

# AKT1-mediated Lamin A/C degradation is required for nuclear degradation and normal epidermal terminal differentiation

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Nuclear degradation is a key stage in keratinocyte terminal differentiation and the formation of the cornified envelope that comprises the majority of epidermal barrier function. Parakeratosis, the retention of nuclear material in the cornified layer of the epidermis, is a common histological observation in many skin diseases, notably in atopic dermatitis and psoriasis. Keratinocyte nuclear degradation is not well characterised, and it is unclear whether the retained nuclei contribute to the altered epidermal differentiation seen in eczema and psoriasis. Loss of AKT1 function strongly correlated with parakeratosis both in eczema samples and in organotypic culture models. Although levels of DNases, including DNase1L2, were unchanged, proteomic analysis revealed an increase in Lamin A/C. AKT phosphorylates Lamin A/C, targeting it for degradation. Consistent with this, Lamin A/C degradation was inhibited and Lamin A/C was observed in the cornified layer of AKT1 knockdown organotypic cultures, surrounding retained nuclear material. Using AKT-phosphorylation-dead Lamin A constructs we show that the retention of nuclear material is sufficient to cause profound changes in epidermal terminal differentiation, specifically a reduction in Loricrin, Keratin 1, Keratin 10, and filaggrin expression. We show that preventing nuclear degradation upregulates BMP2 expression and SMAD1 signalling. Consistent with these data, we observe both parakeratosis and evidence of increased SMAD1 signalling in atopic dermatitis. We therefore present a model that, in the absence of AKT1-mediated Lamin A/C degradation, DNA degradation processes, such as those mediated by DNase 1L2, are prevented, leading to parakeratosis and changes in epidermal differentiation.

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Nuclear degradation is a key stage in keratinocyte terminal differentiation and the formation of the cornified envelope that comprises the majority of epidermal barrier function.<sup>1–3</sup> Parakeratosis, the retention of nuclear material in the cornified layer of the epidermis, is a common histological observation in many skin diseases, but most notably in the epidermal barrier-defective diseases eczema and psoriasis.<sup>4,5</sup> Mechanisms of nuclear degradation in the epidermis have not yet been well characterised and it is not known whether the retained nuclei contribute to the altered epidermal differentiation programmes seen in these skin diseases.<sup>6,7</sup>

It is surprising that, for such a critical component of epidermal terminal differentiation, relatively few molecular mechanisms inducing parakeratosis have been investigated. The caspase-14 knockout mouse develops parakeratotic plaques upon chemical barrier disruption<sup>8</sup> and has subtle defects in epidermal terminal differentiation, including filaggrin processing,<sup>9</sup> whereas the DNase 1L2 knockout mouse showed constitutive nuclear retention in hair and nails, which led to structural abnormalities in the hair shaft.<sup>10,11</sup> Parakeratosis also occurs during wound healing.<sup>12</sup> Nuclei are retained in the scab of healing wounds, and this correlates with the expression of different keratins and altered structural protein expression in this region.<sup>13</sup>

Although taken together this is suggestive that retained nuclei can influence epidermal and adnexal differentiation by signalling to these structures, there is no direct evidence that this is the case. We have already identified AKT1 as an important signalling molecule in epidermal terminal differentiation. Loss of AKT1 causes cornified envelope fragility, and reduces the barrier function of the cornified layer.<sup>14,15</sup> We therefore wanted to test the hypothesis that AKT1 caused this fragility by preventing nuclear degradation in the cornified layer. Organotypic culture of keratinocytes, in which AKT1 has been silenced by specific shRNA, retained nuclei in the cornified layer. We show here that shRNA knockdown (kd) of AKT1 prevents phosphorylation and subsequent degradation of nuclear lamins. Furthermore, expression of non-degradable lamins leads to upregulation of BMP2-SMAD1-mediated signalling and keratinocyte terminal differentiation changes.

## Results

**Parakeratosis in eczema samples correlates with AKT1 activity loss, and AKT1 loss causes parakeratosis and altered keratinocyte differentiation.** We examined the

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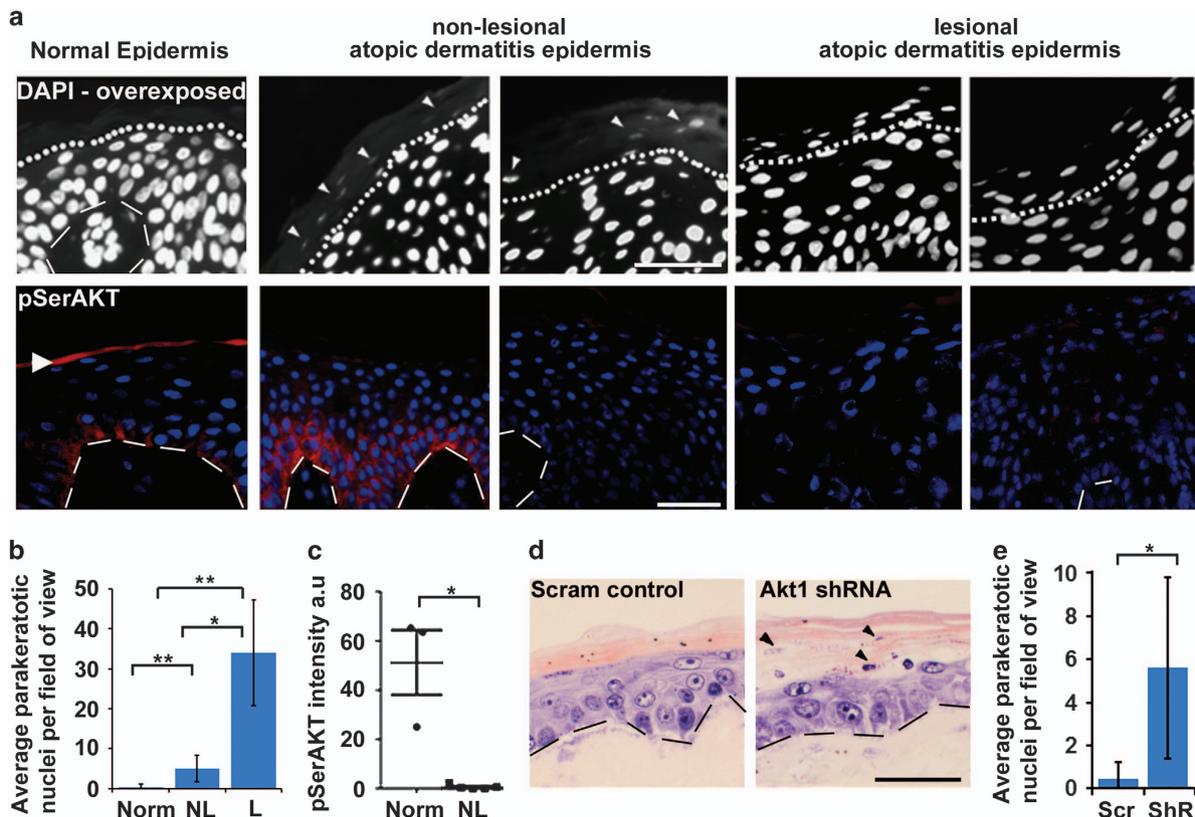
**Abbreviations:** AD, atopic dermatitis; AKT, murine thymoma viral oncogene homologue (protein kinase B); BMP, bone morphogenetic protein; DAPI, 4',6-diamidino-2-phenylindole; E18.5, embryonic day 18.5 (mating plug counted as day 0.5); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kd, knockdown; PBS, phosphate buffered saline; REK, rat epidermal keratinocyte; RIPA, radioimmunoprecipitation assay buffer; SDS, sodium dodecyl sulphate; SMAD, mothers against decapentaplegic homologue

