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## An adult passive transfer mouse model to study desmoglein 3 signaling in pemphigus vulgaris

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### Abstract

Evidence has accumulated that changes in intracellular signaling downstream of desmoglein 3 (Dsg3) may play a significant role in epithelial blistering in the autoimmune disease pemphigus vulgaris (PV). Currently, most studies on PV involve passive transfer of pathogenic antibodies into neonatal mice which have not finalized epidermal morphogenesis, and do not permit analysis of mature hair follicles (HFs) and stem cell niches. To investigate Dsg3 antibody-induced signaling in the adult epidermis at defined stages of the HF cycle, we here developed a model with passive transfer of the monospecific pathogenic Dsg3 antibody AK23 into adult 8-week-old C57Bl/6J mice. Validated using histopathological and molecular methods, we found that this model faithfully recapitulates major features described in PV patients and PV models. Two hours after AK23 transfer we observed widening of intercellular spaces between desmosomes and EGFR activation, followed by increased Myc expression and epidermal hyperproliferation, desmosomal Dsg3 depletion and predominant blistering in HFs and oral mucosa. These data confirm that the adult passive transfer mouse model is ideally suited for detailed studies of Dsg3 antibody-mediated signaling in adult skin, providing the basis for investigations on novel keratinocyte-specific therapeutic strategies.

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### Conflict of interest

The authors state no conflict of interest.

## Introduction

PV is a severe autoimmune blistering disease characterized by suprabasal blisters in skin and mucous membranes (Stanley and Amagai, 2006). On average, 90% of PV patients exhibit autoantibodies against Dsg3 (Amagai *et al.*, 1999a; Ishii *et al.*, 1997), an intercellular adhesion molecule and component of desmosomes (Garrod *et al.*, 2002). Although the pathogenic mechanism leading to blister formation is not well understood, intracellular signaling has been found to be both involved and necessary in this process. A new paradigm was therefore brought forward that Dsg3 and other possible molecular targets in PV govern outside-in signaling (Getsios *et al.*, 2010; Müller *et al.*, 2008).

Over a decade ago, Kitajima and collaborators reported on PV IgG-induced signaling events such as the rapid activation of PKC and PLC (Esaki *et al.*, 1995; Osada *et al.*, 1997). More recently, lack of responsiveness to PV IgG in keratinocytes deleted for the adhesion and signaling molecule plakoglobin (PG) underscored the necessity of a signaling response in PV (Caldelari *et al.*, 2001; de Bruin *et al.*, 2007). To date, passive transfer of PV IgG into neonatal mice in combination with chemical inhibitors has confirmed a number of signaling effectors contributing to PV pathophysiology, including epidermal growth factor receptor (EGFR), Myc, p38, PLC/PKC and Src (Berkowitz *et al.*, 2006; Chernyavsky *et al.*, 2007; Pretel *et al.*, 2009; Sanchez-Carpintero *et al.*, 2004; Williamson *et al.*, 2006). These observations suggest that inhibition of specific signaling molecules represents a potent therapeutic strategy in PV (Getsios *et al.*, 2010).

Myc expression is critical for normal skin homeostasis, where overexpression in combination with stem cell depletion can lead to epidermal hyperproliferation, hair loss and spontaneous wounds (Watt *et al.*, 2008). Human PV patients exhibit increased Myc expression and hyperproliferation in epidermis and HFs (Williamson *et al.*, 2006), the sites of Dsg3 expression in human skin (Hanakawa *et al.*, 2004). Moreover, they suffer from increased hair loss (Koch *et al.*, 1998) (personal communication Dr. Michael Hertl, University of Marburg, Germany). Hair loss due to blisters in the resting (telogen) HF are predominant features of Rag2<sup>-/-</sup> mice after adoptive transfer of Dsg3<sup>-/-</sup> splenocytes or AK23 hybridoma cells (producing a Dsg3-specific antibody), as well as of *Dsg3* null mice (Amagai *et al.*, 2000; Koch *et al.*, 1998; Tsunoda *et al.*, 2003). Due to these striking observations, it is of interest to address the consequences of disrupted Dsg3 function in HFs and epidermal stem cell niches in PV.

Currently, neonatal mice are the model system to test PV antibody pathogenicity *in vivo* (Anhalt *et al.*, 1982). However, the morphogenesis of murine epidermis and its appendages including HFs is not finalized until postnatal day 15 (Schneider *et al.*, 2009). Furthermore, after adoptive transfer, adult Rag2<sup>-/-</sup> mice typically develop blisters over a prolonged incubation time, precluding defined time course studies on antibody-triggered primary signaling events (Amagai *et al.*, 2000; Tsunoda *et al.*, 2003). Hence, the goal of this study was to establish a mouse model for prospective studies on Dsg3-antibody initiated signaling in adult skin at defined stages of the HF cycle.

Here we validate the passive transfer of the pathogenic monospecific Dsg3 antibody AK23 into 8-week-old C57Bl/6J mice (which are in the prolonged synchronized telogen HF stage) by focusing on major ultrastructural, biochemical and signaling parameters observed in PV patients, neonatal mice, human tissue models and cultured keratinocytes. Our data reveal that this adult mouse model recapitulates major findings in PV, and demonstrate that passive transfer into adult mice represents an ideal model both to study Dsg3 signaling in adult skin including HFs and stem cell niches, and to test novel therapeutic strategies in PV.

## Results

### AK23- and AK23/PF-induced changes in neonatal mice

To validate the response of AK23-injected adult mice against the neonatal model, we first tested whether neonatal mice treated with AK23 recapitulate previous findings obtained with PV IgG (containing Dsg1 antibodies) such as EGFR, p38 and c-Myc activation (Berkowitz *et al.*, 2006; Pretel *et al.*, 2009; Williamson *et al.*, 2006). AK23 was injected alone, and as a control, with a half pathogenic dose of pemphigus foliaceus (PF) patients IgG (containing Dsg1 antibodies; AK23/PF) (Mahoney *et al.*, 1999).

