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Human keratinocyte differentiation requires translational control by the eIF2α kinase GCN2

Ann E. Collier[1], Ronald C. Wek[1*], Dan F Spandau[1, 2*]

Departments of Biochemistry & Molecular Biology[1] and Dermatology[2], Indiana University School of Medicine, Indianapolis, IN, United States

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[*]Co-corresponding Authors:

Dan F Spandau
1044 West Walnut Street, Rm W274
Herman B. Wells Center for Pediatric Research
Indianapolis, IN 46202
TEL: 317 274-7115
FAX: 317 278-2815
Email: dspanda@iupui.edu

Ronald C. Wek
635 Barnhill Drive, Room 4067
Van Nuys Medical Science
Indianapolis, IN 46202-5121
TEL: 317 274-0549
FAX: 317 274-4686
Email: rwek@iu.edu

Abbreviations: activating transcription factor 4 (ATF4), C/EBP homologous protein (CHOP), cycloheximide (CHX), doxycycline (DOX), eukaryotic initiation factor 2 alpha (eIF2α), phosphorylated eukaryotic initiation factor 2 (eIF2α-P), growth arrest and DNA damage protein 34 (GADD34), general control non-derepressible 2 (GCN2), Integrated Stress Response (ISR), PKR-like endoplasmic reticulum kinase (PERK), Unfolded Protein Response (UPR), tunicamycin (TM)

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ABSTRACT

Appropriate and sequential differentiation of keratinocytes is essential for all functions of the human epidermis. While transcriptional regulation has proven to be important for keratinocyte differentiation, little is known about the role of translational control. A key mechanism for modulating translation is through phosphorylation of the α subunit of eIF2. A family of different eIF2α kinases function in the integrative stress response to inhibit general protein synthesis coincident with preferential translation of select mRNAs that participate in stress alleviation. Here we demonstrate that translational control through eIF2α phosphorylation is required for normal keratinocyte differentiation. Analyses of polysome profiles revealed that key differentiation genes, including involucrin, are bound to heavy polysomes during differentiation, despite decreased general protein synthesis. Induced eIF2α phosphorylation by the GCN2 protein kinase facilitated translational control and differentiation-specific protein expression during keratinocyte differentiation. Furthermore, loss of GCN2 thwarted translational control, normal epidermal differentiation, and differentiation gene expression in organotypic skin culture. These findings underscore a previously unknown function for GCN2 phosphorylation of eIF2α and translational control in the formation of an intact human epidermis.

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INTRODUCTION

In human skin, differentiation of keratinocytes is required to form a stress-resistant, impermeable barrier that protects against infection, water loss, UV damage, and other environmental stresses (Bikle *et al.*, 2012; Fuchs, 2007). The process of keratinocyte differentiation involves reprogramming of gene expression and cell morphology (Bikle *et al.*, 2012; Green *et al.*, 1982). Undifferentiated epidermal keratinocytes are attached to the cutaneous basement membrane that separates the epidermis and the underlying dermis. These basal layer keratinocytes actively divide until select progeny receive a signal to exit the cell cycle, detach from the basement membrane, and begin to migrate to the upper layers of the epidermis. During this process, cells begin to synthesize differentiation-specific proteins, including involucrin (*IVL*), loricrin (*LOR*), filaggrin (*FLG*), and various keratins (*KRT1*, *KRT10*) that are essential for changes in cell morphology and function (Abhishek and Palamadai Krishnan, 2016; Moll *et al.*, 1982; Steven *et al.*, 1990; Warhol *et al.*, 1985). Disruption of normal keratinocyte differentiation results in a diminished capacity of the epidermis to maintain barrier function, a hallmark of skin diseases such as psoriasis, atopic dermatitis, and non-melanoma skin cancers (Bouwstra and Ponec, 2006; Menon *et al.*, 1994). Although changes in transcriptional and epigenetic networks during keratinocyte differentiation are well documented (Botchkarev, 2015), little is known about the contributions of translational control.

Recent studies have suggested that posttranscriptional regulation has a role in skin development. Changes in amino acid incorporation into proteins were reported between the layers of the epidermis, and a recent genome-wide analysis of psoriatic tissue suggested

increased expression of the translational machinery (Swindell *et al.*, 2015; Zhao *et al.*, 2005). Furthermore, markers of the Unfolded Protein Response (UPR), which is an adaptive response to endoplasmic reticulum (ER) stress that features translational control, were suggested to be increased in the upper layers of normal epidermis and decreased in psoriasis and squamous cell carcinoma tissues (Sugiura *et al.*, 2009). Therefore, we hypothesized that translational control occurs during normal keratinocyte differentiation.

Signaling pathways that modify translation have been shown to participate in cellular differentiation processes (Masciarelli *et al.*, 2010; Yang *et al.*, 2016) and regulation of translation is an important means by which eukaryotic cells adapt to a variety of environmental stresses (Baird and Wek, 2012; Schwanhausser *et al.*, 2011; Sonenberg and Hinnebusch, 2009). Cells repress global protein synthesis to conserve resources, coincident with preferential translation of mRNA transcripts that confer stress resistance. An important mechanism directing translational control during stress features phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2α-P). eIF2α-P decreases initiation of global translation through a reduced ability of eIF2α to combine with GTP and transport initiator Met-$tRNA_i^{Met}$ to ribosomes for mRNA translation (Baird and Wek, 2012; Wek *et al.*, 2006). Four mammalian protein kinases phosphorylate serine-51 of eIF2α, each activated by distinct types of stress. Because a variety of stresses regulate eIF2α-P, this pathway is referred to as the Integrated Stress Response (ISR) (Harding *et al.*, 2003). Key eIF2α kinases include general control nonderepressible 2 (GCN2/EIF2AK4), which is activated by amino starvation and UV irradiation, and PKR-like endoplasmic reticulum kinase (PERK/EIF2AK3/PEK) that responds to ER stress and participates in the UPR. In addition to repressing global translation, eIF2α-P enhances translation of a subset of cytoprotective gene transcripts, such as activating transcription factor 4 (ATF4/CREB2) and its downstream target

C/EBP homologous protein (CHOP/GADD153/DDIT3) by mechanisms involving upstream open reading frames (uORFs) in the 5'-leaders of these mRNAs (Harding *et al.*, 2000; Lee *et al.*, 2009; Palam *et al.*, 2011; Vattem and Wek, 2004; Young *et al.*, 2016; Young and Wek, 2016). Additionally eIF2α-P induces the transcriptional and translational expression of growth arrest and DNA damage-inducible protein 34 (GADD34/PPP1R15A), which facilitates the dephosphorylation of eIF2α-P and resumption of translation (Connor *et al.*, 2001; Novoa *et al.*, 2001; Young *et al.*, 2015).

Here we demonstrate the importance of translational control mediated through eIF2α-P in the differentiation of human keratinocytes. We show that repression of translation initiation occurs during keratinocyte differentiation, and differentiation specific genes such as *IVL* are resistant to translation inhibition by eIF2α-P. Strikingly, the eIF2α kinase GCN2 is activated and is required for proper formation of the human epidermis, as visualized by a three-dimensional (3D) *in vitro* organotypic epidermal model. These results demonstrate that translational control by the ISR is required for proper keratinocyte differentiation during the formation of human skin.

