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Regulators of integrin α6β4 function

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Lisa te Molder

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Regulators of integrin α6β4 function

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Table of Content

Chapter 1	General introduction and thesis overview	7
Chapter 2	The opposing roles of laminin-binding integrins in cancer	13
Chapter 3	PKD2 and RSK1 regulate integrin β4 phosphorylation at threonine 1736	41
Chapter 4	EGFR-dependent tyrosine phosphorylation of integrin β4 is not required for downstream signaling events in cancer cells	65
Chapter 5	Tetraspanin CD151 and integrin α3β1 contribute to the stabilization of integrin α6β4-containing cell-matrix adhesions	89
Chapter 6	Comparative interactomics analysis reveals potential regulators of α6β4 distribution in keratinocytes	115
Chapter 7	Summary and General Discussion	133
Addendum		147

References
Nederlandse samenvatting voor leken
Curriculum Vitae
List of Publications
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CHAPTER 1

General introduction and thesis overview

Cell adhesion and signaling by integrin α6β4

Epithelia are continuous sheets of epithelial cells covering the surfaces and cavities of the body. Epithelial cells can be organized in a single layer of cells as in the intestine but also in multiple layers of cells as in the skin (Fig. 1). They form a barrier between the internal and external environment to prevent excessive water loss and to provide protection against bacteria, viruses and other harmful agents from entering the body. To perform their barrier function, the epithelial cells must adhere to and communicate with one another and with the extracellular matrix (ECM) proteins in the basement membrane (BM). Different types of cell-cell and cell-matrix junctions are formed in epithelial cells, such as focal adhesions, adherens junctions and (hemi)desmosomes, that mediate adhesion of these cells to their environment

Hemidesmosomes (HDs; Fig. 1) are a unique and essential type of cell-matrix junction that mediates firm adhesion of basal epithelial cells to the underlying BM. Two types of HDs can be distinguished: Type I HDs and Type II HDs. Type I HDs are found in stratified (multilayered) epithelia such as the skin and consist of the transmembrane proteins integrin α6β4, BP180 and tetraspanin CD151, and the cytoplasmic plakin-family members plectin and BP230 [1–3] (Fig. 1). Type II HDs are present in simple epithelia such as the intestine and consist only of the integrin α6β4, CD151 and plectin [4].

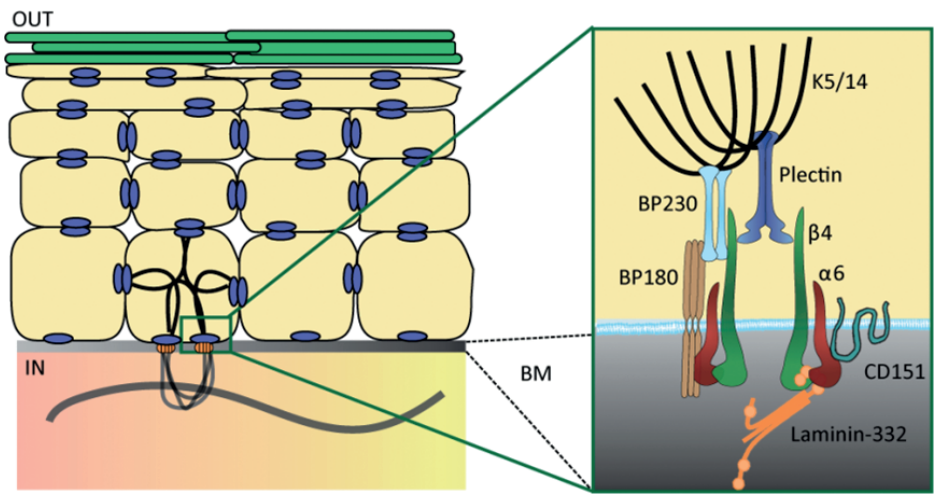


Figure 1 Hemidesmosomes mediate cell-matrix adhesion in skin *(Made after [5]).*

Integrin α6β4 is the core protein of HDs and a unique member of the integrin family due to the unusually long cytoplasmic tail of the integrin β4 subunit (1088 amino acids) and the many proteins that can interact with its cytoplasmic domain. The cytoplasmic tail of β4 harbors a membrane-proximal Na^{+}-Ca^{2+} (CalX) exchange motif, followed by two pairs of fibronectin type III (FnIII) domains separated by a connecting segment (CS) and ends with the C-terminal tail (Fig.2). Plectin binding to the cytoplasmic tail of