In agreement with our former results (Williamson *et al.*, 2006), only AK23/PF-injected neonatal mice exhibited epidermal lesions after 24 hours (hrs), while AK23/PF and AK23-treated animals showed PV-like lesions in lip and hard palate (data not shown). Independently of PF IgG, proliferation was increased in epidermis of all AK23-treated mice as quantified on non-lesional skin sections labeled for the proliferation marker Ki67 (Figure 1a). Back skin protein extracts further revealed significant EGFR activation in AK23/PF- and AK23-treated mice (Figure 1b and 1c). Phosphorylation sites previously addressed in PV were investigated (Heupel *et al.*, 2009; Pretel *et al.*, 2009). In AK23/PF-treated animals we observed increased phosphorylation of Tyr845, a Src kinase substrate, and its phosphorylation was reported to result in receptor activation and mitogenesis in fibroblasts and human breast cancer cells (Ishizawar and Parsons, 2004) (Figure 1b). Phosphorylation of Tyr992 and to a minor extent Tyr1173 was also increased. P-Tyr992 is a high affinity binding site for PLC $\gamma$  and has been involved in MAPK signaling (Morandell *et al.*, 2008). P-Tyr1173 has been linked to phosphatidylinositol-3 kinase (PI3K)/Akt signaling, which results in stabilization of factors such as c-Myc, and further activates EGFR dephosphorylation and silencing (Morandell *et al.*, 2008; Segrelles *et al.*, 2006). In AK23-treated animals, EGFR steady-state protein levels were reduced, suggesting receptor activation and internalization (Sorkin and Goh, 2009) consistent with a significant increase in EGFR phosphorylation on Tyr1173. Phosphorylation of Tyr845 and Tyr992 was not significantly increased at this time point.

In contrast to a previous report (Berkowitz *et al.*, 2006), phosphorylation of p38 was not enhanced in AK23/PF- or AK23-treated neonatal skin at 24hrs, but c-Myc steady-state levels were significantly increased (Figure 1b and 1c). In non-junctional (Triton X-100 soluble) lysates, Dsg3 together with PG was increased in AK23/PF-treated and Dsg3 in AK23-treated animals (Figure 1d and 1e). In contrast, Dsg3 was markedly depleted from desmosomal (Triton X-100 insoluble) extracts in all AK23-treated mice while other components such as Dsc3, Dsg1/2 and desmoplakin remained unchanged.

Consistent with findings in PV patients and PV IgG-injected neonatal mice (Pretel *et al.*, 2009; Shu *et al.*, 2007; Williamson *et al.*, 2007a; Williamson *et al.*, 2006; Williamson *et al.*, 2007b), AK23 alone induced hyperproliferation in neonatal mice which correlated with EGFR activation and c-Myc overexpression as well as depletion of Dsg3 from desmosomes. Interestingly, AK23-induced molecular changes were largely similar with or without additional PF IgG, identifying injections with AK23 alone as a suitable test system to address the feasibility of adult mice for prospective studies on Dsg3 antibody-mediated signaling.

### **AK23-injected 8-week-old mice predominantly develop lesions in telogen HFs and oral mucosa**

AK23-induced changes were then addressed in adult 8-week-old C57Bl/6J mice. After 24hrs, wide-spread PV-like blisters were observed in all AK23-treated animals in areas of mechanical stress such as hard palate, esophagus and lip/snout skin (Figure 2a and b). Blisters were further present in the stem cell niche of the telogen HF known as the bulge, between the inner layer and the outer root sheath (Figure 2c) as reported for *Dsg3* null mice (Koch *et al.*, 1998). 75% follicles were affected at 24hrs, rising to 80% after 48hrs (Figure 2d). Macroscopically, this correlated with loosely anchored hair revealed by tape stripping (Figure 2e). In contrast, spontaneous clinical blisters rarely formed on back skin, in spite of evenly bound AK23 antibody in the basal cell layer (Figure 2a and b). However widening of intercellular spaces, the earliest sign of loosening of intercellular contacts, was occasionally observed. Indicative of reduced food intake due to blisters in the oral cavity, AK23-treated mice exhibited weight loss and were sacrificed at 48hrs.

Eight-week-old Rag2<sup>-/-</sup> mice responded similarly to AK23 than C57Bl/6J mice, establishing that blister formation in response to AK23 does not involve a B- and T-cell-mediated immune response (Supplementary Figure S1).

### **AK23 induces hyperproliferation in 8-week-old mice**

Proliferation in the epidermis of 8-week-old AK23- or mIgG-injected C57Bl/6J mice was assessed by BrdU incorporation and Ki67 staining. Using flow cytometry, 35% more BrdU positive cells were measured in AK23-treated mice as compared to control animals 24hrs after a single BrdU injection (Figure 2f), and 60% more BrdU positive cells at 48hrs after four consecutive BrdU injections (Figure 2g). Immunofluorescence microscopy visually confirmed increased BrdU incorporation and more numerous Ki67 positive cells in the basal layer of the epidermis and in HFs (Figure 2h).

Taken together, these results recapitulate the hyperproliferation in neonatal mice (shown here) and PV patients' epidermis (Williamson *et al.*, 2006).

### **AK23-treated 8-week-old mice exhibit EGFR activation, Myc upregulation, and increased non-junctional Dsg3**

We then addressed the status of EGFR, p38 and Myc in Triton X-100 soluble protein fractions from back-skin of 8-week-old C57Bl/6J mice together with Dsg3, E-cadherin and PG protein levels. Compatible with receptor activation (Sorkin and Goh, 2009), EGFR

steady-state levels were already reduced 2hrs after AK23 injection, and the relative levels of mitotically active p-Tyr845 EGFR were on average increased by twofold (Figure 3a). Furthermore, phosphorylation of Tyr992 was weakly detectable in two animals out of four (but not measurable, data not shown) and Tyr1173 was unchanged. At 48hrs, phosphorylation of Tyr845 was no longer detectable while that of Tyr1173 had increased in 2/2 C57Bl/6J mice and 4/4 mice of a different genetic background (Figure 3a, Supplementary Figure S2). EGFR phosphorylation correlated at 2hrs with an increase in cyclin D1, expressed in actively dividing cells, and at 48hrs with increased c-Myc and N-Myc but not L-Myc (Figure 3b). The latter changes were not yet seen at 24hrs (data not shown). At 48hrs, a tendency towards increased phosphorylation of p38 was observed. In the same lysates, 80% more non-junctional Dsg3 protein was measured at 2hrs in AK23-treated animals, which then returned to base levels (Figure 3c). No changes were observed for E-cadherin and PG.

