Keratins Stabilize Hemidesmosomes through Regulation of β4-Integrin Turnover

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Epidermal integrity and wound healing depend on remodeling of cell-matrix contacts including hemidesmosomes. Mutations in β4-integrin and plectin lead to severe epidermolysis bullosa (EB). Whether mutations in keratins K5 or K14, which cause EB simplex, also compromise cell-matrix adhesion through altering hemidesmosomal components is not well investigated. In particular, the dependence of β4-integrin endocytosis and turnover on keratins remains incompletely understood. Here, we show that the absence of keratins causes loss of plectin-β4-integrin interaction and elevated β4-integrin phosphorylation at Ser1354 and Ser1362. This triggered a caveolin-dependent endocytosis of β4-integrin but not of other integrins through Rab5 and Rab11 compartments in keratinocytes. Expressing a phospho–deficient β4-integrin mutant reduces β4-integrin endocytosis and rescues plectin localization in keratin–free cells. β4-integrin phosphorylation in the absence of keratins resulted from elevated Erk1/2 activity downstream of increased EGFR and PKCα signaling. Further, increased Erk1/2 phosphorylation and altered plectin localization occur in keratin–deficient mouse epidermis *in vivo*. Strikingly, expression of the K14-R125P EBS mutant also resulted in plectin mislocalization and elevated β4-integrin turnover, suggesting disease relevance. Our data underscore a major role of keratins in controlling β4-integrin endocytosis involving a plectin-Erk1/2-dependent mechanism relevant for epidermal differentiation and pathogenesis.

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INTRODUCTION

Epidermal integrity, wound healing, and malignant progression are highly dependent on cell-matrix contacts formed by basal keratinocytes (Ridley *et al.*, 2003). To maintain epidermal homeostasis, a continuous remodeling of the two major keratinocyte cell-matrix contacts, keratin-associated hemidesmosomes (HDs) and actin-dependent focal adhesions (FA), is required. Although the turnover of the major FA integrins β3 and β1 is relatively well understood (Puklin-Faucher and Sheetz, 2009), little is known about the turnover of β4-integrin (β4-integrin subunit), which together with α6 represents major transmembrane components of hemidesmosomes. Extracellularly, α6β4-integrin binds to laminin-332 (Jones *et al.*, 1998; Borradori and Sonnenberg, 1999), intracellularly, β4-integrin binds to plectin, which directly interacts with keratin filaments, forming the major epithelial cytoskeleton (Rezniczek *et al.*, 1998). Keratinocyte–specific type I HD also contains the tetraspanin CD151 and the bullous pemphigoid antigens 180 (BP180) and 230 (BP230) to provide additional protein interactions (Hopkinson *et al.*, 1998; Sterk *et al.*, 2000; Litjens *et al.*, 2006; Bouameur *et al.*, 2013). The importance of the β4-integrin-plectin-keratin linkage is underscored by severe forms of epidermolysis bullosa (EB), resulting from loss-of-function mutations in one of these proteins, causing severe cell-matrix adhesion defects. Mutations in plectin and β4-integrin destabilize cell-matrix adhesion and lead to EBS-MD (EB simplex with muscular dystrophy) and junctional EB with pyloric atresia and skin fragility (Coulombe and Fuchs, 1993; Ashton *et al.*, 2001; Pfendner and Uitto, 2005; Rezniczek *et al.*, 2010), whereas mutations in epidermal keratins K5 or K14 disrupt the cytoskeleton and give rise to EB simplex (EBS), respectively. The most severe K14-R125C mutation accounts for 70% of all EBS cases and compromises cell-matrix adhesion at the intracellular side of desmosomes, whereas the consequence to cell-matrix contacts is not well understood (Porter and Lane, 2003).

Formation and disassembly of HD need to be tightly and dynamically regulated (Tsuruta *et al.*, 2011). HD assembly has been suggested to proceed in an outside-in process in which laminin-332 drives the interaction between plectin and β4-integrin (Koster *et al.*, 2003), although a different study showed that HD can assemble independently from the

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Abbreviations: EB, epidermolysis bullosa; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; FA, focal adhesions; IF, intermediate filaments; HD, hemidesmosome; KO, keratin–deficient knockout; K5, K5/K14 rescue; WT, wild-type; β4, β4-integrin subunit

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extracellular domain of β4-integrin (Nievers *et al.*, 1998). The interaction between β4-integrin and plectin leads to a conformational switch in the intercellular connecting segment (CS) of β4-integrin by binding to plectin (De Pereda *et al.*, 2009). Dissociation of plectin and β4-integrin integrin is correlated with increased β4-integrin phosphorylation mediated by activation of the EGF receptor (EGFR). Subsequently, PKCα, Erk1/2, and PKA become activated and phosphorylate human β4-integrin on cytoplasmic Ser residues S1356, S1360, S1364, and S1424 (Wilhelmsen *et al.*, 2007; Frijns *et al.*, 2010; Li *et al.*, 2013). In addition, phosphorylation of Thr 1736 of β4-integrin is EGFR–dependent, resulting in destabilization of plectin-β4-integrin interactions (Frijns *et al.*, 2012). Whether these events also affect the interaction of HD with the keratin cytoskeleton and how these interactions are precisely regulated during epidermal differentiation and disease have not been addressed so far. We have recently found that keratins are necessary to maintain plectin at hemidesmosomes and demonstrated that in the absence of keratins plectin no longer co-localizes to β4-integrin, resulting in increased migration (Seltmann *et al.*, 2013a).

Here, we address this issue in murine keratinocytes devoid of all keratins, using a combination of genetic and biochemical approaches. We find that the absence of keratins leads to