RESULTS

Translation initiation is repressed during keratinocyte differentiation *in vitro*

Keratinocytes *in vitro* can be induced to differentiate by growing cells to confluence and switching to a growth media containing 2mM Ca^{2+} and 2% FBS for 72 hours (Borowiec *et al.*, 2013) (Figure 1a). This calcium switch protocol is widely accepted as a means to initiate keratinocyte differentiation *in vitro* (Micallef *et al.*, 2009; Pillai *et al.*, 1990; Poumay and Pittelkow, 1995). Differentiated keratinocytes were compared with subconfluent, proliferating cultures of keratinocytes (defined here as undifferentiated). To determine the dynamics of

translation initiation during keratinocyte differentiation, lysates were prepared from undifferentiated and differentiated keratinocytes and subjected to sucrose gradient ultracentrifugation. This method measures the levels of protein synthesis as judged by polysome profiling, which determines the amount of ribosomal loading onto mRNAs at a fixed point in time. Keratinocyte differentiation substantially decreased the level of cellular mRNAs bound to heavy polysomes coincident with an increase in mRNAs associated with 80S monosomes, indicating repression of translation initiation (Figure 1b). Translational efficiency can be quantified by calculating the ratio of mRNAs bound to polysomes and monosomes (p/m); larger p/m values correspond to increased translation. The p/m of differentiated keratinocytes was decreased by six-fold compared to undifferentiated controls. To determine if translational control also impacted the elongation phase of protein synthesis, polysome profiling analyses were performed without the addition of cycloheximide (CHX). If differentiation also lowered the elongation phase of translation, omission of CHX should not significantly change the levels of measured polysomes. However in the absence of CHX, differentiated keratinocytes showed a further decrease in polysomes accompanied by increased levels of monosomes (Figure 1b, blue line), verifying that the repression of translation occurs predominantly at the initiation stage.

While the use of *in vitro* two-dimensional cell culture is a powerful tool to study keratinocytes, this culture condition may not fully represent how intact three-dimensional skin undergoes differentiation. Therefore, 3D organotypic skin equivalents were constructed using primary keratinocytes (Figure 1c) and analyzed by polysome profiling. A monolayer of undifferentiated primary keratinocytes seeded on collagen/fibroblast matrix, the initial step in constructing a skin equivalent, displayed levels of translation similar to that of a keratinocyte monolayer grown on a plastic dish (Figure 1d). However, after seven days of growth at the air-

liquid interface, fully stratified skin equivalents revealed sharply lowered levels of transcripts bound to heavy polysomes coincident with increased numbers of mRNAs associated with 80S monosomes, indicating a repression of translation initiation similar to keratinocytes differentiated in monolayers. Collectively, these results indicate that keratinocyte differentiation is concomitant with lowered translation initiation in a three dimensional tissue.

The Integrated Stress Response is activated in differentiated keratinocytes

To determine whether the ISR is induced in differentiating keratinocytes, eIF2α-P was measured in both undifferentiated and differentiated keratinocytes. Levels of eIF2α-P normalized to total eIF2α were nearly 9-fold higher in differentiated keratinocytes as compared to undifferentiated cells (Figure 2a and b). Of importance, there were increased amounts of the differentiation-specific proteins involucrin (IVL) and keratin 1 (KRT1) (Figure 2a). As a control, keratinocytes were also treated with tunicamycin (TM), a potent inducer of ER stress and the eIF2α kinase PERK. While eIF2α-P was increased following treatment with TM, there were no detectable IVL and KRT1 proteins, indicating that eIF2α-P alone does not induce keratinocyte differentiation. As expected, *IVL* mRNA was significantly elevated with keratinocyte differentiation but not with exposure to TM (Figure 2c). Importantly, eIF2α-P occurred early during differentiation (within 24 hours), was detected concurrently with IVL, and was sustained over the course of the experiment (Figure 2d).

To address whether eIF2α-P occurs during keratinocyte differentiation *in vivo*, full-thickness human skin was obtained from surgical abdominoplasty procedures. The tissue was fixed, paraffin embedded, sectioned, and stained with antibodies to measure eIF2α-P, ATF4, and CHOP (Figure 2e), which are subject to preferential translation in the ISR. Fluorescence marking

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the increased presence of each of these ISR markers was increased specifically in the suprabasal layers of the epidermis, which contain differentiated keratinocytes. By comparison, these protein markers were not visible in the single layer of basal keratinocytes. Staining with an IgG isotype control confirms that the fluorescence is not a result of high background. These results indicate that eIF2α-P and translational control are induced in differentiated keratinocytes, *in vivo* and *in vitro*.

Gene-specific translational control during keratinocyte differentiation

In addition to global translation repression, eIF2α-P leads to enhanced translation of specific mRNA transcripts, such as *ATF4* and *CHOP.* To investigate if gene-specific translational control occurs during keratinocyte differentiation, fractions were eluted and collected from polysome profiles and levels of specific mRNAs were measured by qPCR. The percent of total *ATF4* and *CHOP* mRNAs bound to heavy polysomes (fractions 5-7) was increased by 18% and 27%, respectively, during differentiation of keratinocytes *in vitro* (Figure 3a and b), indicative of preferential translation during eIF2α-P. Average polysome (fractions 5-7) over monosome (fractions 1-3) values are indicated for each gene to further illustrate changes in polysome association during differentiation. Importantly, *IVL* transcripts also shifted 27% toward heavy polysomes during differentiation (Figure 3c). By comparison, *eIF4E* mRNA led to a 12% shift away from heavy polysomes towards monosomes (Figure 4d), which is representative of the large number of genes that are subject to translation repression in the ISR. These results show that individual mRNAs including canonical ISR markers and keratinocyte differentiation-specific transcripts are bound to heavy polysomes despite global repression of translation that occurs during keratinocyte differentiation (Figure 1b).

Inhibition of the ISR response suppresses keratinocyte differentiation

To determine whether eIF2α-P plays a critical role in keratinocyte differentiation, we utilized a doxycycline-inducible system (DOX) to overexpress GADD34 in N-TERT keratinocytes (Collier *et al.*, 2015). Elevated levels of GADD34 lead to robust dephosphorylation of eIF2α, which will halt the ISR and relieve global translational repression. When GADD34 was overexpressed in differentiated keratinocytes, polysome profiling analyses revealed a shift to heavy polysomes alongside a decrease in monosome association (Figure 4a), consistent with GADD34 relieving translation repression in response to keratinocyte differentiation. Importantly, GADD34-induced dephosphorylation of eIF2α reduced the amount of IVL protein over 2-fold in keratinocytes compared to control keratinocytes (Figure 4b and c). GADD34 overexpression also decreased the levels of KRT1 protein (Figure 4b), indicating a widespread effect on differentiation gene expression. Elevated levels of GADD34 lowered the polysome association and resulting p/m ratio for *ATF4* and *CHOP* mRNAs (Figure 4d), and *IVL* transcript (Figure 4e) compared to differentiation in the absence of DOX. By contrast, the p/m value for *eIF4E* was significantly increased upon GADD34 overexpression (Figure 4d). Of note, DOX-reduced levels of eIF2α-P also led to a 2-fold reduction in *IVL* mRNA levels during keratinocyte differentiation, suggesting that translational control also contributes directly or indirectly to the increase in *IVL* transcript (Figure 4f). These results indicate that differentiation-specific protein expression is dependent on eIF2α-P and the induction of the ISR.