On the mRNA steady-state level, *c-Myc* was significantly decreased at 2hrs in AK23-treated mice while *N-Myc* and *cyclin D1* were increased (Figure 3d). *N-Myc* then continued to increase up to 24hrs together with increased expression of *L-Myc* and *cyclin D2*. Except for *N-* and *L-Myc*, mRNA expression levels were back to normal at 48hrs.

In summary, between adult and neonatal AK23-injected mice, the molecular changes were largely similar including an increase in soluble Dsg3, potentially stemming from desmosome remodeling (Aoyama *et al.*, 2010). Furthermore, in adult mice changes in mRNA preceded or coincided with changes in corresponding proteins, indicating *de novo* synthesis. The decrease in *c-Myc* mRNA might suggest a negative feedback loop involving an enhanced turn-over rate following early transcriptional activation (Dai and Lu, 2008).

### **Dsg3 depletion from desmosomes is characteristic for AK23-treated 8-week-old mice**

Desmosomal proteins were quantified in Triton X-100 insoluble fractions of 8-week-old C57Bl/6J mouse skin. Steady-state levels of junctional Dsg3 started to decrease at 24hrs, and were reduced to roughly 30% at 48hrs in all AK23-injected animals, while Dsc3 levels were largely unchanged (Figure 4). Dsg1/2 was not affected at 24hrs but significantly reduced at 48hrs concomitant with a tendency towards a decrease in plaque proteins plakophilin and desmoplakin but not PG. On average, no significant differences in keratin expression were measured between treated and untreated animals. However, three out of four AK23-injected animals exhibited decreased keratin 15 expression whereas keratin 14 levels were above control in two out of four AK23-treated animals at 48hrs, both features of hyperproliferative epidermis (Werner and Munz, 2000).

Comparative immunofluorescence analyses conducted on skin biopsies 24 and 48hrs after AK23 injection revealed no major changes in the expression pattern of epidermal markers, except for a reduction of Dsg1/2 and keratin 15 (Supplementary Figure S3, shows 48hrs). Decreased Dsg1/2 is a consistent feature of PV patients and PV antibody-challenged human organotypic and mouse keratinocyte cultures (van der Wier *et al.*, 2010; Williamson *et al.*, 2006).

## AK23 induces a rapid interdesmosomal membrane detachment

To further correlate the AK23-induced signal activation to changes in desmosomal ultrastructure in 8-week-old C57Bl/6J mice, we performed electron microscopy. Two hours after AK23 injection, antibodies were already bound to the surface of basal keratinocytes, no blisters were observed by routine histology in the epidermis and HF, and only a small blister was present in the hard palate in one out of eight AK23-treated animals (Figure 5a). In spite of an intact epidermis, electron microscopy of the same animals showed basal keratinocytes that were still joined by desmosomes, but most exhibited widening of interdesmosomal spaces (Figure 5b). By 48hrs, microscopic tissue damage largely confined to basal cells indicated skin fragility.

## Discussion

This study describes an adult passive transfer model of 8-week-old C57Bl/6J mice injected with the monospecific pathogenic Dsg3 antibody AK23 (Tsunoda *et al.*, 2003) to enable studies of Dsg3-mediated signaling in adult skin, HF and stem cell niches. We demonstrate that this mouse model reproduces molecular events observed in AK23-injected neonatal mice as well as major features reported in PV.

Histopathologically, PV patients with Dsg3 antibodies (the mucosal dominant phenotype), develop oral blisters with rare clinical lesions in skin (Amagai *et al.*, 1999b) and occasional blister in HFs. Hair loss is a characteristic clinical feature of PV patients, but telogen HF blister may be overlooked because, unlike in mice, the telogen phase is short and human hair grows asynchronously (Koch *et al.*, 1998) (Dr. Michael Hertl, University of Marburg, Germany; personal communication). In line with other adult PV mouse models using adoptive transfer (Amagai *et al.*, 2000; Tsunoda *et al.*, 2003), passive transfer of AK23 into adult mice mimics these clinical phenotypes (Figure 2a).

Our observations are also consistent with previous *in vivo* and *in vitro* reports on Dsg3 depletion from desmosomes in PV (Aoyama and Kitajima, 1999; Calkins *et al.*, 2006; Mao *et al.*, 2009; Shu *et al.*, 2007; Williamson *et al.*, 2006; Yamamoto *et al.*, 2007) which are also seen in AK23-injected neonatal mice shown here. In the adult mouse model, a visible Dsg3 loss commenced at 24hrs without affecting other proteins. It was preceded by a transient increase in Triton X-100 soluble Dsg3, also observed in neonatal mice. This is compatible with the recent suggestion that PV IgG/Dsg3 immune complexes are rapidly excluded from desmosomes without initially affecting other components such as desmoplakin, and are subsequently internalized and degraded (Aoyama *et al.*, 2010). Internalization and degradation of non-junctional Dsg3 is in line with previous studies on cultured mouse and human keratinocytes (Aoyama and Kitajima, 1999; Calkins *et al.*, 2006; Williamson *et al.*, 2006; Yamamoto *et al.*, 2007). Our lysis conditions do not allow us to discriminate between surface exposed and internalized non-junctional Dsg3. Internalization can therefore not be excluded and is indirectly supported by the early widening of interdesmosomal spaces, also described previously in PV IgG-injected neonatal mice (Takahashi *et al.*, 1985), suggesting that non-junctional Dsg3 molecules (and presumably other adhesion molecules) are no longer available for transadhesion outside of desmosomes.



The increase in non-junctional Dsg3 was returned to normal after 48hrs, indicative of degradation.

Consistent with numerous reports on PV IgG-treated keratinocytes, desmosome function was affected around 48hrs in adult mice; the loss of additional desmosomal proteins such as Dsg1/2 and DP correlated with epidermal fragility in basal keratinocytes as underscored by microlesions and tissue damage. In line with the compensation hypothesis (Mahoney *et al.*, 1999), AK23-induced microlesions very rarely evolved into clinical blisters (Figure 2a), except in presence of PF IgG, as seen in neonatal mice. In view of the similar activation of signaling molecules in AK23/PF and AK23-treated mice at the time point analyzed, it appears that blister formation could be determined by either the length and strength of the signal, or by complementary PF IgG-induced signaling. These possibilities can now be pursued further in comparison with the initial signals involved in desmosome remodeling in skin and adult HFs under defined pathological conditions. Together these observations reveal that AK23 triggers a step-wise signal-driven mechanism that results in Nikolsky-positive skin with weakened adhesion.