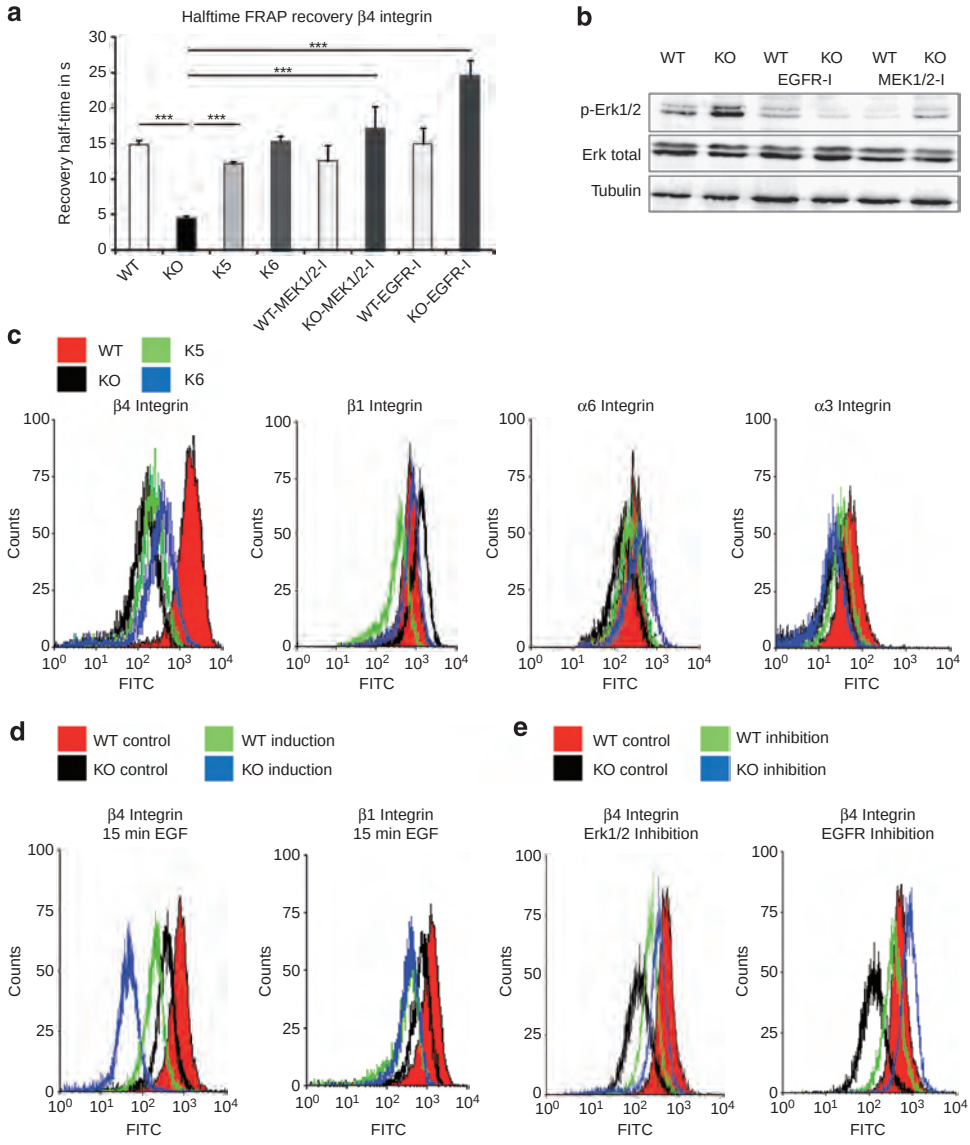


Figure 1. Absence of keratins leads to higher turnover and reduced surface level of β4-integrin. (**a**) FRAP analysis of β4-integrin dynamics with or without inhibitors of MEK1/2 and EGFR. Error bars correspond to five independent experiments with each comprising at least 30 cells. (**b**) Western blot analysis of Erk1/2 phosphorylation of total WT and KO lysates treated with or without EGFR and MEK1/2 inhibitors. (**c**) Flow-cytometric profile of integrin surface expression. Red peak—WT, black—KO, and green/blue—K5 or K6 rescue cells. (**d**) Keratinocytes were starved and then triggered with EGF for 15 minutes. (**e**) Cells were treated with indicated kinase inhibitors. Control cells are illustrated by red and black peaks (WT and KO), whereas green and blue display inhibited WT and KO cells. All experiments were repeated at least three times ($n = 3$). $^{***}P < 0.01$.

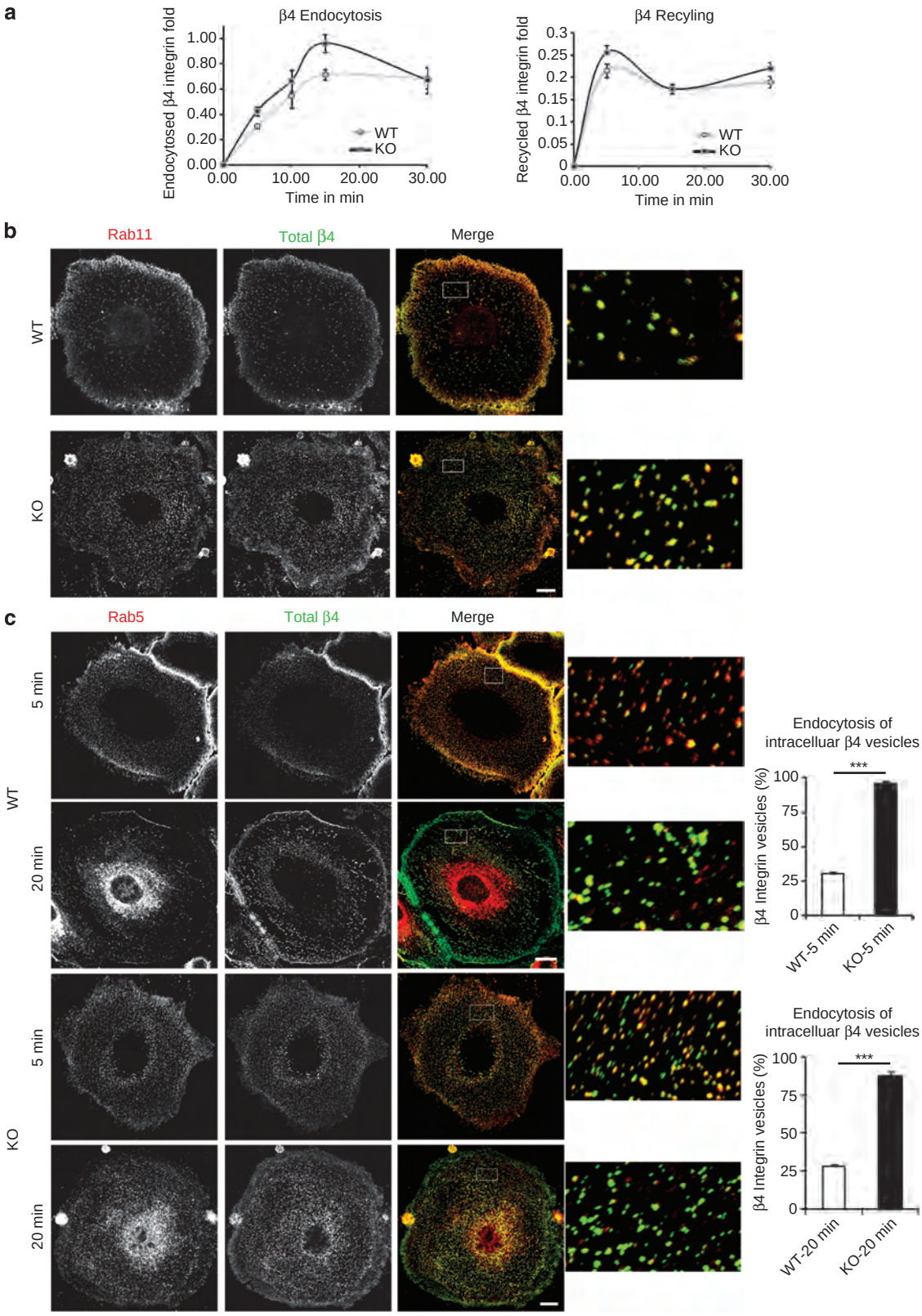


Figure 2. Enhanced short and long-loop endocytosis and recycling of β4-integrin in keratin–deficient cells. (**a**) Endocytosis and recycling of β4-integrin measured with antibody-based plate reader assays at indicated time points. (**b**/**c**) β4-integrin endocytosis was induced for indicated time points and cells were stained for Rab5 and Rab11. Higher magnifications of boxed areas on the left side are shown to detail endocytosis vesicles. Data are expressed as means ± s.e.m. Scale bar = 10 μm. All experiments were repeated at least three times.

Figure 3. Inhibiting phosphorylation reduces caveolin-dependent endocytosis of β4-integrin. (**a**) Immunostaining of caveolin-1 in WT and KO after endocytosis induction. Detailed boxed images with higher magnification are shown on the left side. (**b**) WT and KO stained with caveolin-1 and total β4-integrin antibodies. (**c**) Flow cytometry of β4-integrin surface levels of WT and KO cells with or without treatment with filipin III (inhibitor of caveolin endocytosis). (**d**) After induction of β4-integrin endocytosis, cells were fixed and in immunofluorescence analysis compared WT, KO control, and WT Ras overexpressing cells with treated keratin–free cells. Quantification of intracellular β4-integrin endocytotic vesicles treated or untreated with indicated inhibitors. (**e**) Keratin–free cells were treated with indicated siRNAs and β4-integrin endocytosis was quantified. Data are expressed as means ± s.e.m. Scale bar = 10 μm. ***$P < 0.01$.

HD disassembly downstream of EGFR-mediated hyperphosphorylation of β4-integrin, a process blocked by a phospho-deficient β4-integrin mutant. Thus, keratins stabilize plectin–β4-integrin interactions by limiting β4-integrin phosphorylation and turnover and thereby regulate keratinocyte adhesion and migration.

RESULTS

Keratins specifically stabilize surface expression of β4-integrin

Previously, we reported that keratins stabilize hemidesmosomes through plectin (Seltmann *et al.*, 2013a). In the absence of keratins, β4-integrin and plectin are no longer co-localized, leading to the loss of patchy hemidesmosomal assemblies and significantly increased migration and invasion of corresponding keratinocytes (Seltmann *et al.*, 2013a, 2013b).