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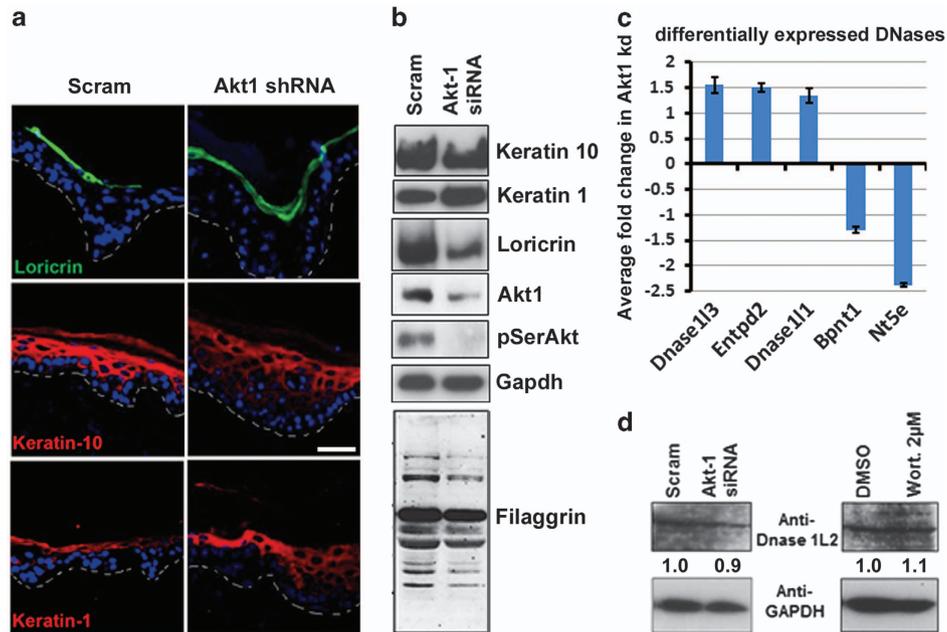
epidermis of five non-lesional and four lesional AD skin samples (Supplementary Figure S1) and compared them with non-AD controls to look for evidence of parakeratosis—that is, retained nuclear material in the cornified layer (Figure 1a). All lesional and non-lesional AD samples demonstrated a significant increase in parakeratosis (Figure 1b), and this correlated with a loss of upper granular layer AKT activity, specifically associated with AKT1 (Figures 1a and c).<sup>14,15</sup> We successfully knocked down AKT1 expression by expressing two separate shRNA plasmids (A1 and A3) in rat epidermal keratinocytes. In this experiment, rat epidermal keratinocytes were used because they differentiate in confluent culture to a greater degree than either human or mouse keratinocytes, do not require calcium switch, and readily form organotypic cultures with a cornified layer highly reminiscent of human epidermis.<sup>14</sup> Both A1 and A3 lines displayed significantly increased numbers of parakeratotic nuclei in the cornified layers of skin-equivalent organotypic cultures (Figures 1d and e). Thus, we examined the expression of the epidermal keratinocyte differentiation markers Keratin 1, Keratin 10, and Loricrin in organotypic cultures grown from the A1 and A3 keratinocyte lines. We found that, although changes were hard to detect by

immunofluorescence in the organotypic cultures (Figure 2a), a reduction of Keratin 10 and Loricrin, and an increase in Keratin 1 in post-confluent cultured kd cells, was clearly detectable by western blot. Furthermore, we found a reduction of filaggrin expression as well as of the mature filaggrin monomer in AKT1-kd cells (Figure 2b). By examination of gene array hybridisation data we could identify no significant change in expression of DNases or nucleotidases in these lines (Figure 2c). Indeed, increased expression of both DNase1L3 and 1L1 was observed. However, no change was detectable in the case of DNase 1L2, a DNase implicated in nuclear degradation in the epidermis,<sup>10,11</sup> and this result was further validated by western blot (Figure 2d). Taken together, these data suggest that the nuclear retention seen in the cornified layer in response to reduced AKT1 expression is not due to a decrease in the expression of DNases.

**AKT1 signalling is required for constitutive Lamin A/C phosphorylation and degradation in keratinocytes.** AKT1 phosphorylates Lamin A/C at Serine 301 and 404, which targets it for degradation.<sup>16,17</sup> As we demonstrated previously, AKT1 is expressed during late terminal differentiation



**Figure 1** Parakeratosis in the cornified layer in eczema correlates with loss of granular layer AKT1 activity. (a) Representative immunofluorescence for pSer473 AKT (pSerAKT), and nuclear material (DAPI) from three normal control skin samples and five non-lesional and four lesional atopic dermatitis skin samples. (b) Graph of counts of parakeratotic nuclei per field of view in normal epidermis ( $n=20$ ) and non-lesional ( $n=5$ ) and lesional ( $n=4$ ) atopic dermatitis samples (\*\* $P<0.0001$ , \* $P<0.001$ , 2-tailed  $t$ -test). (c) Graph of fluorescence intensity of pSerAKT in normal skin and unaffected atopic dermatitis samples in A, \* $P<0.05$ , Mann–Whitney  $U$ -test. (d) Representative histology of organotypic cultures of scrambled control ( $n=7$ ) and AKT1 shRNA-expressing cell lines ( $n=5$  comprising both A1 and A3 lines). (e) Graph of counts of parakeratotic nuclei per field of view in the cornified layer of AKT1 shRNA cultures. Arrowheads indicate parakeratotic nuclei. (e) Bars  $50\ \mu\text{m}$  (a, b and d). Error bars are s.d. (b, c and e). Dotted line indicates the top of the granular layer; dashed line indicates the dermal–epidermal boundary. DAPI, 4',6-diamidino-2-phenylindole