Loss of GCN2 abrogates differentiation gene expression and epidermal formation

eIF2α kinases are activated in response to distinct stress signals (Baird and Wek, 2012). In the case of PERK, accumulating levels of unfolded protein in the ER activates this eIF2 kinase, which is a transmembrane protein situated in this organelle. PERK functions in the UPR in conjunction with other sensory proteins, such as inositol requiring enzyme 1 (IRE1), which directs transcriptional gene expression through cytosolic splicing of x-box binding protein 1 (*XBP1*) mRNA that leads to the expression of an active XBP1(s) transcription factor. To determine if there is activation of the UPR and inferred ER stress during keratinocyte differentiation, levels of mRNA encoding *XBP1*(s) or the ER chaperone, binding immunoglobulin protein (BiP/*GRP78*/*HSPA5*), were measured by qPCR. Keratinocyte differentiation led to lower amounts of both *XBP1*(s) and *GRP78* mRNAs, suggesting that there is minimal activation of the UPR (Supp. Figure 1a and b). In contrast, both UPR markers were robustly induced in keratinocytes treated with TM. While TM also induced robust PERK expression in keratinocytes, there was no increase in PERK protein levels during keratinocyte differentiation, suggesting that PERK is not activated by this type of stress (Supp. Figure 1d).

Since the UPR was not appreciably induced during keratinocyte differentiation, we next tested whether PERK or GCN2 was responsible for differentiation-induced eIF2α-P. Knockdown N-TERT keratinocytes were created using shRNA against either GCN2 or PERK (Supp. Figure 1c and Figure 5a). PERK knockdown had no effect on differentiation-induced eIF2α-P, IVL or KRT1 protein expression (Supp. Figure 1d and e). By comparison, depletion of GCN2 caused a decrease in differentiation-induced eIF2α-P compared to control (shCTRL) (Figure 5b and c). This was confirmed by analysis of two independent shRNA knockdowns of GCN2 targeting either the coding sequence or 3’ UTR. Loss of GCN2 also caused a sharp decrease (5-fold) in IVL and KRT1 protein induced upon differentiation (Figure 5b and c). Of interest, knockdown of

GCN2 also caused a decrease in differentiation-induced *IVL* mRNA expression compared to control (shCTRL) (Figure 5d), similar to that seen with GADD34 overexpression. To test whether lowered levels of *IVL* mRNA in differentiated GCN2-depleted keratinocytes was due to an increase in *IVL* transcript decay, we assayed the stability of *IVL* mRNA. Differentiated keratinocytes were treated with actinomycin D (AD), an inhibitor of transcript synthesis, and harvested at the indicated times following addition of the drug (Figure 5e). There was no significant difference between *IVL* mRNA decay in shCTRL compared to shGCN2 keratinocytes upon differentiation, suggesting that the decrease in *IVL* mRNA levels in shGCN2 cells is due to lowered transcription of the *IVL* gene.

To examine whether GCN2 is directly activated by keratinocyte differentiation, levels of GCN2 phosphorylated on threonine 899 (GCN2-P) were measured by immunoblot. Activation of GCN2 leads to auto-phosphorylation on this residue, releasing auto-inhibitory molecular interactions that enahnce GCN2 phosphorylation of eIF2α (Castilho *et al.*, 2014). Keratinocyte differentiation caused an increase in GCN2-P similar that seen with halofuginone (HF) a known GCN2 activator that inhibits charging of $tRNA^{Pro}$ (Figure 5f). Importantly, the ER stress inducer TM did not induce GCN2-P. These results indicate that GCN2 is activated during keratinocyte differentiation and is required for eIF2α-P and translational control as well as expression of differentiation proteins.

Given the adverse effect of GCN2 knockdown on differentiation in monolayer keratinocytes, we next addressed the impact of GCN2 loss on epidermal differentiation and formation of an intact, stratified tissue. 3D organotypic cultures were constructed using primary keratinocytes expressing shGCN2 or shCTRL. After seven days of induced differentiation, skin equivalents were sectioned and stained with hematoxylin and eosin (H&E) or antibodies against

eIF2α-P, IVL, or Ki67, a well-characterized marker of cell proliferation (El-Abaseri *et al.*, 2006). There was a striking difference in histology of the shCTRL and shGCN2 skin equivalents, as noted by disorganization of the keratinocytes and decreased cornification compared to the control (Figure 6a). As expected, there was decreased immunofluorescence detected using antibodies against IVL protein or eIF2α-P (Figure 6b and c). These findings are consistent with those observed in monolayer tissue culture (Figure 5b). Of interest, there was an increase in Ki67 positive cells in the basal layer, indicating higher numbers of actively proliferating cells, which can be indicative of hyperplasia (Figure 6d). Furthermore, immunofluorescence analysis with an IgG isotype control indicated that these results are not due to non-specific antibodies. These results indicate that GCN2 is required for proper expression of IVL during keratinocyte differentiation and as a consequence is critical for proper formation of an intact epidermis.

DISCUSSION

This study shows that translational control through GCN2 phosphorylation of eIF2α is required for epidermal differentiation. eIF2α-P represses global translation initiation coincident with preferential translational control of genes such as *IVL* during keratinocyte differentiation (Figure 1-3). Loss of either eIF2α-P or GCN2 abrogated this translation regulation as well as caused a decrease in differentiation and accompanying induced expression markers (Figure 4-6). IVL and KRT1 protein levels were decreased sharply in eIF2α-P deficient and GCN2 knockdown cells compared to controls. The significance of GCN2 was further demonstrated when depletion of GCN2 resulted in disorganized epidermal formation and decreased squamous layers in 3D organotypic culture (Figure 6). Of importance, loss of PERK did not have any detectable effect on differentiation-induced eIF2α-P and was not activated during keratinocyte

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differentiation (Supp. Figure 1), indicating that ER stress and induction of the UPR are not critical. Similarly, our data suggest that GCN2 is activated during keratinocyte differentiation (Figure 5). These results show an importance for GCN2 and translational control in normal epidermal formation, as well as point to potential involvement of this signaling pathway in diseases with impaired keratinocyte differentiation such as psoriasis, squamous cell carcinoma, and atopic dermatitis.