To examine the hypothesis that HD disassembly results from an increased β4-integrin turnover, we first performed FRAP of β4-integrin. Compared with controls, the dynamics of β4-integrin in keratin-deficient keratinocytes was elevated three times. Re-expression of the keratin pairs K5/K14 or K6/K16 decreased β4-integrin dynamics (Figure 1a). Moreover, we found a decreased surface level of β4-integrin, whereas other integrins including β1, α3, and α6 remained unaffected in keratin–deficient cells (Figure 1c). The decrease in β4-integrin surface levels was significantly greater than the mild decrease in total protein and mRNA and protein levels in KO cells, arguing against a major role of protein biosynthesis or degradation (Supplementary Figure S1 online).

Keratinocytes lacking keratins have an elevated endocytosis rate of β4-integrin depending on Rab5, Rab11, and caveolin

In light of the reduced β4-integrin surface level and its increased turnover rate, we tested whether endocytosis and recycling were altered in KO keratinocytes. In the absence of experimental data on β4-integrin endocytosis and turnover, a new protocol was established. This revealed a general increase in the endocytosis rate of β4-integrin, with two maxima at 5 minutes and 20 minutes into the endocytotic process in the absence of keratins (Figure 2a). Under these conditions, recycling of β4-integrin was only slightly increased in KO cells, with a maximum after 5 minutes into the recycling process.

Confocal microscopy of the endocytosis process revealed strong enrichment in the number of β4-integrin positive–endocytotic vesicles in KO keratinocytes (Figure 2b, lower panel). In control cells, a ring-like localization pattern at the edge of cells was observed, indicating that β4-integrin resided at the plasma membrane. β4-integrin containing vesicles co-localized with Rab5 5 minutes into endocytosis, whereas after 20 minutes this co-localization disappeared (Figure 2b). Instead, after 20 minutes into endocytosis, β4-integrin vesicles were found in the cytoplasm together with Rab11, a known marker of long-loop endocytosis (Figure 2c). In addition, caveolin-1, a major factor of endocytosis, was co-localized with vesicles at all time points analyzed (Figure 3b). Moreover, total staining of β4-integrin showed an inverse co-localization of patchy HD structures to caveolin in WT keratinocytes, whereas in KO cells a partial co-localization of β4-integrin was noticeable (Figure 3a). Inhibition of caveolin endocytosis with the specific inhibitor filipin III rescued the lower surface level in keratin–deficient cells (Figure 3c). Thus, the absence of keratins significantly enhances endocytosis of β4-integrin.

Loss of keratins leads to enhanced β4-integrin phosphorylation and dynamics mediated by EGFR, PKCα, and ERK1/2

Phosphorylation of β4-integrin mediated by the EGFR has been shown to induce HD disassembly (Frijns *et al.*, 2010). To get a first insight whether keratins act through this pathway, we tested whether EGF treatment affects β4-integrin surface levels. Upon activation with EGF, the amount of β4-integrin at the cell surface was even more reduced in KO compared with WT cells (Figure 1d). At the same time, surface levels of β1 integrin were not influenced by EGF treatment. Conversely, direct inhibition of the EGFR or of its downstream target MEK1/2 kinase resulted in diminished Erk1/2 phosphorylation and reverted β4-integrin dynamics in keratin–deficient cells (Figure 1b). Furthermore, β4-integrin surface levels were enhanced in KO keratinocytes upon the above treatment, whereas they remained largely unaffected in WT controls (Figure 1e). Importantly, EGFR inhibition even eradicated the difference between WT and KO of β4-integrin surface level.

To examine whether phosphorylation is a prerequisite for β4-integrin endocytosis, KO cells were first treated with inhibitors and siRNAs of kinases known to phosphorylate β4-integrin, followed by analysis of the endocytosis rate. Individual inhibition of the EGFR, PKCα, Ras, or MEK1/2 diminished the endocytosis rate by 46–55% in keratin–deficient cells (Figure 3d/e). Conversely, expression of Ras in WT keratinocytes increased β4-integrin turnover through increasing Erk1/2 activity and enhanced migration to the extent found in KO keratinocytes (Figure 3d,Supplementary Figure S2a/b online). Finally, knockdown of caveolin diminished β4-integrin positive vesicles, supporting its major role in β4-integrin endocytosis (Figure 3e). Collectively, these data indicate that phosphorylation regulates the turnover of β4-integrin in a keratin-dependent manner.

To further dissect the role of murine β4-integrin phosphorylation, we raised and extensively characterized (Supplementary Figure S2c/e online) a newly generated phospho-specific

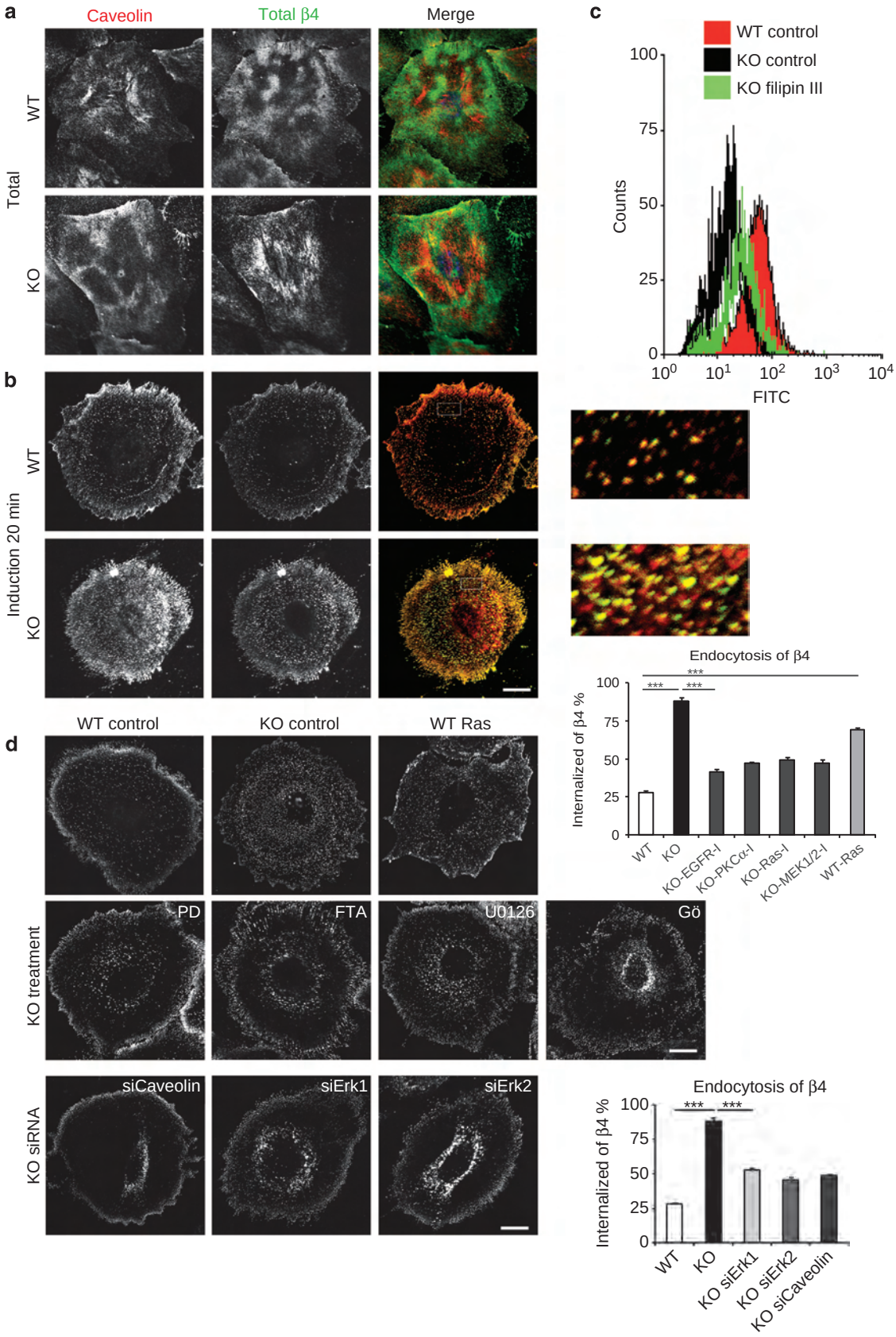

a
Caveolin
Total β4
Merge
Total
WT
KO
b
Induction 20 min
WT
KO
c
WT control
KO control
KO filipin III
Counts
FITC
Endocytosis of β4
Internalized of β4 %
WT
KO
KO-EGFR-I
KO-PKCα-I
KO-Ras-I
KO-MEK1/2-I
WT-Ras
d
WT control
KO control
WT Ras
KO treatment
PD
FTA
U0126
Gö
KO siRNA
siCaveolin
siErk1
siErk2
Endocytosis of β4
Internalized of β4 %
WT
KO
KO siErk1
KO siErk2
KO siCaveolin