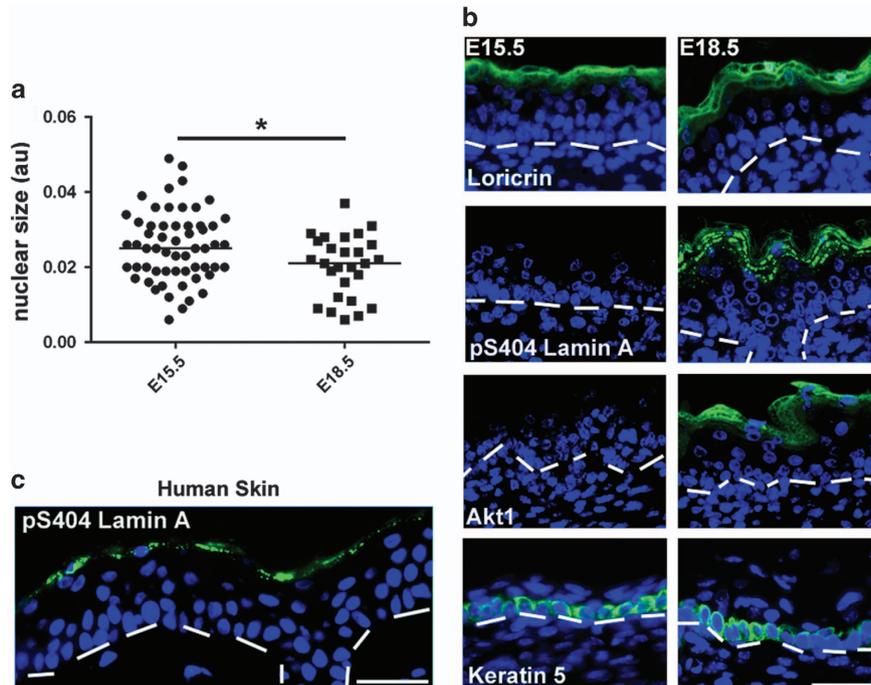
**Figure 2** shRNA kd of AKT1 changes epidermal differentiation marker expression, but does not reduce DNase expression. (a) Representative immunofluorescence of Loricrin, Keratin 10, and Keratin 1 in organotypic cultures ( $n=2$  both lines) of AKT1 shRNA-expressing cell lines A1 and A3. A dashed line indicates the dermal–epidermal boundary. Bar  $50\ \mu\text{m}$ . (b) Representative western blot ( $n=2$  both lines) of lysates of AKT1 shRNA-expressing keratinocytes for Keratins 1 and 10, Loricrin, AKT1, pSerAKT, GAPDH, and filaggrin. (c) Graph of average fold-change of differentially expressed DNases in the gene expression analysis of the AKT1 shRNA-expressing cells. Error bars are the standard deviation ( $n=2$  for both lines). (d) Western blot analysis of DNase1L2 in both AKT1 shRNA-expressing cells and in Wortmannin-treated, AKT1-inhibited cells. GAPDH serves as a loading control in (b and d). Numbers denote the normalised intensity values of DNase1L2. GAPDH, glyderaldehyde-3-phosphate dehydrogenase

in the mouse,<sup>14</sup> and this correlates with increased expression of epidermal terminal differentiation markers between E15.5 and E18.5 of mouse embryonic development.<sup>14</sup> Therefore, we investigated whether nuclear size change and the phosphorylation of Lamin A/C also occurs during this time. We found that nuclear size in terminally differentiated (Loricrin positive) cells was significantly reduced in the epidermis of E18.5 mice compared with E15.5 mice (Figure 3a). Conversely, AKT1 expression was increased in E18.5 mouse epidermis, and this correlated with an increased phosphorylation of Lamin A/C at the Serine 404 site in the terminally differentiated (Loricrin positive) keratinocytes (Figure 3b). This suggested that Lamin A/C undergoes extensive phosphorylation by AKT1 during late terminal differentiation, and this was further supported in adult human epidermis by the expression of phosphorylated Lamin A/C in the uppermost granular layer cells. Although phosphorylated Lamin A/C expression was found to be associated with nuclear material in the upper epidermis, the strongest signal localised in granules throughout the granular layer (Figures 3b and c).

On this basis, we asked whether AKT1 kd leads to changes in Lamin A/C protein phosphorylation and expression. To test this, we compared protein bands differentially represented in the AKT1 kd cells, and analysed them by mass spectrometry (Figures 4a and b). In particular, we identified one band at 60 kDa, which comprised five proteins: heat shock protein 70, prelamin A, DEAD-box polypeptide 1, Keratin 2, and Annexin (Figure 4b). Lamin A/C is expressed as a precursor that is subsequently proteolytically processed to form mature lamins.<sup>18</sup> Using post-confluent keratinocytes we could show that degradation of Lamin A/C to even lower molecular weight

peptides occurred co-incident with AKT Serine 473 phosphorylation in post-confluent (differentiated, Keratin 1 positive) keratinocytes (Figure 4c). Remarkably, inhibition of AKT1 by wortmannin treatment completely prevented Lamin A/C degradation.<sup>19</sup> A similar result was obtained by AKT1 silencing (Figure 4d). Lamin A/C staining was similar in the vital layers of both the control and AKT1-kd organotypic cultures. However, retention of nuclear material in the cornified layer was associated with maintained cornified layer Lamin A/C expression and with a reduction in granular layer phosphorylated Lamin A/C (Figure 4e). Consistent with the above findings in organotypic culture, basal keratinocyte Lamin A/C expression was unchanged in post-confluent keratinocyte culture, whereas Lamin A/C fluorescence intensity was increased in terminally differentiated (Loricrin positive) suprabasal AKT1 kd keratinocytes (Figures 5a–c). In control keratinocytes, the nuclei in these Loricrin-expressing cells were not intact and Lamin A/C expression in the nuclear lamina was disrupted. In contrast, the Lamin A/C expression in Loricrin-positive AKT1-kd keratinocytes was not disrupted. This was associated with reduced Loricrin expression (Figures 5a–c). Nuclear volume in Loricrin-positive cells was higher in controls compared with the AKT1 kd cells. Furthermore, nuclear material extended beyond the disrupted nuclear lamina in controls, whereas the nuclear material was still confined within the intact nuclear lamina in AKT1-kd cells (Figures 5e and f).

**Overexpression of AKT-phosphorylation-dead mutant lamin A causes profound defects in keratinocyte terminal differentiation and filaggrin expression and processing.** Lamin A is a known target of AKT, and AKT phosphorylation