Regulation of *IVL* expression has previously been characterized at the transcriptional level, with extensive focus on promoter elements that control *IVL* mRNA levels (Eckert *et al.*, 2004). To our knowledge, this is the first report of *IVL* regulation at the translational level, as we saw a 27% percent shift of *IVL* mRNA toward heavy polysomes during keratinocyte differentiation (Figure 3c). The resistance of transcripts such as *IVL* to translation inhibition by eIF2α-P ensures appropriate protein expression during keratinocyte differentiation. Lowered general translation would reduce energy and nutrient expenditure and dramatically alter the proteome during the differentiation process. It is noteworthy that total *IVL* mRNA levels were also decreased both in eIF2α-P deficient and GCN2 knockdown keratinocytes compared to controls (Figure 4f and 5d). This finding indicates that transcriptional induction of *IVL* is partially dependent on the ISR. Our laboratory and others have previously characterized a cohort of eIF2α-P-dependent transcription factors, which include ATF3, ATF5, C/EBPβ, and NF-κB (Calkhoven *et al.*, 2000; Deng *et al.*, 2004; Dey *et al.*, 2012; Jiang and Wek, 2005; Jiang *et al.*, 2004; Jiang *et al.*, 2003; Teske *et al.*, 2013; Zhou *et al.*, 2008). The *IVL* promoter contains canonical AP1, SP1, C/EBP, and CRE binding sites, all of which have shown to have some effect on *IVL* transcription (Adhikary *et al.*, 2005; Banks *et al.*, 1998; Crish *et al.*, 2006). It is

anticipated that GCN2/eIF2α-P controls the activity of a transcription factor(s) that modulates *IVL* transcriptional expression, which can also contribute to keratinocyte differentiation.

Importantly, general and gene-specific translational control can also occur during inhibition of mammalian target of rapamycin (mTOR) signaling. Our preliminary data suggest that mTOR signaling is also inhibited during keratinocyte differentiation, and previous work indicates that GCN2 can facilitate sustained mTOR repression during amino acid deprivation (Anthony *et al.*, 2004; Ye *et al.*, 2015). Therefore GCN2 may also be required for inhibition of mTOR signaling during keratinocyte differentiation, and that this could contribute to translational regulation of genes such as *IVL*.

Our data indicate direct activation of GCN2 during keratinocyte differentiation. GCN2 is known to be activated through binding of uncharged tRNAs to an aminoacyl-tRNA synthetase-like domain (Dong *et al.*, 2000; Wek *et al.*, 1989; Wek *et al.*, 1995; Zaborske *et al.*, 2009). Previous research has reported that the composition of tRNAs can be altered in different layers of mouse skin (Zhao *et al.*, 2005), suggesting that the dynamics of tRNA expression and subsequent aminoacylation may also be an activating signal of GCN2 during human keratinocyte differentiation. GCN2 has previously been suggested to participate in the differentiation of other cells types. During an immune response, GCN2 has been shown to respond to the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO), which blocks T-cell differentiation (Munn *et al.*, 2005). In this case, GCN2 was suggested to be activated by tryptophan depletion caused by IDO activation. Other studies suggest that through GCN2, IDO suppresses T-cell differentiation by blocking key enzymes for fatty acid synthesis (Eleftheriadis *et al.*, 2015). Leucine deprivation has also been suggested to inhibit differentiation of myoblasts through GCN2 (Averous *et al.*, 2012) and GCN2 is specifically inhibited by the protein IMPACT

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in differentiated neural cells to allow for high levels of translation (Pereira *et al.*, 2005; Roffe *et al.*, 2013). Based on these earlier observations and our findings, GCN2 is suggested to play critical roles in cellular differentiation among diverse tissues. However, there appear to be important differences in the mechanisms by which GCN2 and translational control can effect cell fates. Additionally, the processes of regulating GCN2 and the ISR in the determination of different cell types are suggested to incorporate changes in tRNA charging, amino acid availability, and/or expression of GCN2 regulatory proteins.

In addition to the importance of the ISR in normal keratinocyte differentiation, our results also point toward potential ISR involvement in skin diseases in which differentiation is dysregulated. Previous reports have shown differential expression of translation-related proteins in psoriasis and squamous cell carcinoma (SCC) tissue, and there are suggestions that SCC-derived cell lines fail to regulate translation in response to calcium (Gibson *et al.*, 1996; Sugiura *et al.*, 2009; Swindell *et al.*, 2015). Combined with studies herein suggesting specific expression of the ISR in differentiated skin layers, we propose that ISR markers such as eIF2α-P, along with ATF4, CHOP, or GADD34 could serve as valuable markers of skin disease. Previous studies have also shown that the action of the cyclosporin A in psoriasis treatment depends on CHOP, indicating that activation of the ISR could alleviate certain skin lesions (Hibino *et al.*, 2011). Multiple pharmacological agents can alter the ISR, and targeting eIF2α in disease has become an increasingly promising option as the importance of translational control continues to be elucidated (Fullwood *et al.*, 2012). Among drugs that activate the ISR is salubrinal, a GADD34 inhibitor, which has been shown to be effective in cell culture and mouse models of neurodegenerative disease and osteoporosis (Sato *et al.*, 2015; Saxena *et al.*, 2009). Another reported GADD34 inhibitor, guanabenz, is a clinically approved α2-adrenergic agonist used to

treat hypertension (Holmes *et al.*, 1983). Alternatively, ISRIB is an ISR inhibitor that can improve memory function in mice (Sidrauski *et al.*, 2013). Altogether, this work has underscored a previously unrecognized importance for translational control through the ISR in keratinocyte differentiation, and we can now begin to explore the application of well-studied pharmacological agents in the context of skin disease.

MATERIALS AND METHODS

Cell culture

Normal human keratinocytes were isolated from neonatal foreskin tissue as described previously (Kuhn *et al.*, 1999). The collection of human skin samples was approved by the Indiana University School of Medicine Institutional Review Board. Cultured cells were treated with 2 µM tunicamycin (Sigma-Aldrich, St. Louis, MO), 10 µM actinomycin D (Sigma), 1 µg/mL doxycycline (Sigma), 100 nM halofuginone (Sigma), or 50 µg/mL cycloheximide (Sigma) as indicated. Stable GADD34 overexpression using a Tet-inducible promoter in N-TERT cells was carried out as previously described (Collier *et al.*, 2015), and doxycycline was added for 24 hours prior to any additional treatments. Tunicamycin was added for 6 hours, but similar experimental results were obtained following 24 hour treatment (data not shown). Knockdown of PERK and GCN2 by shRNA and lentiviral delivery are detailed in the supplement section. 3D organotypic cell culture was performed using primary human keratinocytes and fibroblasts obtained from neonatal foreskin tissue as described previously (Kuhn *et al.*, 1999; Loesch *et al.*, 2016). Details are discussed in the Supplemental Data.

Immunoblot analysis

Immunoblots were performed as described previously (Collier *et al.*, 2015; Teske *et al.*,

2013) and details are discussed in the Supplemental Data.

Measurement of mRNA levels by qPCR

Total mRNA and polysome mRNA levels were measured as previously described (Collier *et al.*, 2015). Details and primer sequences are listed in the Supplemental Data.

Immunofluorescence and Microscopy

Immunofluorescence was performed as described previously (Loesch *et al.*, 2016). Details are discussed in the Supplemental Data. A Nikon 80i microscope with Intensilight epifluorescence and Qimaging camera were used for all imaging purposes. Images were taken using a 20X objective lens at 25°. Qimaging and Nikon Elements software were used for data acquisition.