Figure 4. Increased phosphorylation of β4-integrin in keratin–free cells. (**a**) Quantification of p-β4-integrin normalized to total β4-integrin by western blotting of untreated and treated keratinocytes. (**b**) WT and KO stained for p-β4 and total β4-integrin. (**c**) Western blotting of WT and KO mice skin lysates revealed increased Erk1/2 phosphorylation *in vivo*. (**d**) Quantification of Erk1/2 phosphorylation by 5 minute induction of conditioned media compared with control cells. (**e**) EGF secretion into cell supernatants of keratinocytes was measured by an ELISA assay. (**f**) Western blotting of Erk1/2 phosphorylation of total WT and KO cell lysates treated with or without PKCα (Gö) inhibitor. Immunoprecipation (IP) to determine phosphorylated (activated) PKCα. (**g**) Endocytosis of β4-integrin Ser1354 and Ser1362 Ala phospho–deficient mutant expressed in stable β4-integrin shRNA–knockdown KO cells. **h**) Staining of phospho–deficient β4-integrin Ser1354A and Ser1362A mutant and plectin. Data are expressed as means ± s.e.m. Scale bar = 10 μm. ***$P < 0.01$.

antiserum against pSer1354 and pSer1362 (corresponding to human Ser1356 and Ser1360) involved in HD disassembly. This revealed a 2-fold increase in β4-integrin phosphorylation in KO keratinocytes, correlating with a higher β4-integrin turnover (Figure 4a). Further, β4-integrin phosphorylation was reduced by treatment with EGFR, MEK1/2, and PKCα inhibitors. Localization studies of total and phosphorylated β4-integrin revealed that, in the absence of keratins, the proportion of phosphorylated β4-integrin is greater in hemidesmosomal patches (Figure 4b). Intriguingly, increased Erk1/2 phosphorylation was confirmed in the epidermis of mice lacking all keratins *in vivo* (Bär *et al.*, 2014), using an activation-specific antibody (Figure 4c). Immunostaining of prenatal skin also showed a widening of the basement membrane, persistence of plectin and β4-integrin in the suprabasal layers, and increased endocytosis activity, indicated by enhanced P-caveolin in keratin–deficient mice (Supplementary Figure S3a/b online).

Notably, cell culture supernatants of KO keratinocytes contained elevated EGF levels competent to strongly activate Erk1/2 in WT keratinocytes upon incubation (Figure 4d). Conversely, no p-Erk1/2 was detectable upon incubation of KO keratinocytes with media conditioned by WT keratinocytes. To examine whether EGF secreted by KO keratinocytes was responsible for EGFR-dependent regulation of β4-integrin, conditioned media from KO cells were used and WT were treated with the EGFR inhibitor. Upon EGFR inhibition, Erk1/2 phosphorylation was decreased and no induction occurred by KO cell supernatants (Figure 4d). In addition, secretion of EGF into the supernatant was enhanced in keratin-free cells (Figure 4e). Further, Erk1/2 phosphorylation in KO cells was diminished by PKCα inhibitor treatment, suggesting elevated PKCα activity in KO keratinocytes, consistent with PKCα activity depending on a Rack1-keratin scaffold mechanism (Kröger *et al.*, 2013) (Figure 4f). In support, immunoprecipitation revealed that PKCα phosphorylation was enhanced in keratin–deficient keratinocytes (Figure 4f). Thus, elevated p-Erk1/2 results from increased EGFR and PKCα activities in KO keratinocytes.

To further substantiate kinase inhibitor studies demonstrating that β4-integrin endocytosis depends on its phosphorylation, the phospho–deficient HA-tagged Ser-Ala mutant of β4-integrin Ser1354 and Ser1362 was expressed in a stably transfected β4-integrin knockdown cell line in a keratin–free background (Supplementary Figure S2d/e online). In these cells hardly any β4-integrin endocytosis occurred (Figure 4g). Most importantly, the phospho–deficient mutant rescued the mislocalization of plectin in keratin–deficient keratinocytes (Figure 4h). This suggests that keratins regulate β4-integrin endocytosis through stabilizing plectin at HD and through controlling Erk1/2 activation.

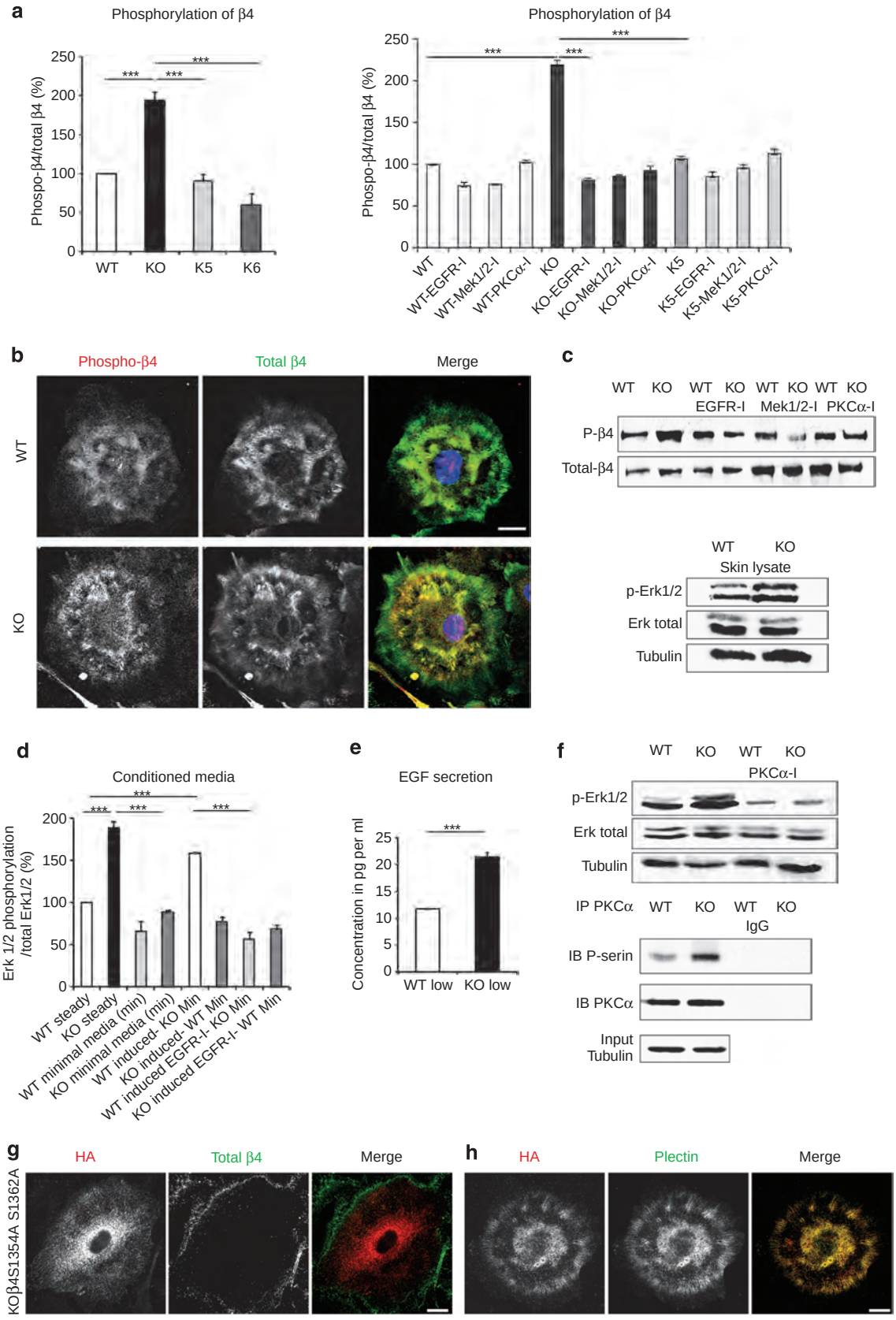
Delocalization of plectin decreases β4-integrin stabilization underlining EBS-mutated K14 R131P phenotype