In addition to desmosomal proteins, we also investigated signaling molecules reported in PV to validate the adult mouse model for analyses on AK23-mediated signal induction. A recent study has suggested that p38 activation correlates with Dsc3 endocytosis and clinical blisters (Mao *et al.*, 2011). We did not observe a decrease in Dsc3 following AK23-treatment of adult or neonatal mice, and p38 showed only a tendency to be hyperphosphorylated at 48hrs. This may support the conclusion by the authors that p38 is not involved in mechanisms leading to Nikolsky positive skin but contributes to clinical lesions. Alternatively, because PF IgG-injected neonatal mice showed a biphasic p38 activation scheme (Lee *et al.*, 2009), it can currently not be excluded that p38 activation occurred outside the time points investigated here.

Compatible with EGFR activation, we also observed increased proliferation and upregulation of Myc isoforms in adult and neonatal mice. While little is known about N-Myc and L-Myc in epidermis, balanced N-Myc expression was found to be essential for HF development and regeneration (Mill *et al.*, 2005). Furthermore, hyperproliferation and increased Myc/c-Myc have been associated with PV pathology and disease progression (Williamson *et al.*, 2007a; Williamson *et al.*, 2006). Previously shown, transcriptional activation of *c-Myc* resulted from PV IgG-mediated nuclear depletion of its repressor PG. Intriguingly, at 2hrs we observed a decrease in *c-Myc* steady-state mRNA while *c-Myc* protein was increased at 48hrs. This might indicate a negative feedback loop involving enhanced *c-Myc* turn-over in response to transcriptional activation (Dai and Lu, 2008) followed by stabilization of *c-Myc* protein through EGFR-mediated PI3K/Akt activation (Segrelles *et al.*, 2006). Indeed, hyperproliferation and Myc overexpression correlated with EGFR activation and phosphorylation of Tyr845 (pro-mitogenic) and Tyr1173 (PI3K/Akt activation) respectively, which is consistent with EGFR activation in PV IgG-treated neonatal mice and various epidermoid cell types (Chernyavsky *et al.*, 2005; Frusic-Zlotkin *et al.*, 2006; Pretel *et al.*, 2009). Our current result on late phosphorylation of EGFR on Tyr1173 could further explain why this event was not observed in human keratinocytes one hour after treatment with PV IgG (Heupel *et al.*, 2009).

In summary, AK23 injection in this adult mouse model induced early molecular changes and widening of intercellular spaces in basal keratinocytes, followed by a step-wise series of changes in intracellular signaling and adhesion molecules reported in PV. Therefore, the adult passive transfer mouse model described here represents a valuable test system to further unravel the initial Dsg3 antibody-induced molecular changes in epidermis, HF and stem cell niches of adult skin and holds great promise as a test system for the validation of novel therapeutic indications in PV.

## Material and Methods

### Mice and passive transfer

Seven- to 8-week-old C57Bl/6J or B6.129S6-Rag2<sup>tm/Fwa</sup>N12 (Taconic) mice received a single subcutaneous injection of 12µg/g body weight AK23 (a kind gift of Dr. Masayuki Amagai, Tokyo; (Tsunoda *et al.*, 2003)) or normal mouse IgG (mIgG; Equitec) at the back as defined in a dose response study ranging from 5µg and 2.5mg/g body weight AK23. Neonatal C57Bl/6J mice received 90µg/g body weight AK23/mIgG with or without 1.5 mg PF IgG as described previously (Williamson *et al.*, 2006). Experiments were approved by the ethics committee, Canton Bern, Switzerland (26/08).

### BrdU incorporation

Mice received 50µg/g body weight BrdU (Sigma, 5-Bromo-2'-Deoxyuridine B5002) once intraperitoneally at the time point of AK23/mIgG injection and were euthanized at 24hrs, or alternatively received four times 50µg/g body weight in 12hrs intervals before euthanasia at 48hrs.

### Cell isolation and flow cytometry

Epidermis was incubated in Trypsin-EDTA/PBS (0.2%/0.08%) (Amimed) 2hrs at 32°C, keratinocytes were scraped off and dissociated in DMEM/10% FCS on a magnetic stirrer for 20min. Cells were filtered through a 70µm then 40µm cell strainer (BD Falcon), washed with CnT-02 (CELLnTEC) and stained with the LIVE/DEAD<sup>®</sup> Fixable Dead Cell Stain Kit BLUE (Molecular Probes). The FITC BrdU Flow Kit was used (BD Pharmingen) following the manufacturer's protocol. Cells were gated for single cells and viability using a BD LSR II (BD Biosciences), and 50'000 cells were analyzed using FlowJo 7.5 (Tree Star, Inc.).

### Immunofluorescence microscopy

Routine histology, BrdU and Ki67 detection were done on paraffin embedded biopsies. Heat-mediated antigen retrieval was performed 3× 5min by microwave in 0.01M sodium citrate buffer, pH 6.0. Sections were blocked with 5% NGS, 4% BSA in PBS<sup>+</sup> (containing 0.2mM CaCl<sub>2</sub>), incubated with BrdU (Clone BU1/75, Abcam) or Ki67 (SP6, Cell Marque) antibodies in PBS<sup>+</sup> containing 2% BSA, 2.5% NGS, 0.2% Triton X-100 at 4°C overnight followed by anti-rabbit/rat IgG Alexa Fluor<sup>®</sup> 488 or 594 (Molecular Probes).

For direct immunofluorescence, frozen sections were prepared from OCT embedded tissue (Tissue Tek, Sakura). Eight µm sections were fixed 10min in 4% PFA at RT, washed with PBS<sup>+</sup> followed by 20mM Glycine in PBS<sup>+</sup> and blocked in PBS<sup>+</sup> containing 2.5% NGS, 1%



BSA, 2% gelatine, 0.1% Triton X-100 for 1hr at RT prior to incubation with anti-mouse IgG Alexa Fluor® 488 1hr at RT.