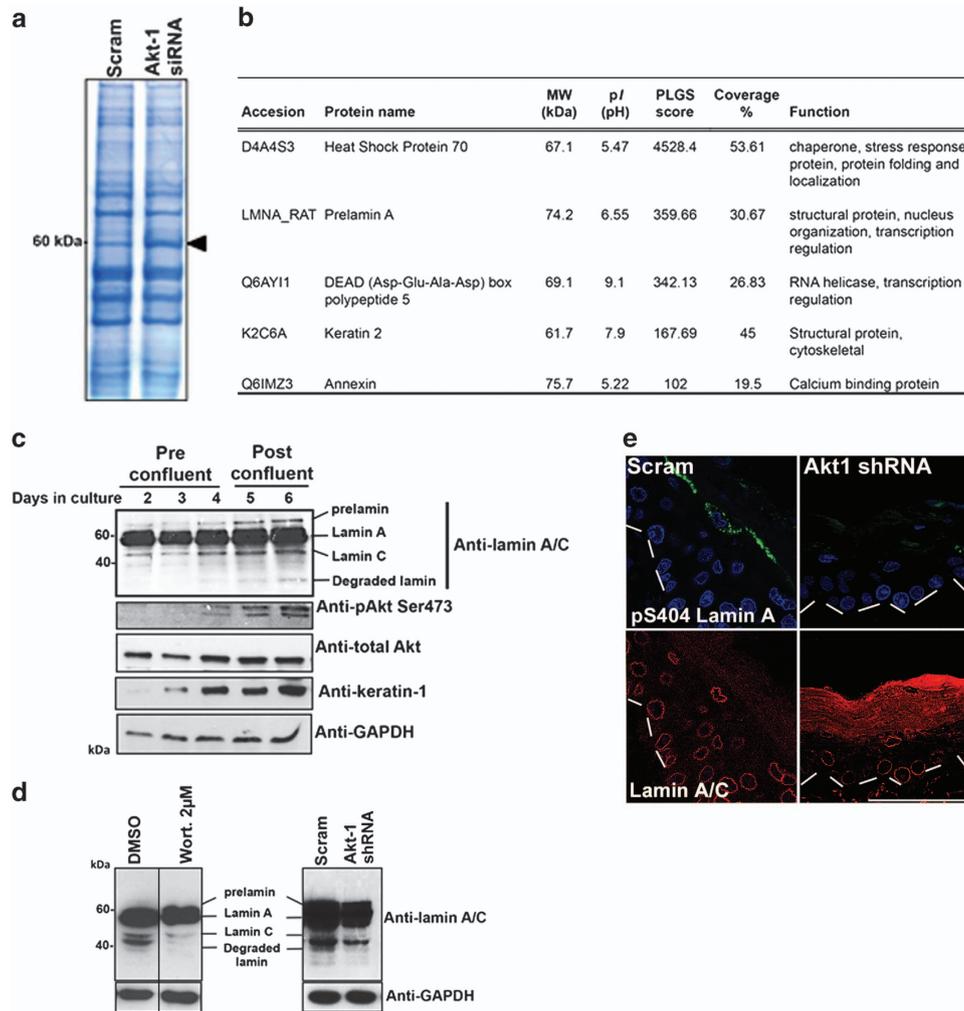


**Figure 3** Lamin A phosphorylation correlates with reduced nuclear size and terminal differentiation in the epidermis. (a) Analysis of nuclear size from  $n = 5$  sections from two different E15.5 and E18.5 mouse embryos. (b) Immunofluorescence of loricrin, AKT1, pS404 Lamin A, and Keratin 5 in E15.5 and E18.5 embryo epidermis. (c) Immunofluorescence of pS404 Lamin A in human epidermis. Bar (b and c)  $50 \mu\text{m}$ .  $*P < 0.05$ , Mann-Whitney  $U$ -test. Dashed line indicates the dermal-epidermal boundary

is required for Lamin A degradation in other tissues.<sup>16,17</sup> To test the consequences of expressing a non-degradable Lamin A on keratinocyte differentiation, we transfected cells with human flag-tagged Lamin A with serine to alanine mutations at positions 301 and 404 (S301A and S404A; Figure 6a). Both prevented degradation of Lamin A, which was shown by the immuno-depletion experiments to be construct specific; that is, the mutant Lamin A did not inhibit or prevent the degradation of the wild-type Lamin A/C present (Figure 6a). Compared with the overexpression of flag-tagged wild-type Lamin A, expression of both mutants led to a reduction in suprabasal keratins (Keratin 1 and Keratin 10), as well as in filaggrin and Loricrin levels. No changes in basal keratin expression (Keratin 5) or AKT activity were observed. We confirmed the reduction on Loricrin expression by immunofluorescence (Figure 6b). The effects were not confined to the transfected, Flag tag-positive cells (Figure 6b), suggesting that the effect on differentiation of non-degradable lamins is not cell intrinsic, and that a secreted factor may be responsible for the differentiation changes. We also observed increased nuclear and nuclear lamin-associated filaggrin in the S301A and S404A Lamin A-expressing cells (Figures 6c and d). Taken together, this suggests that the presence of non-degraded nuclei alone is sufficient to significantly alter the terminal differentiation programme in keratinocytes.

**Non-degraded nuclei caused increased BMP signalling *in vitro* and *in vivo*.** As the changes in epidermal terminal differentiation were not cell intrinsic, we hypothesised that a secreted factor was responsible for the epidermal terminal differentiation changes caused by the expression

of non-degradable Lamin A. Therefore, we examined differential gene expression in the AKT1-kd cells specifically for secreted proteins. We found 13 differentially expressed genes coding for secreted proteins (Figure 7a). We were interested in the upregulation of BMP2, as overexpression of a constitutively active BMP receptor in mouse skin was reported to alter cytokeratin expression and reduce filaggrin expression,<sup>20</sup> in good agreement with our results in the non-degradable Lamin A transfection experiments (Figure 6a). In our AKT1-kd organotypic cultures, hyperkeratotic areas were associated with increased levels of SMAD1 and nuclear SMAD1 expression, suggestive of activated SMAD1 signalling in these regions (Figure 7b). Consistent with these data, both Lamin A S301A- and S404A-expressing keratinocytes had increased BMP2 and SMAD1 expression, and S301A had highly upregulated SMAD1 phosphorylation (Figure 7c). Treatment of post-confluent rat epidermal keratinocytes with BMP2 resulted in a specific decrease in Loricrin expression (Figure 7d). Taken together, these data suggest that retained nuclei in terminally differentiated keratinocytes increase SMAD1 signalling and that this alters epidermal terminal differentiation. If BMP2 signalling through SMAD1 was a consequence of parakeratosis in pathology, we would expect it to occur in areas of parakeratosis in clinical samples. We tested this hypothesis on five non-lesional and four lesional AD skin samples. Higher levels of nuclear SMAD1 were observed in both non-lesional and lesional AD samples (Figures 7e and f). Therefore, we provide evidence both *in vitro* and *in vivo* that the retention of nuclear material in the cornified layer can cause changes in epidermal terminal differentiation through a BMP/SMAD1-mediated mechanism.