To validate the role of plectin in β4-integrin endocytosis, we compared β4-integrin cell surface levels in plectin–deficient with that of keratin–deficient keratinocytes, using identical experimental settings. This revealed a decreased surface level of β4-integrin accompanied by enhanced endocytosis of β4-integrin in plectin–deficient cells, highly similar to keratin deficiency (Figures 5a and b). In further support, plectin–deficient cells displayed an increased β4-integrin phosphorylation at Ser1354 and Ser1362 comparable to keratin–deficient cells as well (Figure 5c). Loss of plectin coincided with increased Erk1/2 phosphorylation (Osmanagic-Myers *et al.*, 2006), underscoring the major role of MAPK kinase signaling in mediating β4-integrin phosphorylation (Figure 5d). Collectively, these data suggest that the absence of keratins mediates dissociation of plectin from β4-integrin and triggers increased β4-integrin phosphorylation and endocytosis by enhancing Erk1/2 activity.

Finally, we asked whether the keratin–dependent regulation of HD is of relevance for pathomechanisms underlying epidermolysis bullosa simplex, caused by missense mutations in K5, K14, or plectin (Coulombe and Lee, 2012; Winter and Wiche, 2013). In this setting, keratin–HD interactions are compromised, leading to intracellular rupture above the level of the basement membrane. Although HD reportedly appear intact at the ultrastructural level in EBS, it is not known whether they are functionally compromised. Analysis of mouse keratinocytes stably expressing mouse K14-R131P, equivalent to the most severe EBS mutant K14-R125P, revealed that plectin was dissociated away from β4-integrin, similar to the absence of keratins (Figure 5e). Intriguingly, plectin was extensively co-localized with K14-R131P-positive keratin aggregates (Figure 5e, magnified box). This coincided with increased β4-integrin endocytosis (Figure 5f) and reduced surface levels in the mutant keratinocyte cell line, similar to keratin–deficient cells (Figure 5g). Thus, in analogy to keratin's role in stabilizing desmosomes through PKCα (Kröger *et al.*, 2013), they contribute to cell-matrix adhesion through a plectin-Erk1/2-dependent mechanism.

DISCUSSION

Whereas or while the turnover of focal contacts and their corresponding actin-associated integrins is relatively clear, the mechanisms underlying β4-integrin recycling and turnover in epithelial cells remain incompletely understood. Existing data


a
Phosphorylation of β4
Phospo-β4/total β4 (%)
WT
KO
K5
K6
Phosphorylation of β4
WT-EGFR-I
WT-Mek1/2-I
WT-PKCα-I
KO-EGFR-I
KO-Mek1/2-I
KO-PKCα-I
K5-EGFR-I
K5-Mek1/2-I
K5-PKCα-I
b
Phospho-β4
Total β4
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c
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Skin lysate
p-Erk1/2
Erk total
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d
Conditioned media
Erk 1/2 phosphorylation /total Erk1/2 (%)
WT steady
KO steady
WT minimal media (min)
KO minimal media (min)
WT induced- KO Min
KO induced- WT Min
WT induced EGFR-I- KO Min
KO induced EGFR-I- WT Min
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EGF secretion
Concentration in pg per ml
WT low
KO low
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IP PKCα
IgG
IB P-serin
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KOβ4S1354A S1362A
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Figure 5. K14 EBS mutation triggers increased, plectin-dependent β4-integrin turnover. (**a**) β4-integrin localization in WT and plectin–knockout cells after endocytosis induction. (**b**) Flow cytometry of β4-integrin surface levels of WT, keratin knockout (KO), WT plectin, and plectin–knockout cells. (**c**) Western blot and quantification of p-β4-integrin in WT, keratin–, and plectin–deficient cells. (**d**) Western blot analysis of Erk1/2 phosphorylation in total cell lysates. (**e**) Staining of keratin 5, plectin, and β4-integrin of keratin 14 (K14) rescue keratinocytes and mutated keratin14 R131P cells. (**f**) Immunostaining of β4-integrin in K14 and K14-R131P cells after endocytosis induction. (**g**) Flow cytometry of surface levels of β4-integrin of WT, KO, K14 cells, and keratin14 R131P cells. Data are expressed as means ± s.e.m. Scale bar = 10 μm. ***$P < 0.01$.

support the role of β4-integrin phosphorylation in HD disassembly (Frijns *et al.*, 2010, 2012; Kashyap and Rabinovitz, 2012). However, the coordination of β4-integrin turnover and its unique linkage to the keratin cytoskeleton are unknown.

Here, we provide strong evidence for a role of keratins in HD stabilization through plectin- and Erk1/2-dependent mechanisms that converge on β4-integrin and are crucial in keratinocyte adhesion and migration. It has been shown that the interaction of plectin with β4-integrin induces a conformational change in the CS segment of the latter. This switch is a prerequisite for stable plectin binding (De Pereda *et al.*, 2009). We suggest that loss of keratins induces a conformational change in plectin and reduces the interaction with β4-integrin. Using a novel phospho-specific antiserum, we demonstrate that this permits S1354 and S1362 hyperphosphorylation and finally results in reduced surface levels of β4-integrin.

In agreement with the known role of EGFR, PKCα, and Erk1/2–dependent pathways in human S1356 and S1364 (related to mouse S1354/ S1362) phosphorylation involved in HD disassembly (Frijns *et al.*, 2010), we found increased keratin–dependent PKCα activity that contributed to elevated Erk1/2 activity in keratin–deficient cells. Second, the Erk1/2 phosphorylation is triggered by increased secretion of EGF in keratin–free cells, a mechanism that requires future investigation. Altogether, this causes enhanced β4-integrin phosphorylation, destabilization of HD, and significantly higher β4-integrin turnover by increased endocytosis. We demonstrated the importance of S1354 and S1362 in β4-integrin regulation, showing that a phospho–deficient Ser → Ala mutant at both sites prevented the dissociation of plectin and β4-integrin in keratin–deficient keratinocytes and decreased endocytosis. The significance of phosphorylation of β4-integrin at these sites for the induction of endocytosis was confirmed upon pharmacological or RNAi-mediated inhibition of EGFR, MEK1/2, PKCα, and Ras, all diminishing β4-integrin endocytosis.

Similar to the well-investigated β1 and β3 integrins, we found that β4-integrin endocytosis follows a time–dependent process dependent on caveolin-1 (Caswell and Norman, 2006). At 5 minutes into the endocytosis process, β4-integrin endocytotic vesicles co-localized with Rab5, whereas, at 20 minutes, when its endocytosis occurs at maximal rates, β4-integrin co-localized with the recycling marker Rab11 (Maxfield and McGraw, 2004). In analogy to known pathways for β1 and β3, β4-integrin internalization occurs through two major routes: a short- Rab5-dependent and a long-Rab11-dependent loop (Caswell and Norman, 2006). The rescue of β4-integrin surface levels by filipin III strongly supports that the caveolin-1 pathway is primarily responsible for β4-integrin endocytosis in keratinocytes.