the β4 subunit is essential for proper formation of HDs. Plectin contains an N-terminal actin binding domain (ABD), followed by a plakin domain, a central rod domain and six C-terminal plakin-repeat domains [1,6]. Plectin binds to the first pair of FnIII domains and part of the connecting segment (CS; residues 1328-1355) of β4 via its ABD, while its plakin domain binds to the C-terminal half of the CS and C-terminal tail of β4 [7–10] (Fig. 2).

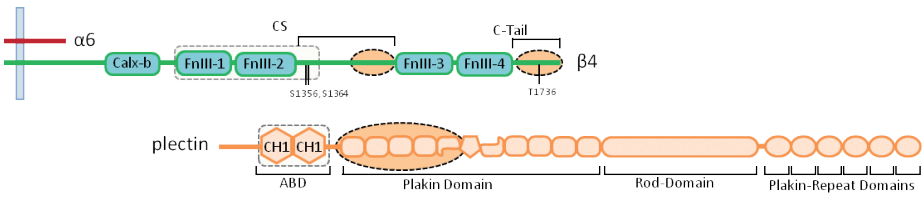


Figure 2 Domain structures and interaction sites of integrin α6β4 and plectin

In addition to β4-plectin interaction, multiple other protein-protein interactions contribute to HD formation: BP230 and BP180 interact with β4 [11–14], BP180 and CD151 interact with α6 [3,15], BP180 and BP230 interact with each other [16] and BP180 and plectin interact with each other [11]. Furthermore, both plectin and BP230 connect the HDs to keratin intermediate filaments [11,17,18], while integrin α6β4 and BP180 mediate interactions of HDs with laminin-332 in the BM [1,19,20] (Fig.1). Laminin-332 is a major component of the BM and interacts directly with collagen type VII, which is the major protein of anchoring fibrils. All these interactions together ensure that the basal layer of epithelial cells is firmly anchored to the dermis.

Mutations in the genes encoding HD components and associated proteins lead to Epidermolysis Bullosa (EB), a disease which involves severe blistering of the skin and mucous membranes [21]. Children affected with EB are better known as butterfly children. The severity of the disease ranges from mild to fatal and is associated with complications such as itchy skin, chronic blisters, gastrointestinal and upper airway complications, blindness, excessive scaring, body deformations and squamous cell carcinoma. The severity of the disease is dependent on the affected gene and on how the mutation affects the associated protein. There are at least 3 subtypes of EB and classification is based upon the location of the cleavage plane in the skin: For example mutations in genes encoding Keratin 5 or 14 results in EB simplex (EBS) in which blistering occurs within the epidermis, while those in the genes encoding in any of the three subunits of laminin-332 causes junctional EB (JEB) in which blistering occurs at the epidermal-dermal junction. Mutations in the gene encoding collagen VII results in dystrophic EB (DEB) in which blistering occurs in the dermis [22]. The loss of integrin α6β4 results in a severe form of EB which is associated with loss of HDs and is usually fatal within the first weeks of life [23–25].

In addition to contributing to cell-matrix adhesion, integrin α6β4 influences intracellular signaling. Various intertwined intracellular signaling pathways determine cell fate by promoting or inhibiting proliferation, migration, or cell death. The contribution of integrin α6β4 to intracellular signaling is thought to occur in concert with receptor tyrosine kinases (RTKs) outside the confines of HDs. The signaling function of α6β4 in relation to tumor development and progression is discussed in detail in chapter 2. Although much knowledge has already been gained concerning the adhesion function of this integrin, the precise molecular mechanisms underlying its signaling function and regulation remain ill defined.

A search to understand regulatory mechanisms of integrin α6β4

This thesis describes our search to identify and understand possible regulatory mechanisms of integrin α6β4 in cell-matrix adhesion and intracellular signaling.

