated amount of time with (white) or without Tet (black) then incubated in presence of a resazurin compound. Reduction of the resazurin was measured in the same cultures over a period of 8 days. Result represents mean ±SD of 4 independent experiments.

(d): qPCR analysis of differentiation genes induced in N/TERT-TR-sh*TRAF3IP2* cells seeded in Poly-HEMA coated plates in presence (black) or not (white) of Tet. Results represent mean+SD of 3 independent experiments. Cells were also seeded in non-coated dish (Ctl) to show the induction of differentiation gene expression induced by suspension.

(e-f): Flow cytometry analysis of cell viability and Ki-67 expression after being seeded on Poly-HEMA coated or normal dishes for 24h in presence (white) or absence (black) of Tet. Results are expressed as percent of live cells or mean fluorescent units (MFI).

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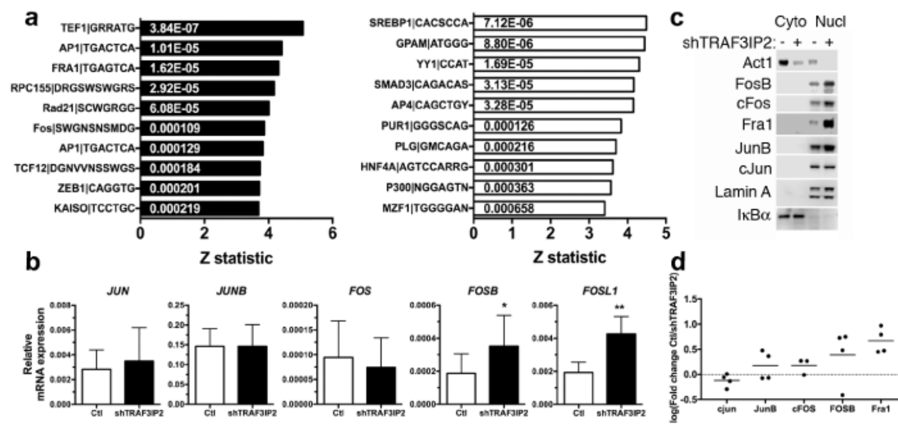

Figure 3. *TRAF3IP2*-silencing increases enrichment of binding motifs and expression of differentiation-related TFs

(a): Bar plots depicting the top 10 Z-scores for TF/TCF binding motifs in the 2kb promoter of genes upregulated (black) or downregulated (white) in *TRAF3IP2*-silenced cells. *p*-values are indicated in the bars.

(b): Relative gene expression of AP1 TF subunits in Ctl or sh*TRAF3IP2* cells. Results are mean+SD of 3 independent experiments and (*) indicates $p<0.05$ and (**) indicates $p<0.005$.

(c): Protein abundance of AP1 subunits in cytoplasmic or nuclear extract of Ctl or sh*TRAF3IP2* cells. Purity of cytoplasmic and nuclear fractions was assessed by detection of IκBα (cytoplasmic) and Lamin A/C (nuclear). Blots are representative of four experiments.

(d): Relative abundance of AP1 subunit nuclear proteins, normalized to LaminA/C. Results are expressed as the base 10 logarithm of fold change between Ctl and sh*TRAF3IP2* from four independent experiments of which one is shown in (**c**).