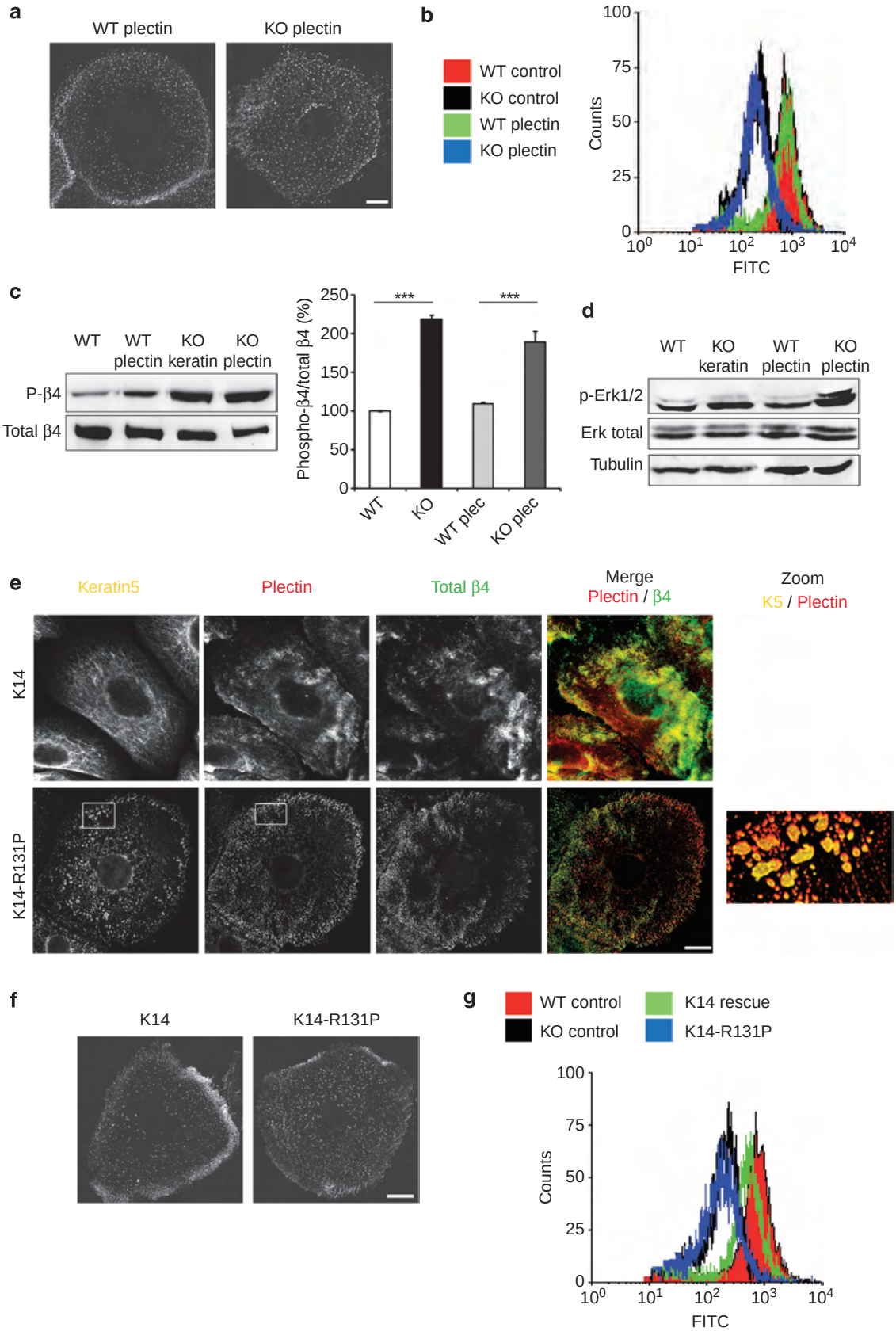
Recently, HDs in *C. elegans* were shown to participate in outside-in mechanotransduction. This involved force transduction initiated by external muscle tension through a tripartite signaling cascade initiated at HD and resulting in parts in intermediate filament hyperphosphorylation and reorganization (Zhang *et al.*, 2011). We have previously shown that the absence of keratins is accompanied by mislocalization of plectin, increased migration, and adhesion of keratinocytes (Seltmann *et al.*, 2013a). In line, HDs are dynamic structures, which contribute to adhesion and migration properties of keratinocytes (Geuijen and Sonnenberg, 2002). Intriguingly, β4-integrin, plectin, and keratin–deficient keratinocytes share a similar phenotype regarding enhanced migration and adhesion (Raymond *et al.*, 2005; Osmanagic-Myers *et al.*, 2006). Our data place keratins upstream of these processes and suggest that keratins could mediate an inside-out signaling to reorganize hemidesmosomes during adhesion and migration. In pathological conditions like EBS our results explain how defects in the keratin cytoskeleton lead to decreased stability of cell-matrix adhesion and rupturing of the skin. In support, we have shown that loss of keratins decreases cell stiffness independent from the actin cytoskeleton and promotes invasion into matrigel (Seltmann *et al.*, 2013b). This is in line with the recently reported participation of hemidesmosomal proteins in various cancers (Raymond *et al.*, 2007; Li *et al.*, 2013; Scartozzi *et al.*, 2013; Chen *et al.*, 2014).

Our data suggest a model in which keratins are needed to stabilize HDs by maintaining β4-integrin surface level (Figure 6). Loss of keratins could lead to conformational or posttranslational changes in plectin, mediating an increased β4-integrin phosphorylation at Ser1354 and Ser1362 by PKCα via activated EGFR and Erk1/2 kinase. The elevated PKCα and Erk1/2 kinase activities in keratin–deficient cells accelerate this process even further. Altogether, we demonstrated that keratins are major factors of migration and adhesion by stabilizing β4-integrin–plectin interaction, which regulates β4-integrin turnover by EGF-PKCα-Erk1/2-dependent mechanism.

MATERIALS AND METHODS

Cell culture and drug treatment

Isolation and culture of mouse keratinocytes of various genotypes was described previously (Vijayaraj *et al.*, 2009; Seltmann *et al.*, 2013a). In brief, cells were grown in a chelex-treated FAD medium supplemented with 10% FCS Gold (PAA, chelex-treated), 0.18 mM Adenine, 0.5 μg ml^{-1} hydrocortison, 5 μg ml^{-1} insulin, 100 pM cholera toxin (all Sigma, Munich, Germany), 10 ng ml^{-1} EGF, 100


a
WT plectin
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KO plectin
P-β4
Total β4
Phospho-β4/total β4 (%)
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WT
KO
WT plec
KO plec
d
WT
KO keratin
WT plectin
KO plectin
p-Erk1/2
Erk total
Tubulin
e
Keratin5
Plectin
Total β4
Merge Plectin / β4
Zoom K5 / Plectin
K14
K14-R131P
f
K14
K14-R131P
g
WT control
KO control
K14 rescue
K14-R131P
Counts
FITC

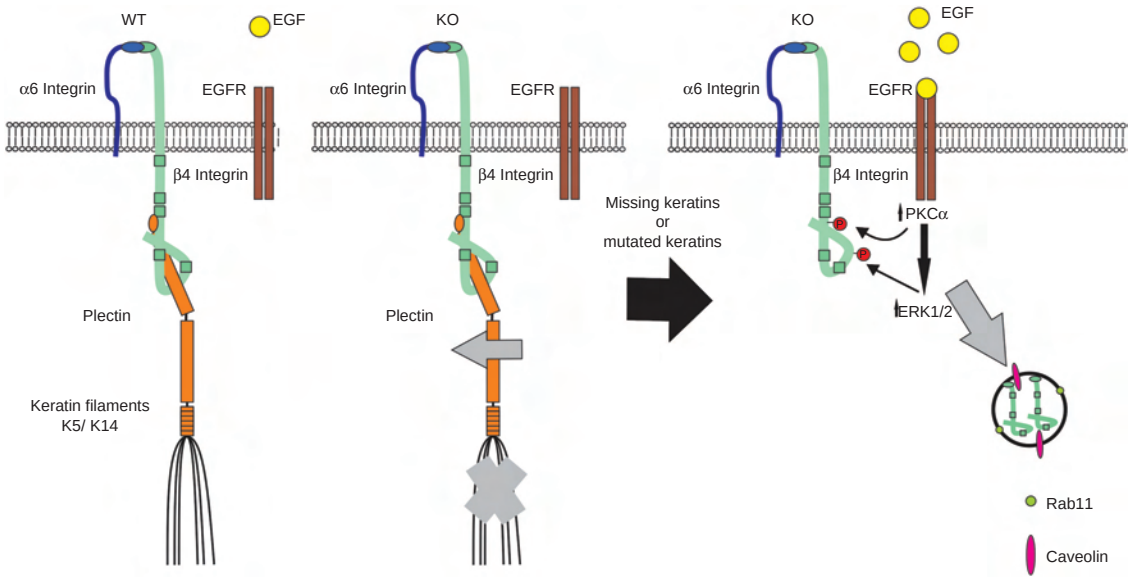

Figure 6. Model for keratin-dependent β4-integrin turnover. Loss of keratins leads to dissociation of plectin and a conformational change in β4-integrin structure. Thereby, phosphorylation of β4-integrin is induced by PKCα and Erk1/2 kinase-dependent on activated EGFR. This results in destabilization of β4-integrin surface levels. Thereby, β4-integrin surface integrin is endocytosed in a Rab5 (short-loop), Rab11 (long-loop), and caveolin–dependent manner.