In **chapter 2** the function of integrin α6β4 as a bidirectional signaling receptor is discussed in the context of tumorigenesis. This literature review discusses the often opposing roles of integrin α6β4 and other laminin-binding integrins in tumorigenesis in various types of cancers and a variety of circumstances. We describe the multitude of factors such as post-translational modifications, complex formation with tetraspanins, and RTKs which affect the function of laminin-binding integrins in cancer. We discuss how integrin α6β4 functions as a tumor promoter and indicate two aspects that are essential for the ability of α6β4 to promote tumorigenesis: On the one hand the disassembly of HDs which releases α6β4 from its predominantly adhesive function and on the other hand the ability of α6β4 to mediate signaling downstream of RTKs, which has been suggested to amplify protumorigenic signaling.

Disassembly of HDs occurs through inhibition of the binding of α6β4 to other complex members such as plectin. It has been shown that phosphorylation of the β4 subunit at different serine and threonine residues (S1356, S1364, T1736; Fig. 2) abolishes α6β4 binding to plectin. This phosphorylation occurs upon activation of the epidermal growth factor receptor (EGFR), which is frequently activated in skin cancer. In **Chapter 3** we investigated which kinases and phosphatases regulate phosphorylation of integrin β4 at threonine 1736 and contribute to the regulation of HD disassembly. We identified RSK1 as the kinase that phosphorylates β4 at T1736 downstream of EGFR activation in normal keratinocytes. Additionally we showed that under certain circumstances (PMA stimulation) PKD2 is also able to phosphorylate T1736.

The β4 cytoplasmic domain contains several tyrosine residues, which are phosphorylated in response to both RTK and α6β4 engagement. These tyrosine phosphorylation sites may serve as docking sites for adaptor proteins containing SH2 and/or PTB domains, and contribute to RTK signaling. However, the function of

β4 tyrosine phosphorylation in promoting tumorigenic signaling is subject of much debate. In **Chapter 4** we investigated the role of β4 and β4 tyrosine phosphorylation in signaling downstream of the EGFR using multiple (cancer) cell lines and integrin β4 mutants. From this study we conclude that β4 tyrosine phosphorylation does not enhance EGFR mediated ERK, Akt and PLCγ signaling, which means that the biological relevance of the phosphorylation of β4 on tyrosine residues is still unknown.

In addition to posttranslational modifications such as phosphorylation, the function of α6β4 might be affected by its association with the tetraspanin CD151. In **Chapter 5** we investigated the contribution of CD151 in keratinocyte adhesion and HD formation. We found that CD151 is not essential for HD formation and does not significantly contribute to the adhesion strength mediated by α6β4. However, through binding to integrin α3β1, CD151 plays a critical role in the stabilization of a novel integrin α6β4 containing adhesion structure located in the center of cells. In this chapter we characterized its composition and gained insights into the formation dynamics of the various HD-like adhesions.

Besides these known modifications and interactors of integrin α6β4, it is possible that other and still undiscovered interactors of integrin α6β4 regulate its functions as well. In **Chapter 6** we used a proteomics approach to identify the interactome of α6β4 in keratinocytes. We identified an interactome of 135 proteins amongst which several FA proteins and the complete CMSC. By unraveling the interactome of integrin α6β4, this study lays the foundations for a whole new era of investigating possible regulators of integrin α6β4 function. Additionally, the identification of the CD151-specific interactome of α6β4 confirmed the findings from Chapter 5. In **Chapter 7** the findings presented in this thesis are summarized and discussed.



CHAPTER 2

The opposing roles of laminin-binding integrins in cancer

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Abstract

Integrins play an important role in cell adhesion by linking the cytoskeleton of cells to components in the extracellular matrix. In this capacity, integrins cooperate with different cell surface receptors, including growth factor receptors and G-protein coupled receptors, to regulate intracellular signaling pathways that control cell polarization, spreading, migration, survival, and gene expression. A distinct subfamily of molecules in the integrin family of adhesion receptors is formed by receptors that mediate cell adhesion to laminins, major components of the basement membrane that lie under clusters of cells or surround them, separating them from other cells and/or adjacent connective tissue. During the past decades, many studies have provided evidence for a role of laminin-binding integrins in tumorigenesis, and both tumor-promoting and suppressive activities have been identified. In this review we discuss the dual role of the laminin-binding integrins $\alpha 3\beta 1$ and $\alpha 6\beta 4$ in tumor development and progression, and examine the factors and mechanisms involved in these opposing effects.