### Protein extraction and western blot analyses

Mouse back skin was minced in lysis buffer (100mM Tris HCl pH 7.4, 150mM NaCl, 1% Triton X-100, 10mM NaF, 10mM  $\beta$ -Glycerophosphate, 10mM  $\text{Na}_3\text{VO}_4$ , 1mM PMSF, and complete protease inhibitor EDTA-free (Roche)) using a Polytron PT 1600E. Lysates were incubated 30min on ice and Triton X-100 soluble and insoluble fractions were obtained after 10min centrifugation at 12000 rpm and 4°C. Insoluble fractions (pellet) were solubilized in 8M Urea, 1% SDS, 10% Glycerol, 60mM Tris pH6.8, 5%  $\beta$ -mercaptoethanol, 1mM PMSF. Equal amounts of total protein were subjected to SDS-PAGE and transferred onto nitrocellulose or PVDF (for EGFR) membranes. Signals were quantified using Odyssey (LiCor). Antibodies used are listed in Supplementary Methods.

### Quantitative RT-PCR

Total RNA was extracted from mouse back skin using the RNeasy Fibrous Tissue kit (Qiagen) according to the manufacturer's instructions and processed as described previously (Kolly *et al.*, 2005). Primers are described in Supplementary Methods.

### Electron microscopy

For cryopreservation back skin samples were processed by a modified version of the Tokuyasu method (Peters *et al.*, 2006) as previously described (Scothern and Garrod, 2008).

### Statistical analyses

Statistical analyses were performed using NCSS (Kaysville, UT). Significant differences between two groups were defined using the Student's t-test with  $P < 0.05$ .

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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### Abbreviations used

<b>Dsg3</b>	Desmoglein 3
<b>PV</b>	pemphigus vulgaris
<b>AK23</b>	mouse monoclonal pathogenic anti-Dsg3 antibody

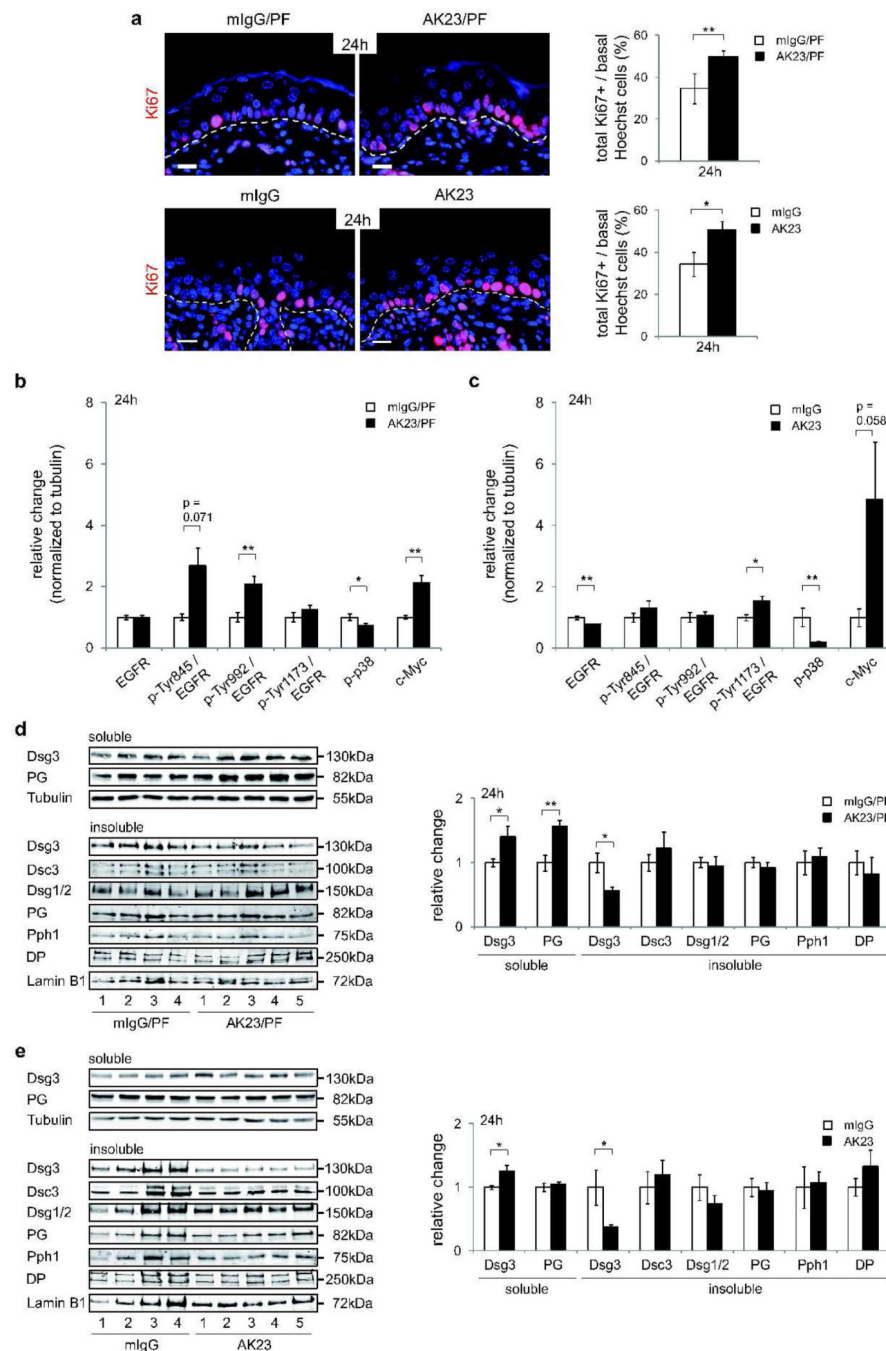
<b>HF</b>	hair follicle
<b>hrs</b>	hours