U ml^{-1} and 100 μg ml^{-1} penicillin/streptomycin, and 2 mM glutamax (all Invitrogen, Karlsruhe, Germany), 5% CO_2 at 32 °C. Cells were cultured on collagen I- (Invitrogen) coated cell culture dishes. Cells were treated overnight with indicated inhibitors (Supplementary Table online). K14 and K14-R131P keratinocytes were generated by lentiviral transfection in analogy to Kröger *et al.*, 2013 JCB and will be described in detail elsewhere (Homberg *et al*, in preparation). The tissue preparations and immunostainings were described earlier (Vijayaraj *et al.*, 2009; Bär *et al.*, 2014). Animal care and experimental procedures were in accordance with the institutional and governmental guidelines.

Antibodies

Antibodies used were mouse anti-α3 integrin (R&D Systems), anti-HA (Covance, Wiesbaden, Germany) Rab5, rat anti-β4-integrin, rat anti-β1 integrin (all BD Bioscience, Freiburg, Germany), mouse anti-alpha tubulin (Sigma-Aldrich, Munich, Germany), rabbit anti-β4-integrin (Santa Cruz Biotechnology, Heidelberg, Germany), rabbit Total and phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), Rab11 and p-/total caveolin-1 (Cell Signaling, Frankfurt, Germany), mouse anti-plectin (HD 1, kind gift from Prof. Katsushi Owaribe, Japan), and guinea pig anti-keratin 5, Magin lab. Secondary antibodies conjugated to fluorochromes and PromoFluor-488-conjugated phalloidin were purchased from Dianova (Hamburg, Germany) and Promokine (Heidelberg, Germany). Polyclonal guinea pig antibody specific for β4-integrin phosphorylated at residue S1354 and S1362 was raised against a synthetic peptide, CVLRSPASSQRPSVS, in which the underlined serine residues contained phosphate groups.

Immunofluorescence analysis

Staining conditions and image processing was described previously (Seltmann *et al.*, 2013a). In brief, cells were fixed for 10 minutes in 4% paraformaldehyde and incubated with primary antibodies for 1 hour. All antibodies were diluted in TBS containing 1% BSA. Afterward, cells were incubated with the secondary antibody for 30 minutes and mounted with mounting medium (Dianova). Images were acquired using an AxioImager Z2 equipped with Zeiss Plan-Apochromat ×63/1.46 oil or a Zeiss LSM 780 confocal microscope with 40/1.3 NA or 63 /1.46 NA. Image analysis and processing were performed using the AxioVision version 4.8 and Zen Software 2010 (Zeiss, Jena, Germany).

Integrin endocytosis and recycling assay

A modified protocol was used for endocytosis assay described previously (Arjonen *et al.*, 2012). In brief, cells were labeled with an Alexa-488-conjugated β4-integrin antibody in cell culture media for 1 h on ice. Afterward, endocytosis was induced by lifting the cells in a water bath at 37 °C for indicated time points. Quenching of the surface β4-integrin was done by adding an anti-Alexa Fluor 488 antibody for 1 h on ice (Invitrogen). Next, cells were fixed for 10 minutes with 4% paraformaldehyde on ice in all endocytosis assays, permeabilized, and stained for additional endocytosis markers. For quantitative assays, fluorescent intensities were measured using an Infinite200 (Tecan, Crailsheim, Germany). Fluorescence intensities were normalized against nuclear Hoechst staining. For integrin recycling, additional steps followed after the same procedure like for 20 minute endocytosis. After quenching, cell plates were again incubated in the water bath for indicated time points and re-quenched afterward followed by paraformaldehyde fixiation.

FACS

Primary antibodies used were labeled to Alexa-488 (Life Technologies, Munich, Germany). Isotype control staining was performed using FITC-conjugated mouse anti-rat IgG1 antibody (Santa Cruz). Trypsinized cells were incubated with primary antibodies diluted 3% BSA/PBS for 1 hour on ice. FACS analysis and data acquisition were

performed using the BD FACSCanto flow cytometer and BD FACSDiva-based software (BD Biosciences). Cell debris and dead cells were excluded from the analyses on the basis of their scatter signals.

Immunoblotting and immunoprecipation

SDS–PAGE and western blot were performed as described (Vijayaraj *et al.*, 2009). In brief, total proteins were extracted in SDS–PAGE sample buffer under repeated heating (95 °C) and sonication cycles. Separation of total protein extracts was carried out by 8–12% SDS–PAGE gels and followed by western blotting. For immunoprecipitation experiments, cells were washed twice with phosphate-buffered saline (PBS) and lysed with 500 µl of cold RIPA buffer (10 mM Na2HPO, 150 mM NaCl, 5 mM EDTA, 2 mM EGTA, 1% Triton X-100, 0.25% SDS, 1% sodium deoxycholate, 0.1 mM DTT pH 7.5, protease, and phosphatase inhibitor cocktails (Pierce, Rockford, IL)). Lysates were clarified by centrifugation at 16.000*g* for 10 minutes. Supernatants were incubated with appropriate primary antibodies overnight at 4 °C. A 25-µl volume of protein-G agarose beads (Pierce) was added and tubes were rotated at 4 °C for 2 h or overnight. Beads were washed three times with 500 µl RIPA buffer, boiled, and sonicated in 50 µl Laemmli sample buffer.

Knockdown of β4-integrin and generating β4-integrin mutants

β4-integrin integrin knockdown was generated using MISSION shRNA lentivirus (Sigma). In brief, keratinocytes were seeded in six-well dishes, the following day they were infected with lentivirus encoding β4-integrin-specific shRNAs with using a 3 ml of fresh lentivirus supernatant supplemented with 8 µg ml^{-1} polybrene. Two days after transduction, cells were selected with 8 µg ml^{-1} puromycin to generate stable lines for stable transfectants. Knockdown efficiency of β4-integrin shRNA was analyzed by western blotting. The generation of full-length β4-integrin cDNA has been described previously (Niessen *et al.*, 1997). Mutants of β4-integrin were created by site-directed mutagenesis using the PCR-based overlap extension method and Phusion DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany). Wild-type and mutant HA-tagged β4-integrin cDNA was cloned into the pcDNA3 vector (Invitrogen).