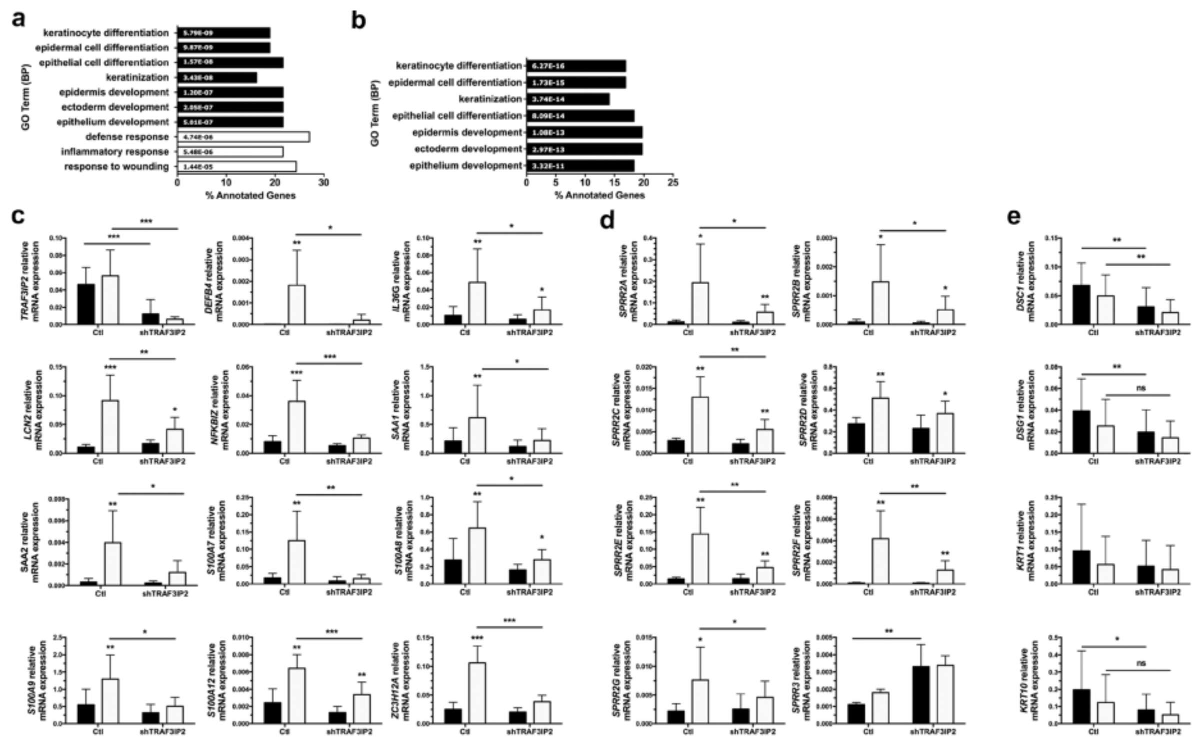

Figure 4. *TRAF3IP2* silencing affects IL-17-induced genes involved in host defense and keratinocyte differentiation

(a, b) Bar plots representing the percentage of IL-17-induced genes associated with various GO BP terms related to keratinocyte differentiation (black) or host defense (white) in control cells **(a)** or *TRAF3IP2*-silenced cells **(b)**. *p*-values are indicated within the bars.

(c, d, e) qPCR analysis of N/TERT-TR-sh*TRAF3IP2* cells stimulated with PBS (black bars) or with IL-17 (10ng/mL, white bars) for 24h after growth to confluence in the presence or absence of Tet. Panel (**c**) depicts genes involved in host defense responses. Panels (**d** and **e**) depict keratinocyte differentiation genes (late and early, respectively). Bars represent mean +SD of 3 experiments, which were independent from the ones used for RNA-seq (* indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.005$). Asterisks over open bars indicate significance with respect to IL-17 treatment; asterisks over horizontal lines indicate significance with respect to *TRAF3IP2* silencing.

(f) ELISA measurement of protein secretion in the supernatant during 24h of IL-17 stimulation in presence or absence of Tet. Graphs represent mean+SD of 3 independent experiments and * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.005$.