**Figure 4** Lamin A/C phosphorylation and degradation is reduced in AKT1 shRNA-expressing keratinocytes. (a) Representative Commissie blue stain of the two shRNA-expressing lines. An overrepresented band is present in the AKT1 shRNA-expressing line (arrowhead). This band was excised and subjected to mass spectrometry analysis. (b) The table shows the detected proteins, including prelamin A, molecular weight, % coverage, and function of protein species. (c) Western blot of pre- and post-confluent keratinocytes for Lamin A/C, pSerAKT, total AKT, Keratin 1, and GAPDH. Note that degradation of Lamin A/C occurred concomitant with phosphorylation of AKT. (d) Western blot analysis of Lamin A/C in both AKT1 shRNA-expressing cells (representative data of  $n=2$  both lines) and in Wortmannin-treated, AKT1-inhibited cells. GAPDH serves as a loading control in (c and d). (e) Representative data of co-immunofluorescence of pS404 Lamin A and Lamin A/C in AKT1 shRNA-expressing organotypic cultures and in scrambled controls. Bar 50  $\mu\text{m}$ . (e) Dashed line indicates the dermal-epidermal boundary

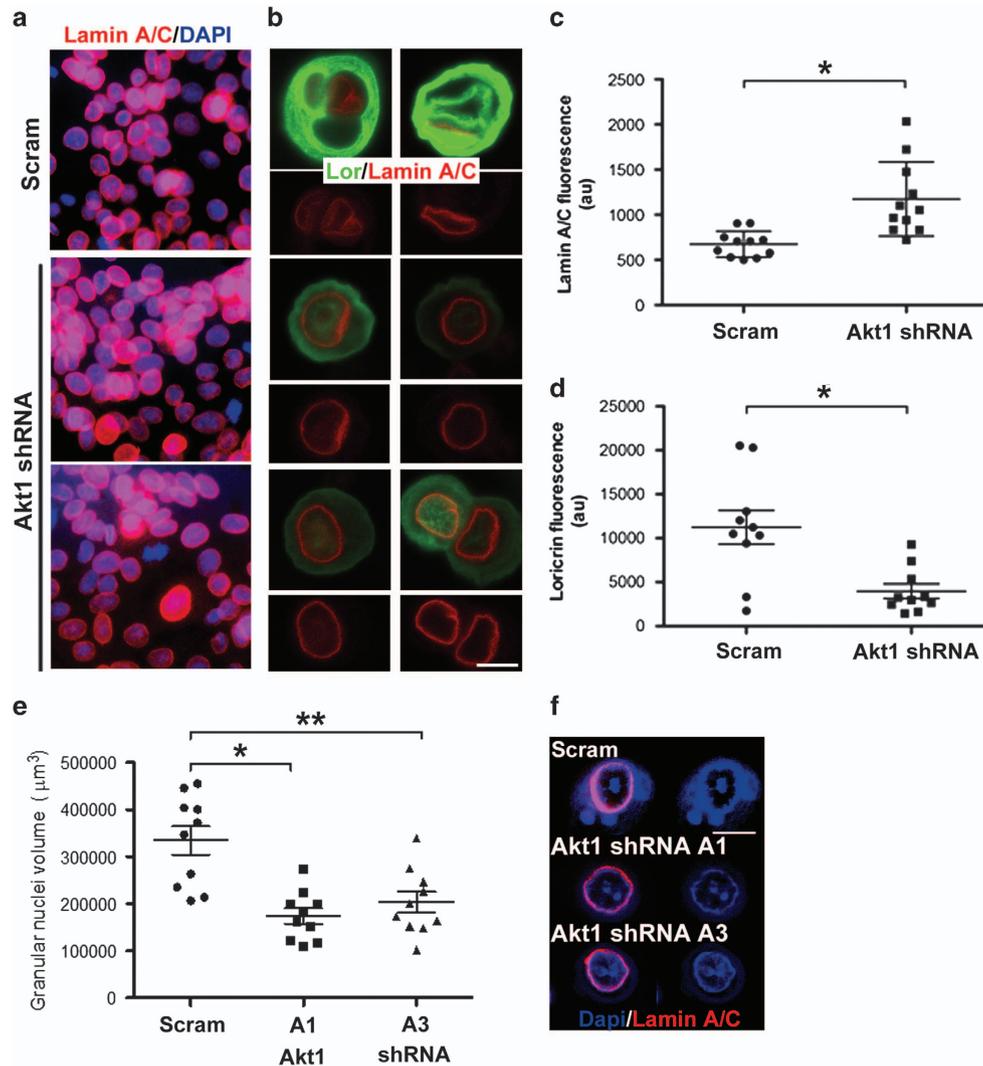
## Discussion

A competent skin barrier depends on proper execution of the keratinocyte terminal differentiation programme resulting in an anucleate cornified layer.<sup>3</sup> Cell death during terminal keratinocyte differentiation is a highly regulated specialised process, with similarities to apoptosis, where DNA is degraded and the nucleus destroyed. Surprisingly little is understood about this process.

The keratinocyte-specific endonuclease, DNase 1L2, has been reported to be essential for nuclear degradation during cornification, and knockdown of DNase 1L2 expression has been reported to cause parakeratosis.<sup>10</sup> However, our data demonstrate that its expression remains unchanged in AKT1 kd cells, suggesting that the observed reduction in nuclear degradation is most likely not due to DNase 1L2. Even though DNase 1L2 is reported to be an important mediator of nuclear degradation during cornification, it lacks a nuclear localisation

signal, hence can only gain access to DNA after the breakdown of the nuclear membrane, which in keratinocytes occurs during terminal differentiation in the transitional stage prior to cornification.<sup>10,21</sup>

Lamin A/C is a major component of the inner nuclear membrane providing scaffolding for the cell nucleus and anchoring chromatin to the nuclear lamina.<sup>22–24</sup> Studies have shown that Lamin A/C degradation leads to nuclear dysregulation and apoptosis.<sup>24,25</sup> Furthermore, functions of Lamin A/C also include maintenance of cell shape and mechanical properties; ectopic expression of Lamin A results in stiffness of nuclei and increased mechanical strength, hence making them more resistant to disintegration.<sup>26</sup> Our data demonstrate that Lamin A/C degradation was reduced in both AKT1 shRNA-expressing cells and AKT-inhibited cells. AKT1-mediated phosphorylation of Lamin A occurs extensively in the upper epidermal layers. We therefore propose that the