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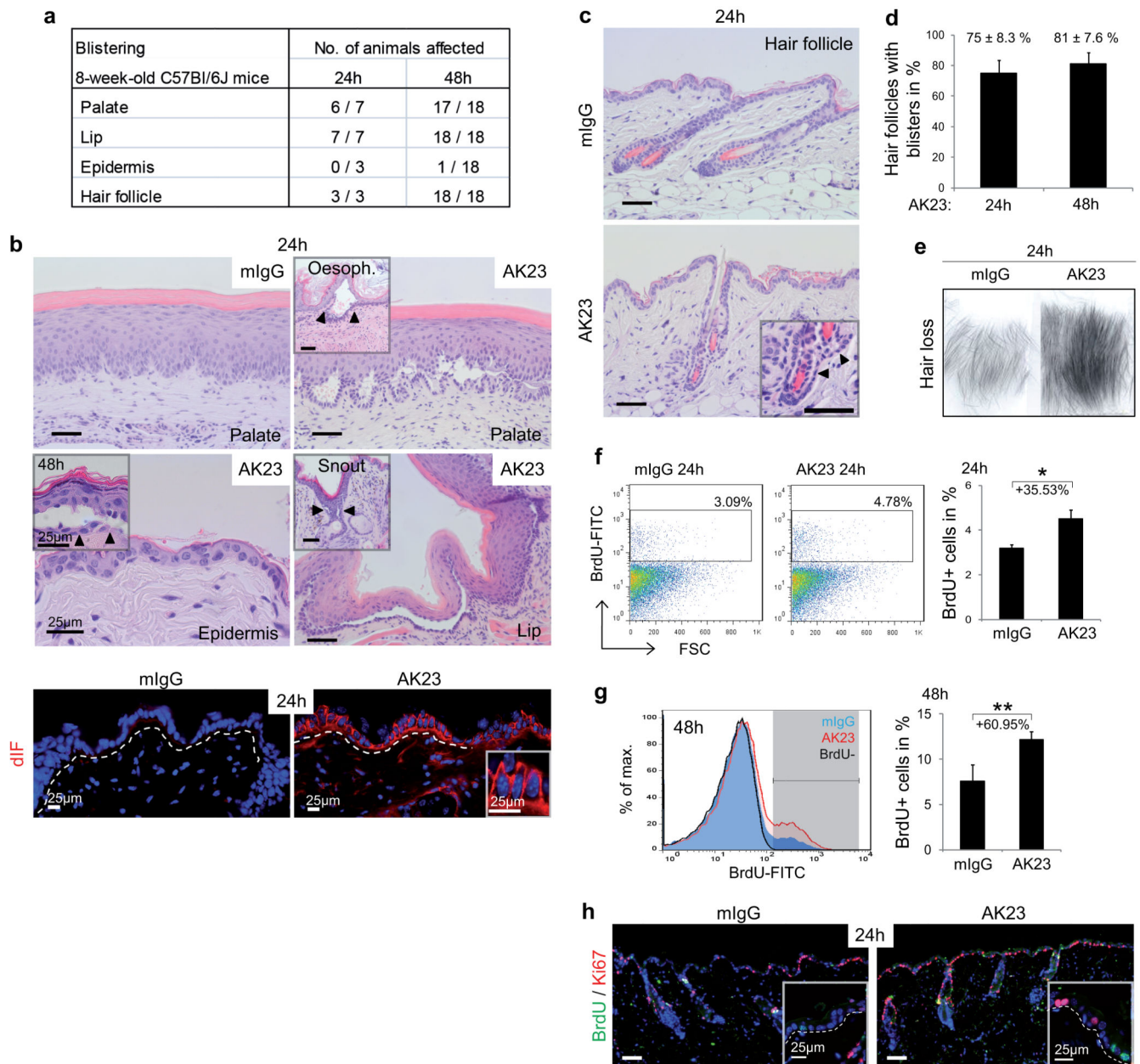


**Figure 1. AK23/PF IgG and AK23-treated neonatal mice show hyperproliferation, EGFR activation, c-Myc upregulation and depletion of Dsg3 from desmosomes**

(a) Shown are micrographs of immunofluorescence microscopy for Ki67 and graph of total Ki67+ cells in basal keratinocytes counted on micrographs of neonatal mice injected with AK23 or control mouse IgG (mIgG), with or without a half-maximal dose of PF IgG as indicated. Counted were >1000 cells per animal. Scale bars: 25  $\mu$ m. (b+c) Graphs depict average quantitative results of immunoblots from indicated proteins in Triton-X 100 soluble fractions at 24hrs. Signals on each blot were quantified, normalized to tubulin and plotted

relative to mIgG set as 1. **(d+e)** Immunoblots and graphs of indicated proteins from Triton-X 100 soluble and insoluble fractions. Lanes indicate different animals. Blots were normalized to tubulin (soluble fractions, upper panel) or lamin B1 (insoluble fractions, lower panel; shown is lamin B1 of blots probed for **d**: Dsg3/Dsc3/PG/Pph1, **e**: Dsg3/Dsg1/2/PG). Pph1: plakophilin, DP: desmoplakin. Data are mean  $\pm$  SEM. ( $n(\text{mIgG}/\text{PF}; \text{mIgG}) = 4$ ,  $n(\text{AK23}/\text{PF}; \text{AK23}) = 5$  animals), \*P 0.05, \*\*P 0.01.





**Figure 2. AK23-treated 8-week-old C57Bl/6J mice exhibit typical PV lesions and epidermal hyperproliferation**

(a) Blister sites and number of affected/tested animals are summarized. (b) H&E-stained paraffin sections of indicated tissues show lesions and direct immunofluorescence microscopy depicts AK23 binding to basal epidermal keratinocytes (dIF). Inserts show lesions (arrow heads) in oesophagus and snout skin and a rare lesion in the epidermis. (c) Histology and (d) percentage of HF blisters (insert, arrow head) in AK23-treated animals. Data are mean ± SDM. HF analyzed ( $n(24h) = 115$ ;  $n(48h) = 455$ ). (e) Hair loss by tape stripping. (f) Representative flow cytometry blots for forward scatter (FSC) and BrdU-labeled viable cells gated for BrdU-positive cells, and graph of average results. Data are