Polysome Profiling by Sucrose Gradient Ultracentrifugation

Polysome profiling was performed as described previously (Collier *et al.*, 2015; Palam *et al.*, 2011; Teske *et al.*, 2013). Additional details are discussed in the Supplemental Data.

CONFLICT OF INTEREST

The authors have no conflicts of interest to report.

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visualization, and writing. Drs. Wek and Spandau contributed to resources, software, review and editing. Support for this work was supplied by the National Institute of Environmental Health Sciences (ES020866 to DFS and F31ES026517 to AEC), the National Institute on Aging (AG048946 to DFS), and the National Institute of General Medical Sciences (GM049164 to RCW). There are no conflicts of interest to report.

FIGURE LEGENDS

Figure 1. Global translation initiation is repressed during keratinocyte differentiation. (a) Phase-contrast images of undifferentiated (undiff) and differentiated (diff) N-TERT keratinocytes generated by switching confluent N-TERT keratinocytes to a media containing 2mM Ca^{2+}/2% FBS for 72 hours. Polysome profiles for these conditions are shown in (b). No CHX (blue) indicates that cycloheximide was omitted from the protocol. (c) 3D organotypic cultures were fixed at day 0 (undiff) and 7 (diff) after raising to the air-liquid interface and stained with H&E. Polysome profiles were generated for these conditions compared to an undifferentiated monolayer control are shown in (d). For all polysome profiles, ratio of polysomes to monosomes (p/m) is indicated to the right of each sample label. Scale bars = 50µm.

Figure 2. The Integrated Stress Response is activated in differentiated keratinocytes. (a) Undifferentiated (undiff), differentiated (diff), and tunicamycin (TM) treated N-TERT keratinocytes were subjected to immunoblot analysis to measure levels of the indicated proteins. Levels of eIF2α-P normalized to total eIF2α as measured by densitometry are indicated in (b). Alternatively, RNA was collected from samples and total mRNA levels were measured for *IVL* (c). Keratinocyte differentiation was also monitored for the indicated number of days and

subjected to immunoblot analysis (d). Full-thickness skin was stained for antibodies against eIF2α-P, ATF4, CHOP, or an IgG isotype control (e). The basement membrane is demarcated with a gray line. Scale bars are: large image = 50 µm, inset = 25µm. Error bars = mean +/- SD.

Figure 3. Gene-specific translational control during keratinocyte differentiation. RNA was isolated from sucrose fractions taken from polysome profiles in Figure 1b and used to generate cDNA. qPCR was used to measure mRNA levels of (a) *ATF4*, (b) *CHOP*, (c) *IVL*, and (d) *eIF4E* in each fraction. Levels of mRNA were normalized to a spike-in luciferase transcript, and represented as a percent total for each mRNA so as to omit changes in gene transcript levels. Arrows represent shifts toward (green) or away from (red) heavy polysomes (fractions 5-7), and the total percentage of each gene transcript that shifts during differentiation are indicated. Error bars = mean +/- SD.

Figure 4. Inhibition of the ISR suppresses keratinocyte differentiation. (a) Polysome profiles were generated for undifferentiated (undiff), differentiated (diff+vehicle), and differentiated during GADD34 overexpression (diff+DOX) N-TERT keratinocytes grown in monolayer culture. Polysome/monosome (p/m) ratios are listed beside each sample. (b) Alternatively, lysates were subjected to immunoblot analysis to measure the indicated protein levels. Measurement of eIF2α-P normalized to total eIF2α and IVL proteins are indicated in (c). (d) p/m values were calculated for each indicated gene in differentiated cells treated with vehicle or DOX to induce GADD34 overexpression that sharply lowers eIF2α-P by dividing the percent of the gene transcript in polysome (5-7) by monosome (1-3) sucrose fractions. (f) Alternatively, total

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RNA was isolated from cells and qPCR was performed to measure levels of *IVL* mRNA. *, $p<0.05$, error bars = mean +/- SD.

Figure 5. Loss of GCN2 abrogates differentiation gene expression. (a) GCN2 mRNA knockdown efficiency was measured by qPCR. (b) Undifferentiated (undiff), differentiated (diff), or tunicamycin (TM) treated N-TERT keratinocytes with control (shCTRL) or shGCN2 shRNA were subjected to immunoblot analysis to measure the indicated proteins. Measurement of eIF2α-P normalized to total eIF2α and IVL proteins are indicated in (c). (d) RNA was also isolated from these treatments and *IVL* mRNA levels were measured by qPCR. (e) An mRNA half-life assay was performed by treating shCTRL or shGCN2 cells that had been allowed to differentiate for 24 hours with 10μg/mL actinomycin D (AD). RNA was isolated from each sample after the indicated number of hours, and qPCR was performed to measure levels of *IVL* mRNA. (f) N-TERTs were treated with 2μM TM or 100nM halofuginone (HF) for 6 hours or were differentiated. Lysates were then subjected to immunoblot analysis to measure GCN2 activation. *, $p<0.05$. Error bars = mean +/- SD.

Figure 6. GCN2 is required for proper epidermal differentiation. 3D organotypic cell cultures were made using shCTRL and shGCN2 primary human keratinocytes that were seeded on a collagen-fibroblast matrix and raised to the air-liquid interface in order to differentiate and stratify. After 7 days the samples were fixed, sectioned and stained with (a) H&E or antibodies against (b) eIF2α-P, (c) IVL, (d) Ki67, or (e) an IgG isotype control. Relative GCN2 knockdown efficiency in primary keratinocytes was measured by qPCR in (f). Scale bar = 25μm for every image.