Laminin-binding integrins, what they are and what they do

Laminins are large heterotrimeric extracellular matrix (ECM) glycoproteins that contain an α, a β, and a γ chain. They are major components of the basement membrane (BM) that separates the nervous system, epithelial, endothelial, fat and muscle cells from adjacent connective tissue [26]. The BM, however, is not just a physical barrier; it also contributes to the adhesion, proliferation, migration and survival of cells. Integrins are heterodimeric transmembrane glycoproteins that function as adhesion receptors for ligands in the extracellular matrix (ECM) and transduce mechanical signals from the ECM into biochemical signals within the cell. Four integrins recognize laminins as their extracellular ligands: α3β1, α6β1, α7β1 and α6β4 (reviewed in [27]). Their specificity and affinity for binding to various laminin isoforms differ considerably [28–30] (Table 1). Alternative mRNAs splicing of the α3, α6 and α7 subunits further increases the functional diversity of these laminin-binding integrins by generating evolutionary conserved isoforms with different affinities for ligand binding and signaling activities [31]. The α6 and α7 subunits have distinct isoforms that differ in both their extracellular (X1 and X2) and cytoplasmic domains (A and B), while the α3 subunit only exists as two distinct cytoplasmic variants [32–39]. The expression of these isoforms is tissue specific and developmentally regulated [40–43], however a full understanding of their role is still lacking.

Integrin α6β4 is expressed at the base of most epithelial cells, but also by a subset of endothelial cells [44] and by perineural fibroblasts and Schwann cells in peripheral nerves [45,46]. It mediates cell adhesion to laminins and plays a crucial role in the formation of specific cell-matrix complexes, i.e. hemidesmosomes (HDs) [13]. Hemidesmosomal dysfunction is associated with a group of inherited disorders called epidermolysis bullosa, symptoms of which are severe blistering of the skin and mucosal membranes [25]. Mice lacking either the integrin α6 or β4 subunit display very similar defects in skin and mucosal membranes, and die perinatally [47–49].

Despite sharing the common β1 subunit, the integrins α3β1, α7β1 and α6β1 have unique functions and distinct distribution patterns. Integrin α3β1 is most abundant in skin, kidneys, lungs, intestine, bladder and stomach. In these tissues, it mediates adhesion of epithelial cells to laminin-332 and -511 in the BM, and plays a role in the maintenance of cell-cell contacts. Recently, mutations in the gene encoding the α3 subunit (ITGA3) have been identified in patients suffering from a congenital nephrotic syndrome, interstitial lung disease and a mild form of epidermolysis bullosa [50–52]. Similar symptoms have been previously described in genetically engineered mice lacking α3β1 [53]. Notably, the skin defects observed in the absence of α3β1 occur early in life and are associated with micro-blisters and a disorganized BM. Later in life, these defects are no longer observed [54,55].

Laminin isoform specificity		Gene	Mouse Phenotype		Human Disease
-332 -511/521	α6β4	ITGB4	LETHAL, Perinatal	Severe skin blistering	Epidermolysis bullosa
		ITGA6	LETHAL Birth	Severe skin blistering, defects in cerebral cortex and retina	Epidermolysis bullosa
-111, -332, -511/521 -211/221, -411	α6β1				
		ITGB1	LETHAL E 5.5	Inner cell mass deterioration	Lethal
-511/521, -332 -211/221	α3β1	ITGA3	LETHAL Birth	Defects in kidneys, lungs, skin, and cerebral cortex. Disorganization of the BM	Congenital nephrotic syndrome, interstitial lung disease, and epidermolysis bullosa
-211/221 -111, -511/521	α7β1	ITGA7	VIABLE Fertile	Muscular dystrophy	Congenital myopathy

Table 1 *The ligand-binding specificity of the laminin-binding integrins (bold printed – laminin isoforms reported to bind with the highest affinity) and reported phenotypes of mice and human diseases linked to non-functional integrins*

Integrin α7β1 is most prominently expressed in cardiac and skeletal muscles, where it connects muscle fibers to laminin-211/221 in the BM of the myotendinous junction. In line with its function, patients with a loss-of-function mutation in the gene encoding the α7 subunit (ITGA7) suffer from congenital myopathy [56], and mice lacking α7β1 develop muscular dystrophy [57].