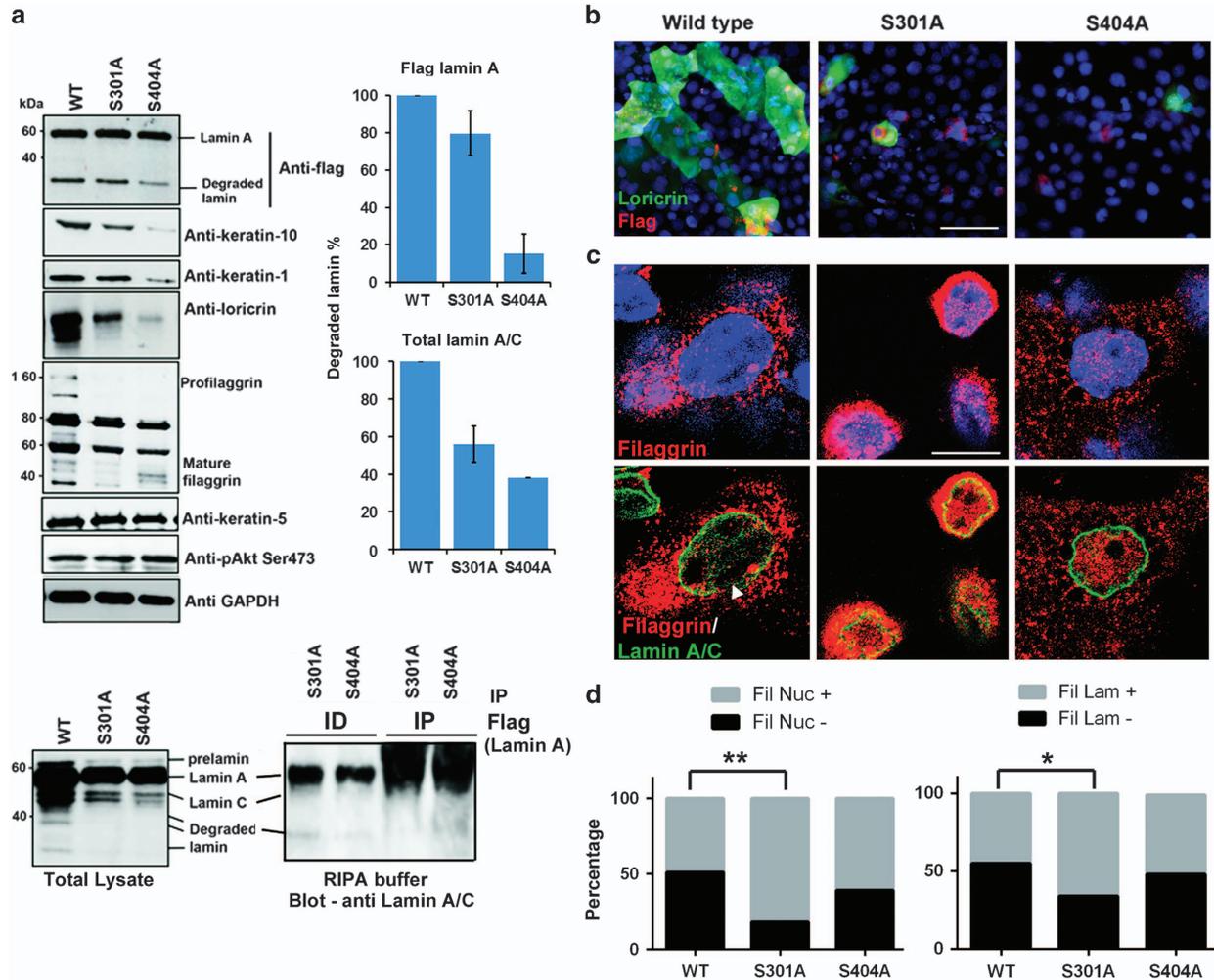
**Figure 5** Maintained Lamin A/C expression in AKT1 kd cells reduced loricrin expression and prevented escape of nuclear material. (a) Immunofluorescence of Lamin A/C in AKT1 shRNA-expressing cells and in scrambled controls. Arrowheads point to disrupted nuclear Lamin A in controls and intact nuclear lamina in AKT1 kd cells. (b) Co-immunofluorescence of loricrin and Lamin A/C in AKT1 shRNA-expressing cells and scrambled controls. (c and d) Graph of image analysis of lamin A/C fluorescence (c) and loricrin fluorescence (d) in loricrin-expressing cells from AKT1 kd cells and scrambled controls. (e) Volocity analysis of nuclear volume in loricrin-positive cells in AKT1 kd cells and scrambled controls. (f) Representative confocal image of Lamin A/C and nuclear material (DAPI) from scrambled controls and AKT1 shRNA-expressing lines. Bars (a, b and f) 10  $\mu\text{m}$ . \* $P < 0.05$ , \*\* $P < 0.05$  Mann-Whitney *U*-test

AKT-mediated phosphorylation of Lamin A is necessary to initiate the disruption of the nuclear lamina to allow entry of DNases. This would be consistent with previous data where a tetracycline-inducible Keratin 5 promoter, driving high levels of Lamin A expression in epidermal keratinocytes, caused hyperkeratosis and parakeratosis.<sup>27</sup>

AKT phosphorylates lamin A/C at the sites S301 and S404, which upon phosphorylation triggers Lamin A/C degradation coinciding with nuclear lamina breakup.<sup>16</sup> Changes in filaggrin processing for the S301A and S404A lamins, and in the expression of important keratinocyte differentiation markers such as Loricrin, Keratin 10, and Keratin 1, suggested an important role for Lamin A/C degradation in regulating not only nuclear degradation but also epidermal differentiation. Filaggrin has been shown to be associated with nuclear degradation and keratin intermediate filament aggregation.<sup>28,29</sup> Studies have reported that keratohyalin granules with

profilaggrin aggregates localise to the perinuclear region sometimes forming deep invaginations at the areas of association.<sup>28,29</sup> This is thought to facilitate entry of cleaved N-terminal domain of profilaggrin into the nucleus, where it favours nuclear degradation.<sup>28</sup> One potential explanation for the altered processing of filaggrin, partially supported by our data, is that filaggrin interacts with nuclear lamins and therefore the persistence of Lamin A/C increases its association with filaggrin intermediates at the nuclear lamina, preventing its processing to more mature forms. Alternatively, the observation of nuclear filaggrin in the S404A- and S301A-expressing cells could be due to the non-degraded nuclei preventing proper access to the protein by cytoplasmic proteases.

Parakeratosis is frequently described as pathology, but little is known about whether the nuclear material in the parakeratotic cornified layer is actually transcriptionally active.



**Figure 6** Expression of non-phosphorylatable Lamin A/C changes keratinocyte differentiation. (a) Western blot analysis of lysates from rat epidermal keratinocytes transfected with flag-tagged WT, S301A, or S404A Lamin A for Flag-tag, Keratins 5, 1, and 10, Loricrin, Filaggrin, pSerAKT, and Lamin A/C. Lamin A/C expression was also assessed in immunodepleted (ID) and immunoprecipitated (IP) lysates from the S301A and S404A lamin A-transfected cells. GAPDH serves as a loading control. Graphs show normalised band intensity for the degraded Lamin species in the Flag-tagged lamin and total lamin ( $n = 2$  experiments). Bars are s.d. (b) Co-immunofluorescence for Loricrin and Flag in post-confluent WT and mutant Lamin A/C-expressing keratinocytes. (c) Co-immunofluorescence for filaggrin and Lamin A/C in post-confluent WT and mutant Lamin A/C-expressing keratinocytes. Arrowhead shows typical disruption of Lamin A/C seen in WT but not in mutant Lamin A/C-expressing keratinocytes. (d) Graph of percentage on nuclear filaggrin (Fil Nuc) and filaggrin co-localising with lamin A/C (Fil lam)  $*P < 0.05$ ,  $**P < 0.005$  ( $\chi^2$  test  $n > 30$  separate cells in each analysis). Bars  $50 \mu\text{m}$  (b) and  $10 \mu\text{m}$  (c)

The transcription factor activator protein 1 proteins (AP-1), composed of jun and fos proteins that form homodimers (jun/jun) and heterodimers (jun/fos) that bind to AP-1 factor DNA binding sites, have been implicated in keratinocyte proliferation and differentiation.<sup>30–32</sup> Indeed, phosphorylated c-Jun is present in the nuclei of transitional cells in these studies. Interestingly it has been reported that c-fos is sequestered in the nuclear envelope in a Lamin A/C-dependent manner, which effectively reduces AP-1 factor DNA binding activity.<sup>33</sup> It is tempting to speculate that continued AP-1-mediated transcription in the nuclear remnants is necessary for the increased BMP2 expression;<sup>34</sup> and that parakeratosis itself can alter keratinocyte terminal differentiation.