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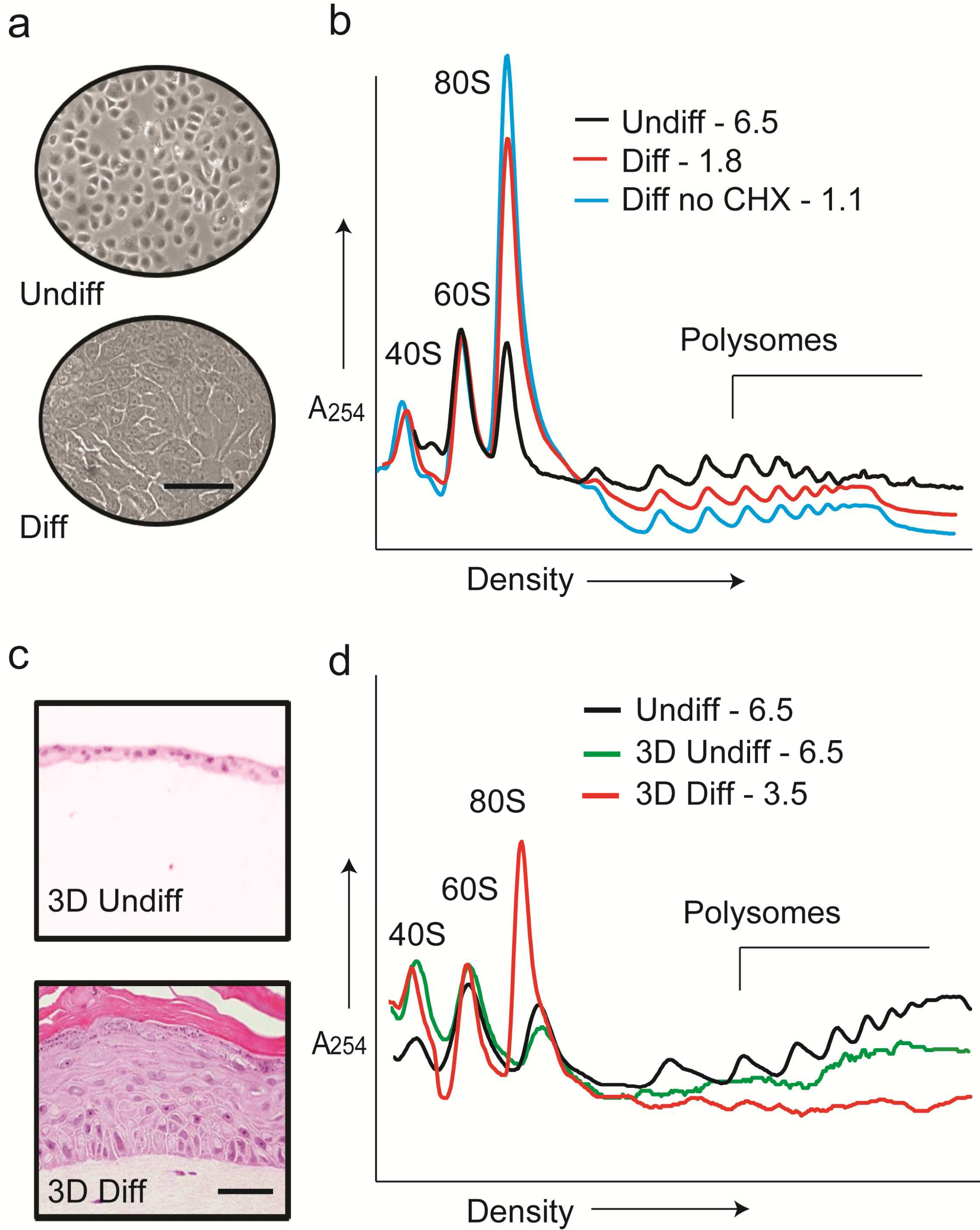

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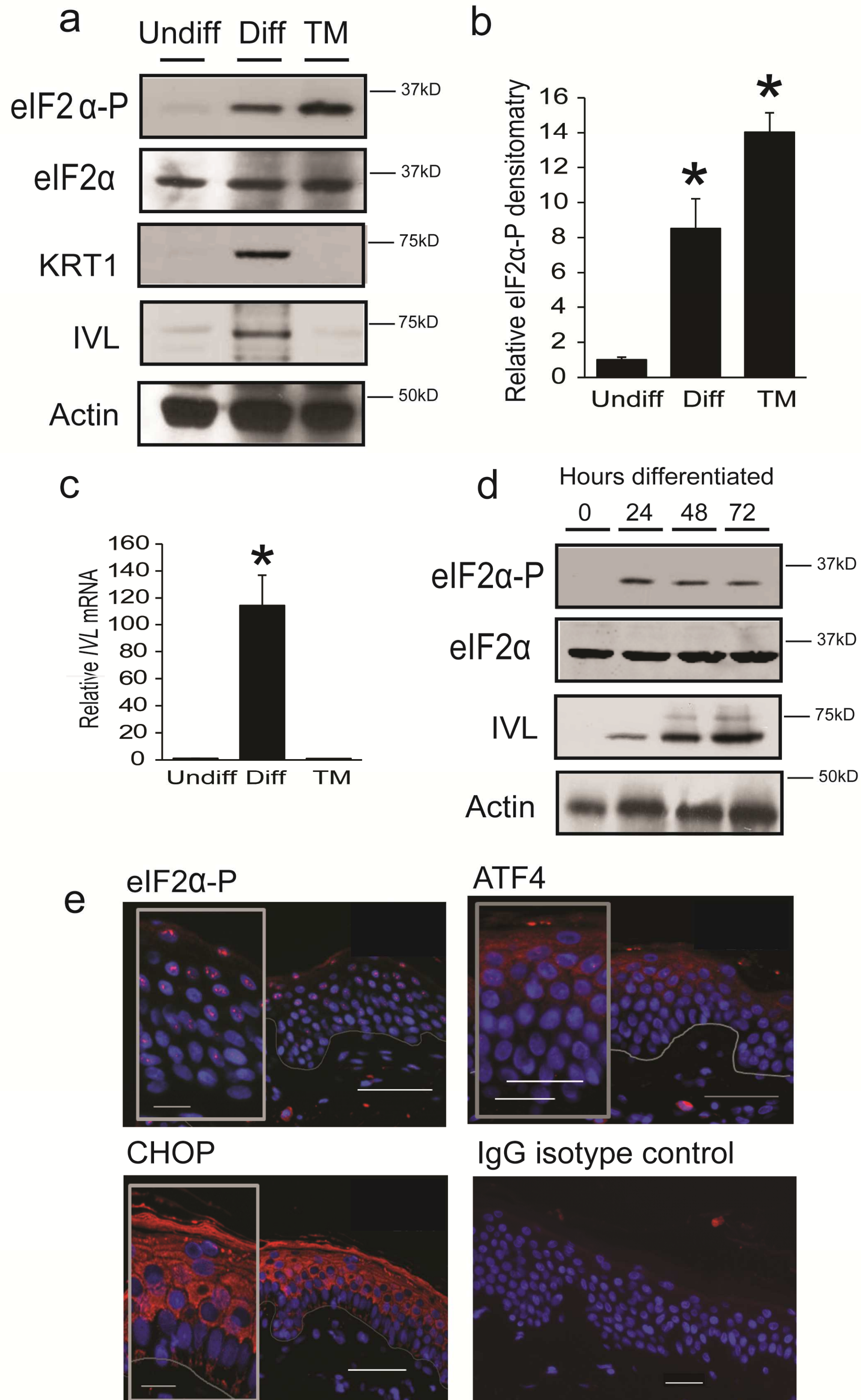