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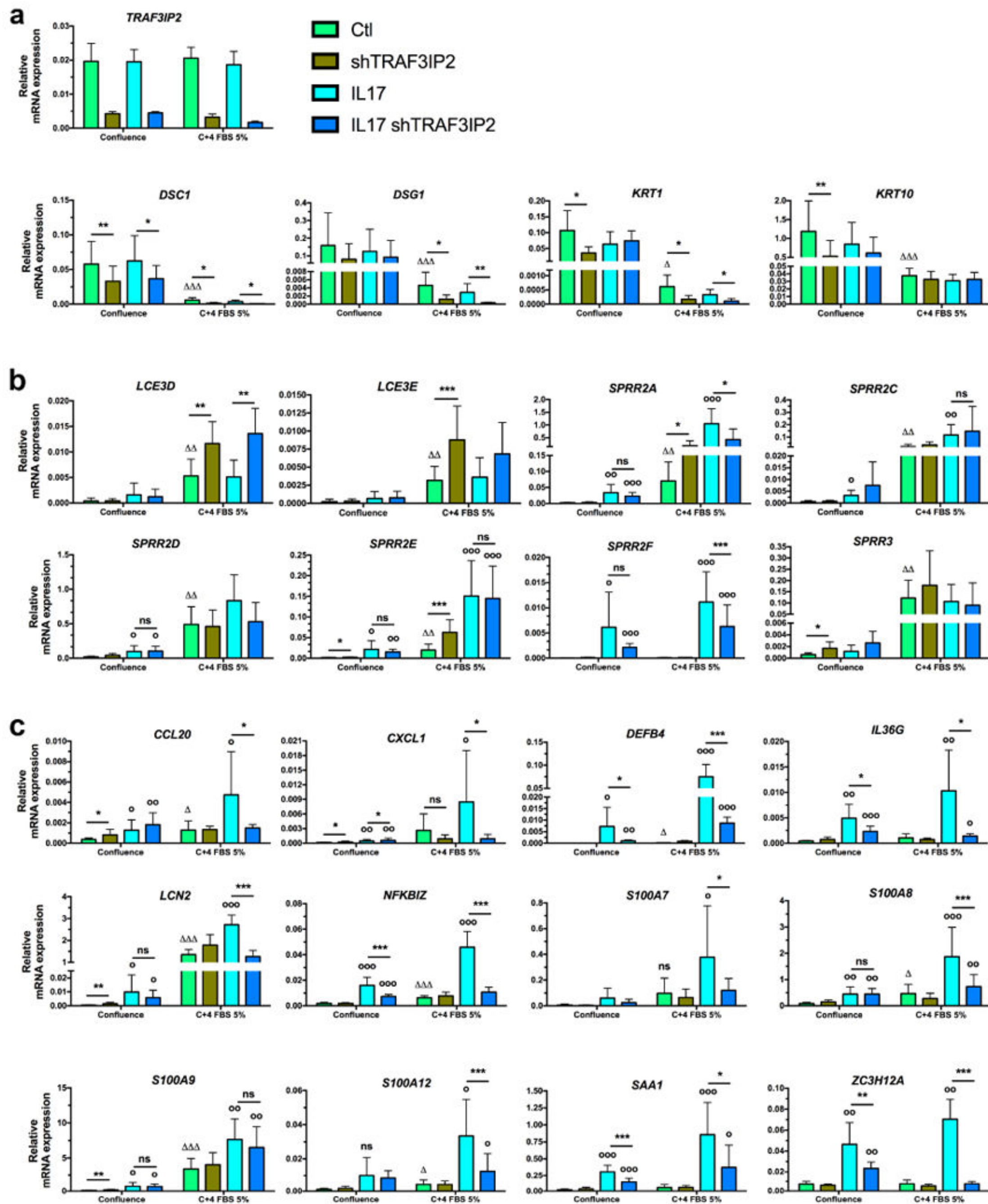

Figure 5. *TRAF3IP2* silencing in an inflammatory environment preferentially affects IL-17-induced genes involved in host defense, compared to epidermal differentiation genes

N/TERT-TR-sh*TRAF3IP2* cells were stimulated with IL-17 (10ng/mL, 24h) when confluent or after being kept for 4 days post-confluence in presence of FBS. Expression of genes related to early keratinocyte differentiation **(a)** late differentiation **(b)** or host defense **(c)** was then assessed by qPCR. Graphs represent mean+SD of 3 independent experiments. *p*-values are designated as follows: o, $p<0.05$, oo, $p<0.005$ for IL-17 stimulation vs. controls. *,

p<0.05, **, *p*<0.005 and ***, *p*<0.001 in *TRAF3IP2*-silenced vs. non-silenced cells. ,
p<0.05, , *p*< 0.001 relative to expression at confluence versus postconfluence + FBS.

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