#### Materials and Methods

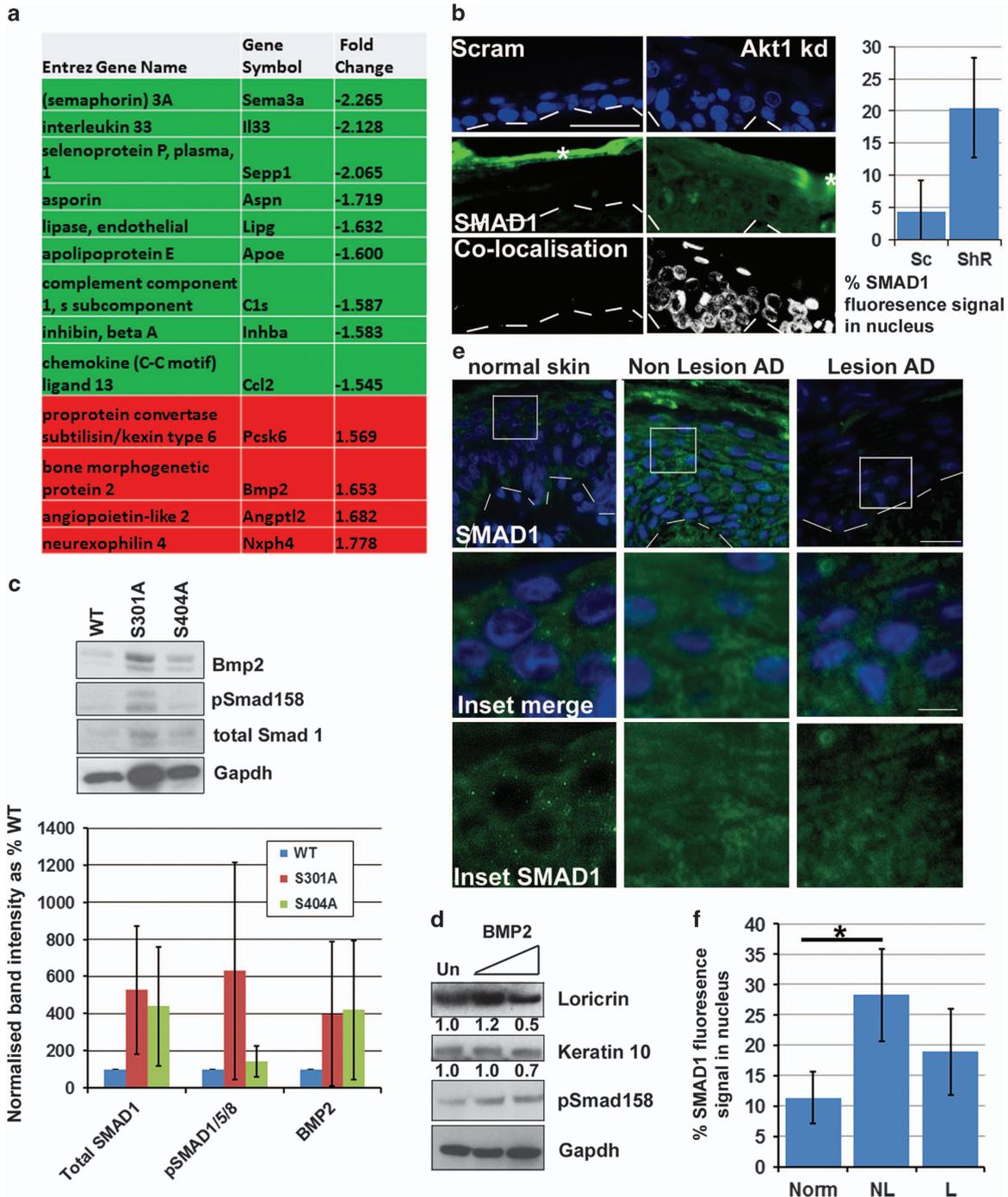
**siRNA kd, cell and organotypic culture, mouse tissue.** Four shRNA plasmids (Qiagen, Manchester, UK) were used to knock down AKT1 expression (shRNA1:

5'-GCACCGCTCTTTGCCAACAT-3'; shRNA2: 5'-AAGGCACAGGTCGCTACTAT-3'; shRNA3: 5'-GAGGCCCAACACCTTCATCAT-3'; shRNA3: 5'-GCTGTTCGAGCTC ATCCTAAT-3'), and of these 1 and 3 were used for further experiments. AKT1 kd plasmids were transfected into rat epidermal keratinocyte (REK) cells, using lipofectamine (Invitrogen), according to the manufacturer's instructions. The S404A and S301A Lamin A constructs have been previously described<sup>16,17</sup> and transfected into REK cells in an identical fashion. Cells were cultured and selected for two weeks in  $100 \mu\text{M}$  G418 (Gibco). BMP2-treated keratinocytes were treated for 24 h with recombinant BMP2 (Life Technologies, Paisley, UK) at 25 and 125 nm concentrations. The organotypic cultures were made by growing rat epidermal keratinocytes on a de-epidermalised dermal scaffold for 10 days at the air-medium interface as previously described.<sup>14,32</sup> Organotypic cultures were paraffin embedded prior to sectioning. E15.5 and E18.5 CD1 mouse embryo tissue was obtained from timed matings, with the skin processed for paraffin embedding.