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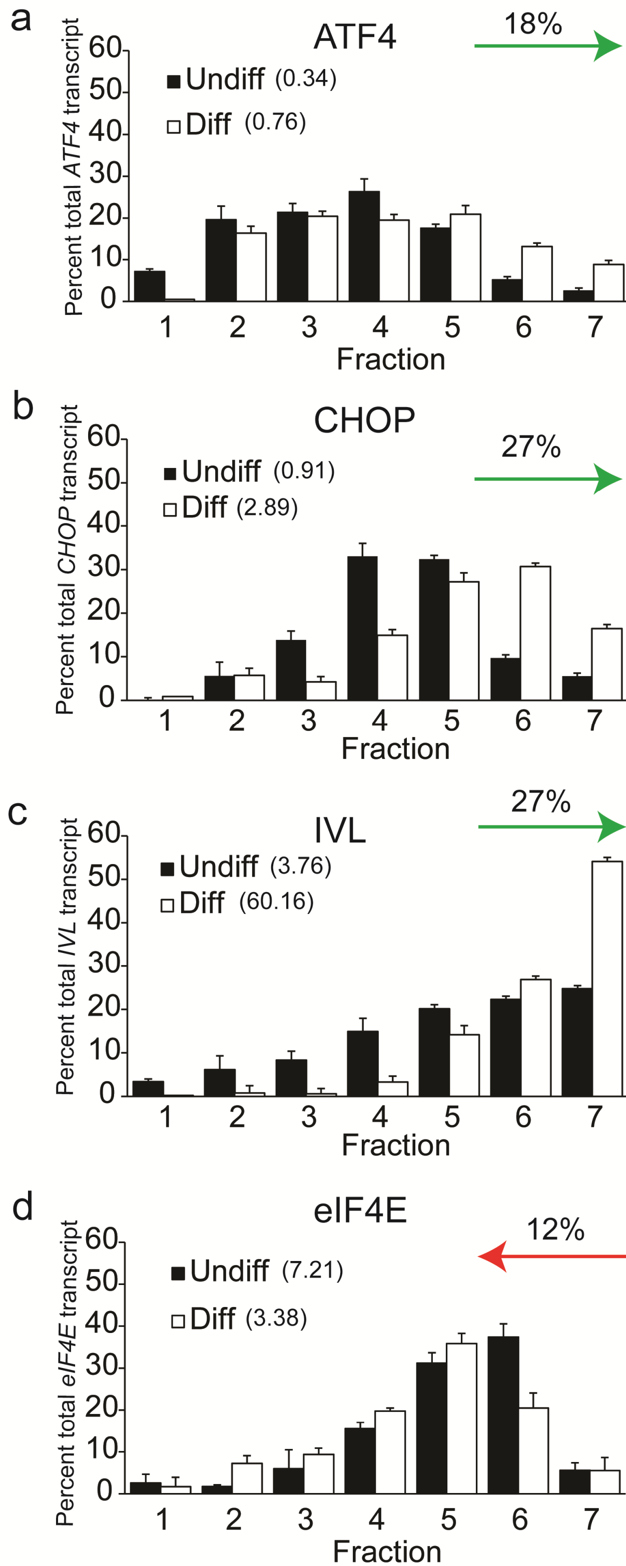