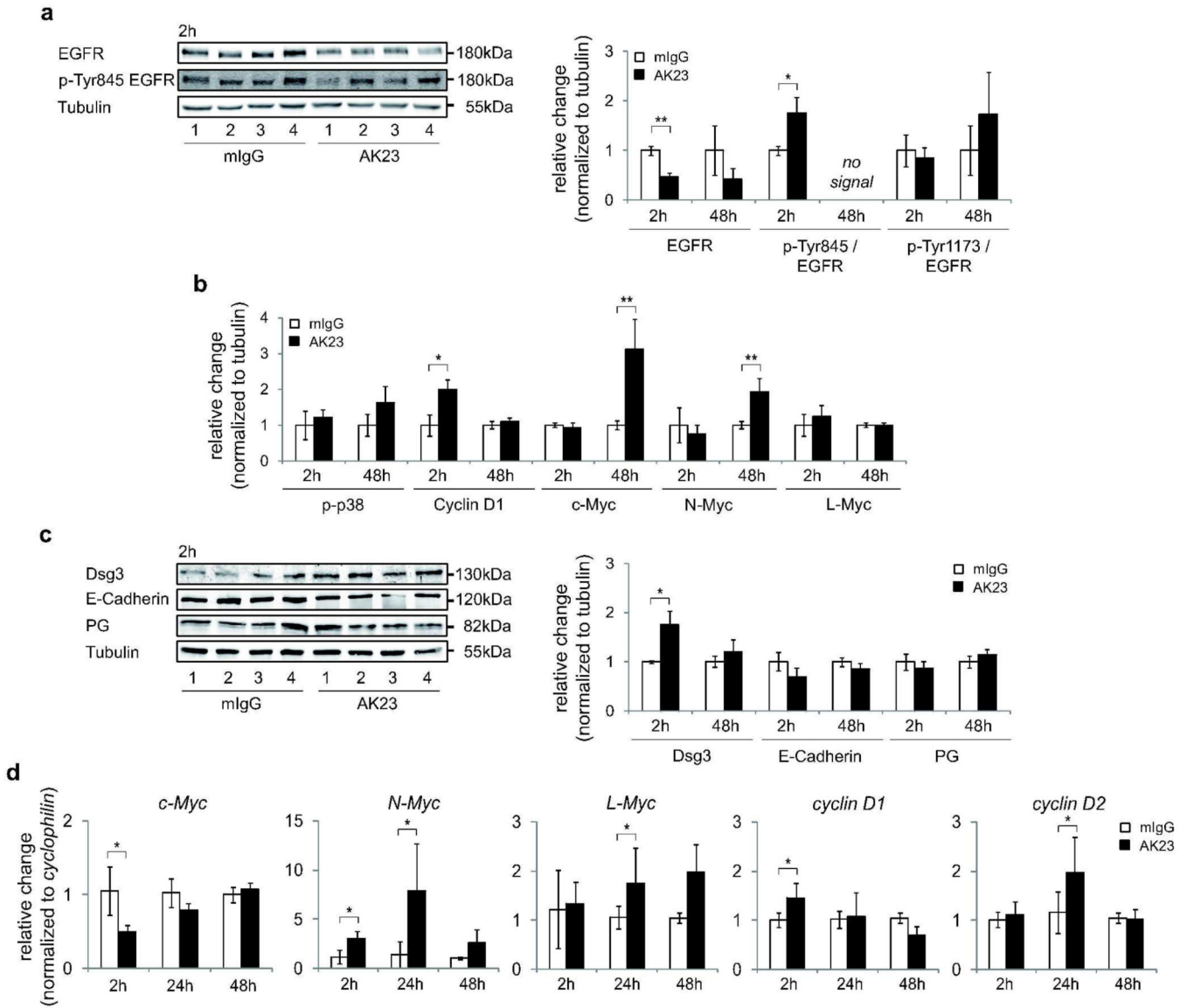
mean  $\pm$  SDM. ( $n = 2$  animals/group; 2 experiments), \* $P < 0.05$ . **(g)** Representative flow cytometry histogram for BrdU-labeled cells and graph of average results. Data are mean  $\pm$  SDM. ( $n = 4$  animals/group). \*\* $P < 0.01$ . **(h)** Immunofluorescence microscopy depicting the distribution of BrdU+ and Ki67+ cells in epidermis (Inserts: close up of epidermis). Scale bars: 50  $\mu\text{m}$  or as indicated.