**Western blot and antibodies.** Keratinocyte protein lysates were prepared by boiling in a denaturing SDS buffer (2% 2-mercaptoethanol, 2% SDS, 10 mM Tris pH 7.5) for 10 min. Immunoprecipitation with an agarose-conjugated goat anti-DDDDK tag antibody (Abcam) ( $20 \mu\text{g}$  for 18 h) was performed on protein extracts prepared from cells by lysis in RIPA buffer (150 mM sodium chloride, 1.0% igequal,

0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0)). Primary antibodies used were as follows: rabbit anti-filaggrin (Santa Cruz Biotechnologies 1 : 500), mouse anti-FLAG (1 : 100, Cell Signaling Technologies, Danvers, MA, USA), rabbit

anti-pSerine 473 AKT (Cell Signaling Technologies, 1 : 500), mouse anti-AKT1 (Cell Signaling Technologies, 1 : 500), rabbit anti-total AKT (1 : 1000, Cell Signaling Technologies), rabbit anti-Keratin 1 (Covance, 1 : 1000), rabbit anti-Keratin 10



**Figure 7** BMP2 expression increased in AKT1 kd and non-phosphorylatable Lamin A/C cells. Parakeratosis correlated with increased BMP signalling in eczema samples. (a) Table of differentially expressed genes encoding secreted proteins in AKT1 shRNA-expressing cells. (b) Representative immunofluorescence of SMAD1 in scrambled control (Scram) and AKT1 shRNA expression organotypic cultures. Asterisk shows regions of non-specific staining in the cornified layer. Dashed line indicates the dermal-epidermal boundary. (c) Western blot analysis of lysates from rat epidermal keratinocytes transfected with either flag-tagged WT, S301A, or S404A Lamin A for BMP2, pSMAD158, and SMAD 1. Graph shows average band intensity values compared with WT controls. (d) Western blot analysis of rat epidermal keratinocytes treated with Bmp2 or vehicle (Un) for Loricrin, Keratin 10, and pSMAD158. In (c) and (d) GAPDH serves as a loading control, and in (d) the numbers denote normalised values compared with controls. (e) Representative SMAD1 fluorescence in normal skin ( $n = 8$ ), non-lesional AD skin ( $n = 4$ ), and lesional AD skin ( $n = 4$ ). Inset detail shows nuclear expression of SMAD1 in AD skin. (f) Graph of the nuclear SMAD1 staining intensity as a percentage of total epidermal SMAD1 signal in normal, non-lesional, and lesional AD samples. \* $P < 0.05$  paired  $t$ -test. Bars (b and f) 50  $\mu\text{m}$  (e, insets, 10  $\mu\text{m}$ )

(Covance, 1 : 1000), rabbit anti-Keratin 5 (Covance, 1 : 1000), rabbit anti-Loricrin (Covance 1 : 1000), mouse anti-Lamin A/C (Santa Cruz Biotechnologies 1 : 100), rabbit anti-BMP2 (1 : 100 Bioss), rabbit anti-pSMAD158 (1 : 100, Cell Signaling Technologies), and rabbit anti-total SMAD1 (1 : 100 Epitomics).

Primary antibody incubations were in PBS+0.1% Tween-20 or in TBST (100 mM Tris HCl, 0.2 M NaCl, 0.1% Tween-20 (v/v)) containing either 5% bovine serum albumin (Sigma, Gillingham, UK) or 5% skimmed milk powder either overnight at 4°C or for 1–2 h at room temperature, whereas secondary antibody incubations were in 5% skimmed milk powder for 1 h at room temperature. The following concentrations were used: swine anti-rabbit HRP (DakoCytomation) 1 : 3000 and rabbit anti-mouse HRP (DakoCytomation) 1 : 2000. Protein was visualised using the ECL plus kit (Amersham). Densitometry of bands was determined using thresholded images in the ImageJ suite (<http://imagej.nih.gov/ij/>).

**Immunofluorescence and eczema samples.** Clinical material was obtained with written informed consent from patients attending dermatology clinics at Great Ormond Street Hospital; ethical approval was granted by the local research ethics committee. Immunofluorescence and histology on paraffin or frozen sections or on cultured keratinocytes was by standard techniques. Antibodies used were rabbit anti-pSer 473 AKT (1 : 10, Cell Signaling), mouse anti-AKT1 (1 : 10; Cell Signaling), mouse anti-Flag (1 : 10, Cell Signaling Technologies), rabbit anti-filaggrin (1 : 50, Santa Cruz Biotechnologies), rabbit anti-Loricrin (1 : 100; Covance), mouse anti-Lamin A/C (1 : 200, Santa Cruz Biotechnologies), and rabbit anti-SMAD 1 (1 : 25 Epitomics). The rabbit anti-lamin A phospho-serine 404 antibody was described previously<sup>16,17</sup> and used at 1 : 25. Primary antibodies were detected using Alexa 488 and 594-conjugated goat anti-mouse and anti-rabbit (Invitrogen, 1 : 500). Cells and sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma). Images were taken with a Leica Upright Microscope with either ×20 (NA 0.4) or ×40 (NA 1.40) objectives, using a Coolsnap digital camera (MediaCybernetics, Bethesda, MD, USA), with ImagePro 6.0 software (MediaCybernetics). Co-localisation analysis was performed using the co-localisation plug-in of the ImageJ suite (<http://imagej.nih.gov/ij/>) comparing thresholded images for both the DAPI and SMAD1. The same images were used to determine the SMAD1 signal as a percentage of total epidermal SMAD1 fluorescence.

**RNA extraction and microarray analysis.** RNA (0.1 mg) was extracted from two scrambled REK lines, and two biological replicates of each AKT1 shRNA kd, and poly-A+RNA was selected using the Oligotex system (Qiagen). Second-strand cDNA was synthesised using the Superscript II kit (Invitrogen, Carlsbad, CA, USA) after the RNA was annealed with a T7 promoter-poly-T primer (Genset, Evry, France). Biotin-labelled cRNA was made from this cDNA (Enzo Diagnostics, Farmingdale, NY, USA). The whole probe was hybridised to the exon array rat genome chip (Affymetrix, Santa Clara, CA, USA) according to the manufacturers' specifications. The scrambled control cells were the baseline in all analyses. Genes that were tagged as present and altered in expression in all four AKT1 kd samples by 1.5-fold or more with a *P*-value of ≤0.05 by Mann–Whitney analysis, and a *P*-value <0.05 after Benjamini-Hochberg's false-discovery rate correction. Secreted genes were identified from the list of differentially expressed genes using DAVID (Database for Annotation, Visualization and Integrated Discovery. <http://david.abcc.ncifcrf.gov/>).

**Mass spectrometry analysis of overexpressed protein species in AKT1 kd cells.** Total lysates as described above were run on a polyacrylamide gel and stained for 2 h in colloidal Coomassie blue (Fisher), and then destained overnight in high-purity (HPLC-grade) water (Sigma). The bands were excised using a clean scalpel and then in-gel tryptic digestion was performed and the peptides analysed by ESI-QTOF. Peptides were then identified by comparison with the UniPROT database.

**Confocal microscopy and nuclear volume calculations.** Confocal images were obtained using a Zeiss LSM 510 (Zeiss, Cambridge, UK) laser confocal microscope with a Plan Apochromat ×63 NA 1.40 oil immersion lens. One micrometre optical sections were taken for confocal images of filaggrin and lamin co-localisation. For the nuclear volume calculations, confocal Z-stacks of control and AKT1 kd REKs immunostained for loricrin and Lamin A/C were acquired with Z-stacks, each consisting of 65 optical slices. Once images were acquired, threshold settings and background elimination were performed using the Fiji Image J software suite. The files were then converted to eight-bit images in greyscale and were uploaded to Volocity3D Image Analysis Software. Nuclear volumes were thus

determined setting the parameters to find objects based on intensity and exclude objects smaller than 3000 μm<sup>3</sup>.

## Conflict of Interest

The authors declare no conflict of interest.

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