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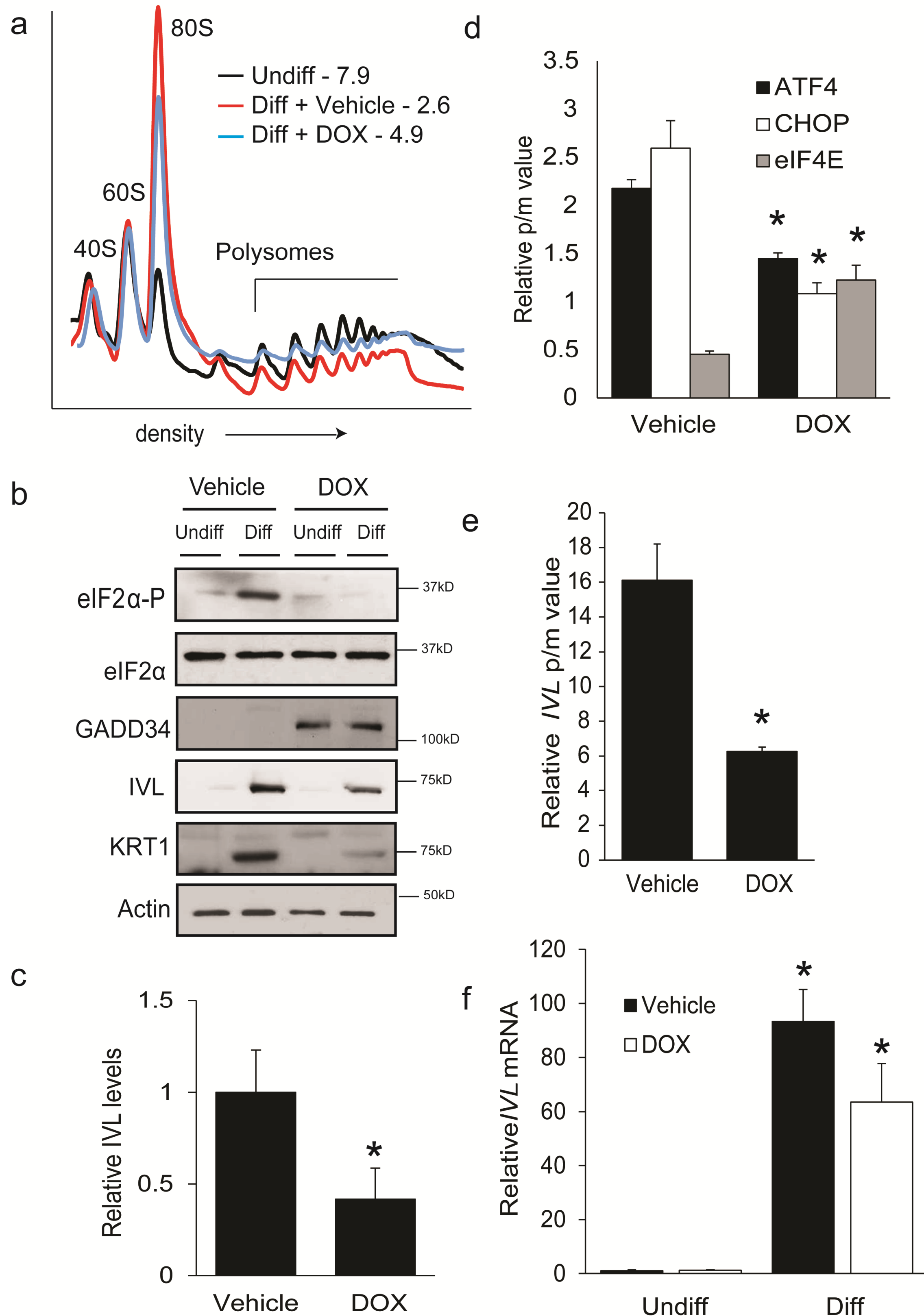
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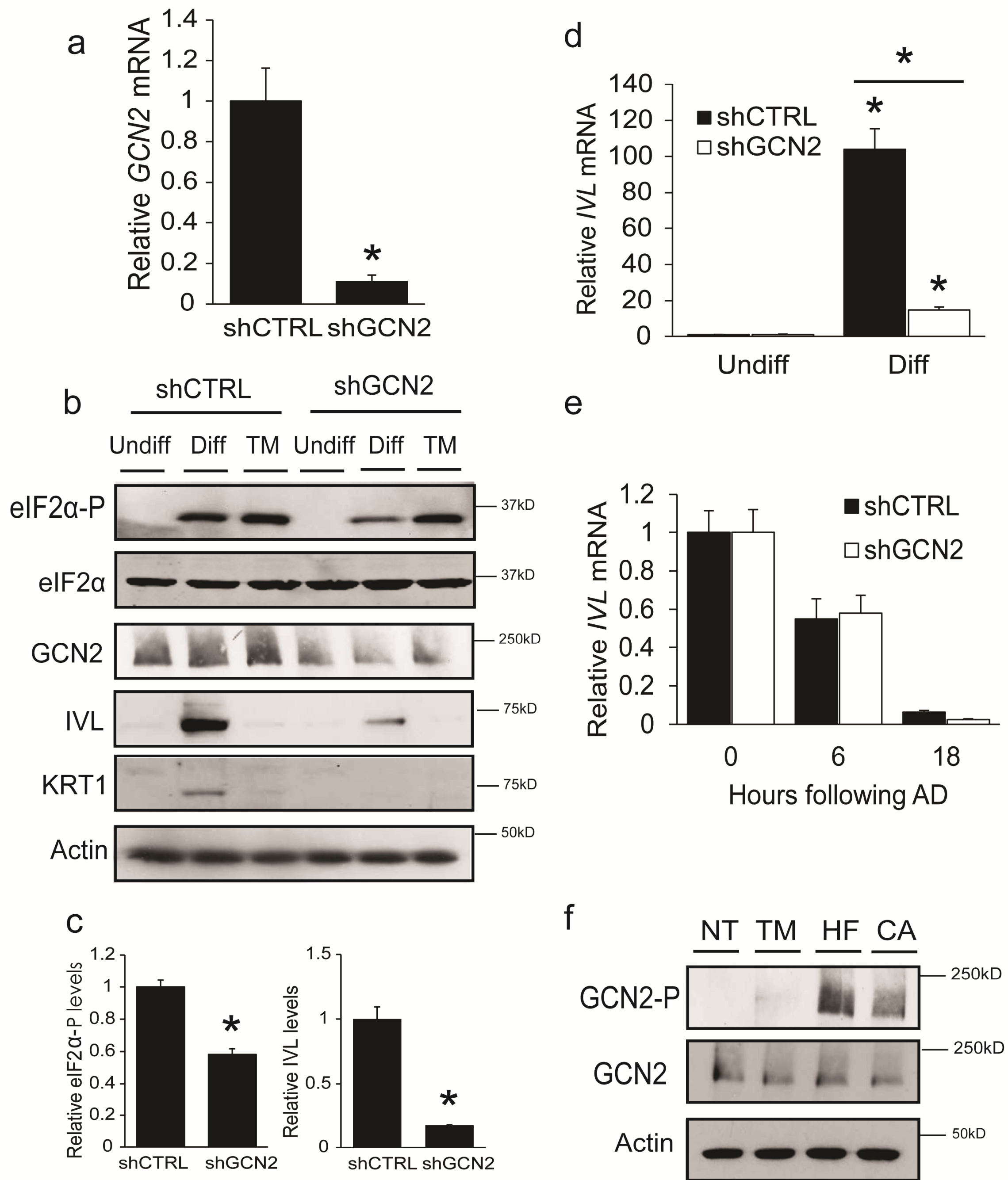
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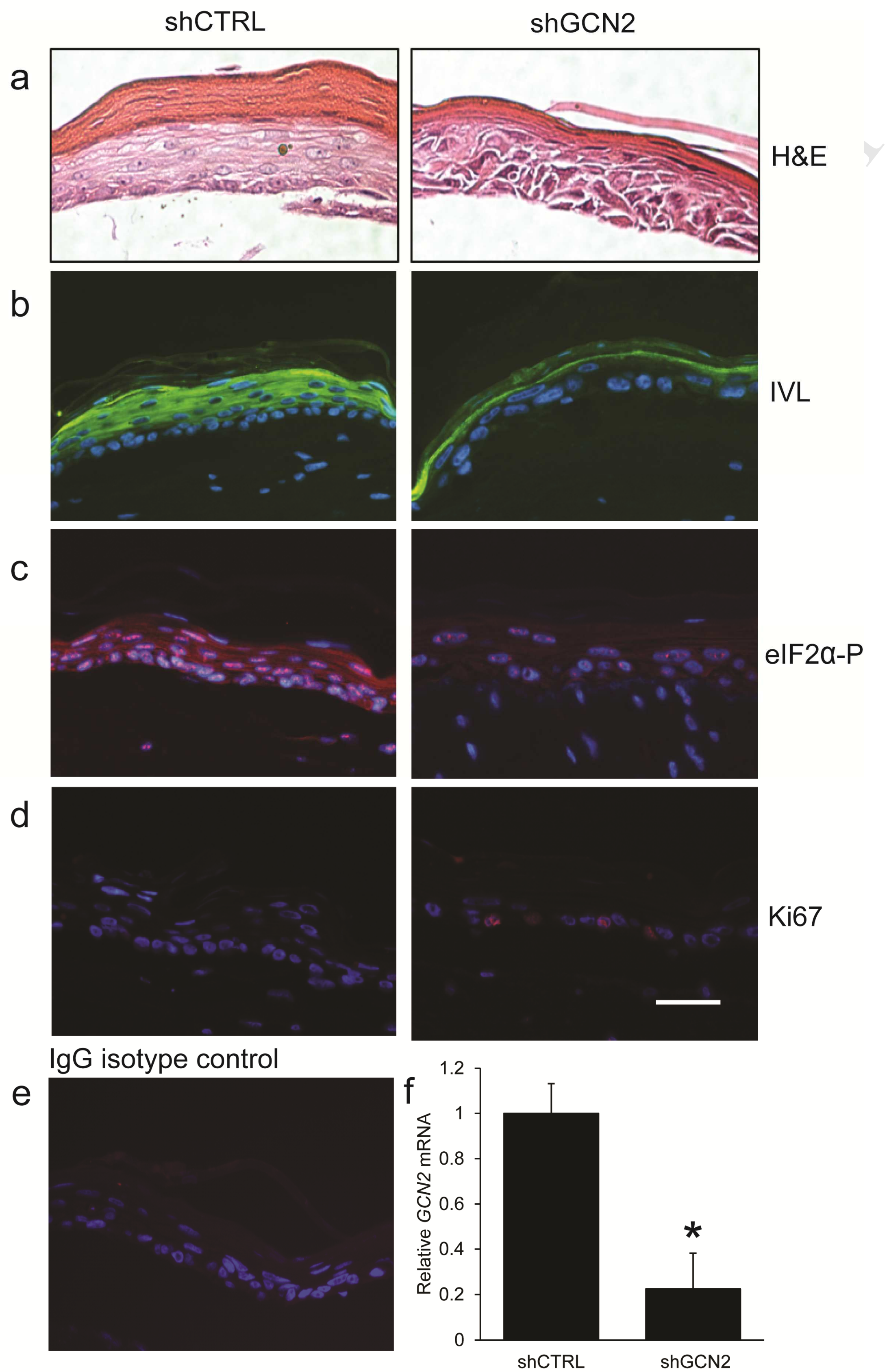

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