FRAP

Live-cell analysis in FRAP (fluorescence of after photobleaching) measurements were performed on a Zeiss LSM 780 (Plan apochromat 63 ×/1.4 numeric aperture [NA] oil immersion objective) with a climate chamber at 32 °C, 5% CO_2. For preparation, cells were seeded on 35-mm collagen I-coated glass-bottom dishes and transfected with GFP-tagged expression plasmids. Defined regions of HDs were bleached at full laser power (100% power, 100% transmission for 20 seconds) using the 488 nm line of the argon laser. Image acquisition was continued in 500 ms intervals of a period of at least 180 s at low laser power. Quantification of FRAP experiments was performed measuring the fluorescence intensity of the bleached area before, directly after and during recovery from bleaching and subtracted by a corresponding background trace measured within the same cell, far from the bleached area. Half-life time was calculated according to the following equation, $t_{1/2} = \ln(0.5)/(-\text{tau})$, and diffusion coefficient was calculated according to the following equation, $D = 0.88 * w^2/(4*t_{1/2})$ Image analysis and data analysis of the FRAP data were performed using ImageJ.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Arjonen A, Alanko J, Veltel S *et al.* (2012) Distinct recycling of active and inactive β1 integrins. *Traffic* 13:610–25

Ashton GHS, Sorelli P, Mellerio JE *et al.* (2001) α6β4 integrin abnormalities in junctional epidermolysis bullosa with pyloric atresia. *Br J Dermatol* 144: 408–14

Bär J, Kumar V, Roth W *et al.* (2014) Skin fragility and impaired desmosomal adhesion in mice lacking all keratins. *J Invest Dermatol* 134: 1012–22

Borradori L, Sonnenberg A (1999) Structure and function of hemidesmosomes: more than simple adhesion complexes. *J Invest Dermatol* 112: 411–8

Bouameur JE, Favre B, Borradori L (2013) Plakins, a versatile family of cytolinkers: roles in skin integrity and in human diseases. *J Invest Dermatol* 134:885–94

Caswell PT, Norman JC (2006) Integrin trafficking and the control of cell migration. *Traffic* 7:14–21

Chen Q, Xu R, Zeng C *et al.* (2014) Down-regulation of Gli transcription factor leads to the inhibition of migration and invasion of ovarian cancer cells via integrin β4-mediated FAK signaling. *PLoS One* 9:e88386

Coulombe PA, Fuchs E (1993) Epidermolysis bullosa simplex. *Semin Dermatol* 12:173–90

Coulombe PA, Lee CH (2012) Defining keratin protein function in skin epithelia: epidermolysis bullosa simplex and its aftermath. *J Invest Dermatol* 132:763–75

Frijns E, Sachs N, Kreft M *et al.* (2010) EGF-induced MAPK signaling inhibits hemidesmosome formation through phosphorylation of the integrin β4. *J Biol Chem* 285:37650–62

Frijns E, Kuikman I, Litjens S *et al.* (2012) Phosphorylation of threonine 1736 in the C-terminal tail of integrin β4 contributes to hemidesmosome disassembly. *Mol Biol Cell* 23:1475–85

Geuijen CAW, Sonnenberg A (2002) Dynamics of the alpha6beta4 integrin in keratinocytes. *Mol Biol Cell* 13:3845–58

Hopkinson SB, Findlay K, deHart GW *et al.* (1998) Interaction of BP180 (type XVII collagen) and alpha6 integrin is necessary for stabilization of hemidesmosome structure. *J Invest Dermatol* 111:1015–22

Jones JC, Hopkinson SB, Goldfinger LE (1998) Structure and assembly of hemidesmosomes. *BioEssays News Rev Mol Cell Dev Biol* 20: 488–94

Kashyap T, Rabinovitz I (2012) The calcium/calcineurin pathway promotes hemidesmosome stability through inhibition of β4 integrin phosphorylation. *J Biol Chem* 287:32440–9

Koster J, Geerts D, Favre B *et al.* (2003) Analysis of the interactions between BP180, BP230, plectin and the integrin alpha6beta4 important for hemidesmosome assembly. *J Cell Sci* 116:387–99

Kröger C, Loschke F, Schwarz N *et al.* (2013) Keratins control intercellular adhesion involving PKC-α-mediated desmoplakin phosphorylation. *J Cell Biol* 201:681–92

Li Q, Yang XH, Xu F *et al.* (2013) Tetraspanin CD151 plays a key role in skin squamous cell carcinoma. *Oncogene* 32:1772–83

Litjens SHM, de Pereda JM, Sonnenberg A (2006) Current insights into the formation and breakdown of hemidesmosomes. *Trends Cell Biol* 16: 376–83

Maxfield FR, McGraw TE (2004) Endocytic recycling. *Nat Rev Mol Cell Biol* 5: 121–32

Niessen CM, Hulsman EH, Oomen LC *et al.* (1997) A minimal region on the integrin beta4 subunit that is critical to its localization in hemidesmosomes regulates the distribution of HD1/plectin in COS-7 cells. *J Cell Sci* 110: 1705–16

Nievers MG, Schaapveld RQ, Oomen LC *et al.* (1998) Ligand-independent role of the beta 4 integrin subunit in the formation of hemidesmosomes. *J Cell Sci* 111:1659–72

Osmanagic-Myers S, Gregor M, Walko G *et al.* (2006) Plectin-controlled keratin cytoarchitecture affects MAP kinases involved in cellular stress response and migration. *J Cell Biol* 174:557–68

De Pereda JM, Lillo MP, Sonnenberg A *et al.* (2009) Structural basis of the interaction between integrin alpha6beta4 and plectin at the hemidesmosomes. *EMBO J* 28:1180–90

Pfendner E, Uitto J (2005) Plectin gene mutations can cause epidermolysis bullosa with pyloric atresia. *J Invest Dermatol* 124:111–5

Porter RM, Lane BE (2003) Phenotypes, genotypes and their contribution to understanding keratin function. *Trends Genet* 19:278–85

Puklin-Faucher E, Sheetz MP (2009) The mechanical integrin cycle. *J Cell Sci* 122:179–86

Raymond K, Kreft M, Janssen H *et al.* (2005) Keratinocytes display normal proliferation, survival and differentiation in conditional beta4-integrin knockout mice. *J Cell Sci* 118:1045–60

Raymond K, Kreft M, Song JY *et al.* (2007) Dual Role of alpha6beta4 integrin in epidermal tumor growth: tumor-suppressive versus tumor-promoting function. *Mol Biol Cell* 18:4210–21

Rezniczek GA, de Pereda JM, Reipert S *et al.* (1998) Linking integrin alpha6beta4-based cell adhesion to the intermediate filament cytoskeleton: direct interaction between the beta4 subunit and plectin at multiple molecular sites. *J Cell Biol* 141:209–25

Rezniczek GA, Walko G, Wiche G (2010) Plectin gene defects lead to various forms of epidermolysis bullosa simplex. *Dermatol Clin* 28:33–41

Ridley AJ, Schwartz MA, Burridge K *et al.* (2003) Cell migration: integrating signals from front to back. *Science* 302:1704–9

Scartozzi M, Giampieri R, Loretelli C *et al.* (2013) Role of β4 integrin in HER-3-negative, K-RAS wild-type metastatic colorectal tumors receiving cetuximab. *Future Oncol Lond Engl* 9:1207–14

Seltmann K, Roth W, Kröger C *et al.* (2013a) Keratins mediate localization of hemidesmosomes and repress cell motility. *J Invest Dermatol* 133:181–90

Seltmann K, Fritsch AW, Käs JA *et al.* (2013b) Keratins significantly contribute to cell stiffness and impact invasive behavior. *Proc Natl Acad Sci USA* 110: 18507–12

Sterk LM, Geuijen CA, Oomen LC *et al.* (2000) The tetraspan molecule CD151, a novel constituent of hemidesmosomes, associates with the integrin alpha6beta4 and may regulate the spatial organization of hemidesmosomes. *J Cell Biol* 149:969–82

Tsuruta D, Hashimoto T, Hamill KJ *et al.* (2011) Hemidesmosomes and focal contact proteins: functions and cross-talk in keratinocytes, bullous diseases and wound healing. *J Dermatol Sci* 62:1–7

Vijayaraj P, Kröger C, Reuter U *et al.* (2009) Keratins regulate protein biosynthesis through localization of GLUT1 and -3 upstream of AMP kinase and Raptor. *J Cell Biol* 187:175–84

Wilhelmsen K, Litjens SHM, Kuikman I *et al.* (2007) Serine phosphorylation of the integrin beta4 subunit is necessary for epidermal growth factor receptor induced hemidesmosome disruption. *Mol Biol Cell* 18:3512–22

Winter L, Wiche G (2013) The many faces of plectin and plectinopathies: pathology and mechanisms. *Acta Neuropathol* 125:77–93

Zhang H, Landmann F, Zahreddine H *et al.* (2011) A tension-induced mechanotransduction pathway promotes epithelial morphogenesis. *Nature* 471:99–103