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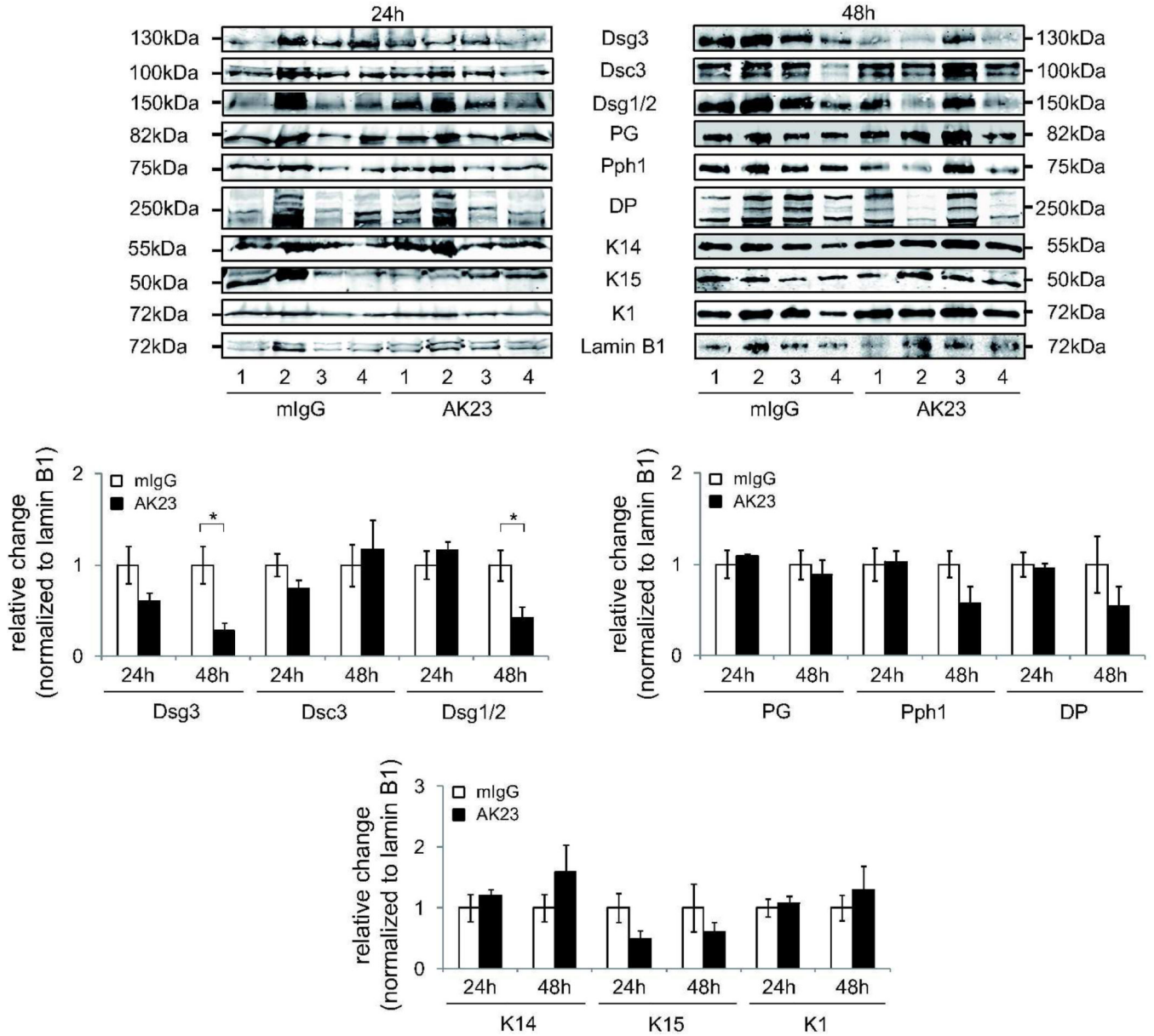
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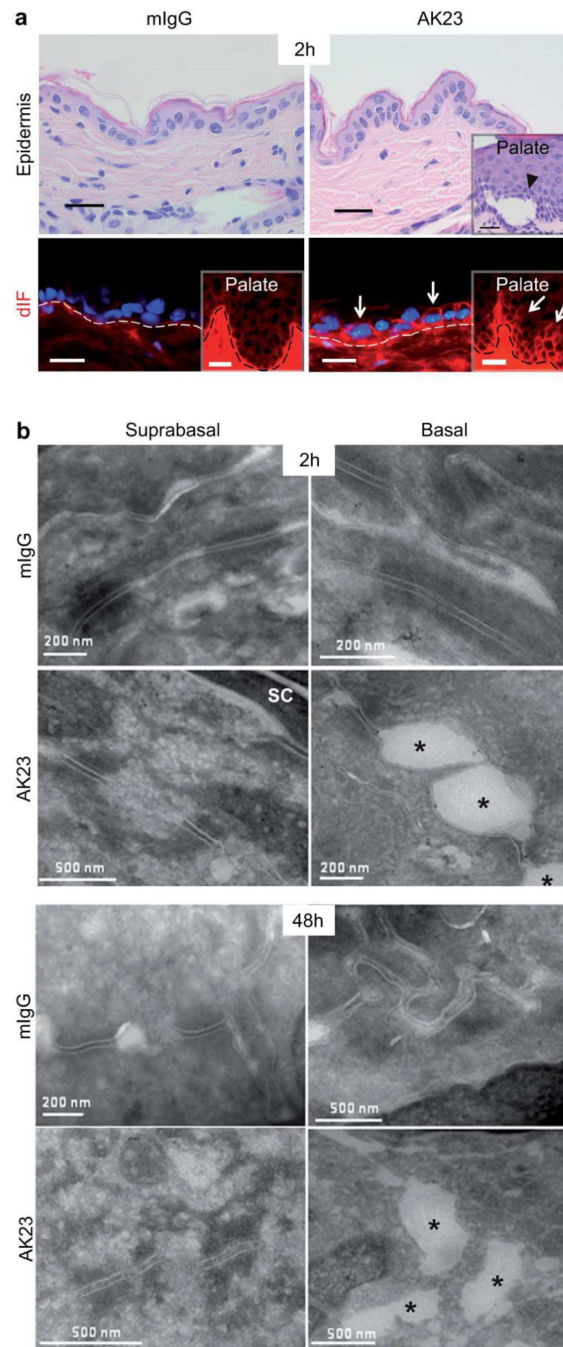
**Figure 3. EGFR is activated and Myc, cyclin D and Dsg3 are significantly increased in Triton X-100 soluble fractions of AK23-treated 8-week-old C57Bl/6J mice**

(a) Immunoblots and graphs of average quantitative results are shown for indicated proteins at 24 and 48hrs. Signals were quantified, normalized to tubulin on each blot and plotted relative to mIgG set as 1. ( $n(2h) = 4$  animals/group;  $n(48h, mIgG) = 4$ ,  $n(48h, AK23) = 2$ ). (b) Graphs like in a, ( $n(2h) = 4$  animals/group,  $n(48h, mIgG) = 8$ ,  $n(48h, AK23) = 6$  animals). (c) like in a, ( $n(2h) = 4$  animals/group,  $n(48h, mIgG) = 8$ ,  $n(48h, AK23) = 6$  animals). (a-c) Data are mean  $\pm$  SEM. \*\*P 0.01, \*P 0.05. (d) Graphs show indicated mRNA steady-state levels obtained by quantitative RT-PCR. Values were normalized to *cyclophilin* and are plotted relative to mIgG set as 1. ( $n(2/24h) = 4$  animals/group,  $n(48h, mIgG) = 8$ ,  $n(48h, AK23) = 7$  animals). Data are mean  $\pm$  SEM.



**Figure 4. Dsg3 is depleted from desmosomes in AK23-treated 8-week-old C57Bl/6J mice**  
 Immunoblot of the Triton-X 100 insoluble fractions are shown for indicated proteins. Numbered lanes correspond to four different animals per group. Each blot was normalized with respect to lamin B1 (shown is the blot probed for Dsg3/K15 at 24hrs and for PG, K15/Pph1 at 48hrs). Signals were quantified, normalized and are plotted relative to mlgG set as 1. Pph1: plakophilin, DP: desmoplakin. Data are mean  $\pm$  SEM. ( $n(24h) = 4$  animals/group;  $n(48h) = 4$  animals/group), \*P 0.05.





**Figure 5. AK23 causes widening of the intercellular spaces after 2hrs and microblisters after 48hrs in basal keratinocytes of AK23-treated 8-week-old C57Bl/6J mice**  
**(a)** Histology (upper panel) and direct immunofluorescence analyses (lower panel) at 2hrs in samples processed for electron microscopy. Only one animal out of 8 showed a microblister in the hard palate (insert) but not in the epidermis. Scale bars: 25  $\mu$ m. **(b)** Electron microscopical pictures at 2 and 48hrs. \*, widened intercellular spaces; SC, stratum corneum. Tissue damage is seen in basal but not suprabasal cells or controls. ( $n(2h/48h) = 2$  animals/group). Scale bars: as indicated.