Finally, integrin α6β1 is predominantly expressed on platelets, leukocytes, gametes and some epithelia. It binds to a wide range of laminin isoforms, with the highest affinity to laminin-111, -511 and -332 [30]. Apart from a defect in laminar organization of the developing cerebral cortex and retina, seen in the α6-deficient mice (but not in β4-deficient mice), no other defects are associated with the absence of this integrin α subunit in mice [49,58]. The β1 subunit is ubiquitously expressed and can bind to as many as 12 different α subunits (reviewed in [2]). Therefore, it is not surprising that its depletion causes a failure of embryonic development [59].

Laminin-binding integrins can be found in two different adhesion complexes, focal adhesions (FAs) and HDs. FAs are dynamic protein complexes that form mechanical links between the ECM and the actomyosin cytoskeleton [60]. The dynamic regulation of FAs and the reorganization of the associated actin cytoskeleton are important determinants for cell migration. HDs are more stable adhesion structures that act as anchoring sites for intermediate filaments (reviewed in [1,2,61,62]). These

adhesions need to be disassembled during migration and several mechanisms have been suggested to contribute to the disassembly of HDs, including endocytosis of HD proteins [63,64], laminin chain processing [65], cleavage of the β4 subunit by calpain or caspases [66,67], and phosphorylation of HD proteins [6,68–72]. Upon dissociation of HDs, α6β4 has been reported to be redistributed to actin-rich filopodia and lamellae [73,74], where it plays a role in the regulation of cell migration. However, the mechanism responsible is poorly understood.

In addition to their role in maintaining structural integrity of tissues, the laminin-binding β1 integrins also function as bidirectional signaling molecules. ''Inside-out'' signaling regulates the binding affinity and/or avidity of the integrin to its ECM ligand, while ''outside-in'' signaling is triggered upon adhesion and results in the transduction of signals into the cell (reviewed in [75–78]). As integrins lack intrinsic enzymatic activity, they signal through direct or indirect interactions of their cytoplasmic domains with numerous intracellular effector molecules (reviewed in [79–81]). Classical integrin outside-in signaling triggers autophosphorylation of focal adhesion kinase (FAK) [82]. Consequently, the FAK/Src complex is activated, resulting in the stimulation of multiple downstream signaling pathways, leading to the activation of effectors such as mitogen-activated protein kinases (MAPKs) ERK1/2 and JNK, as well as the Rho-family of small GTPases Cdc42 and Rac1. Through these effector molecules, the laminin-binding β1 integrins regulate cell polarization, spreading, migration, survival, and gene expression of cells [79]. Interestingly, compared to the β1 integrins that bind to fibronectin, the laminin-binding integrins support strong activation of Rac1 and Cdc42, and a minimal activation of RhoA. It has recently been pointed out by Stipp in his expert review [83] that this particular signaling results in the formation of smaller focal contacts on a laminin matrix as well as in dynamic actin cytoskeleton remodeling and rapid cell migration. Although α6β4 is reported to be involved in the activation of many of the kinases mentioned above, α6β4 and β1-integrins use different signaling mechanisms and the current understanding is that α6β4 needs to be dissociated from HDs to fulfill its role in signaling [84]. Whether α6β4 needs to adhere to laminin-332 in order to signal is unclear, since both adhesion-dependent and adhesion-independent signaling have been reported [85–88]. Data suggests that in transformed cells the β4 cytoplasmic domain is phosphorylated on specific tyrosine residues that serve as a docking platform for various signaling molecules to amplify the signaling output of growth factor receptors [89–91].

Integrin-mediated signaling can be additionally enhanced or modulated through interaction of integrins with integrin-associated proteins (IAPs), e.g. tetraspanins [92], urokinase-type plasminogen activator receptor (uPAR) [93] and several growth factor receptors [94]. Therefore, extensive crosstalk takes place between pathways activated by integrins and other receptors, especially receptor tyrosine kinases (RTKs).

Laminin-binding integrins and cancer

Over the last decades evidence for a role of laminins in cancer has accumulated and been addressed in several excellent reviews [27,95,96]. Laminin-332, -511 and -111 are reported to be particularly important in carcinogenesis and the motility of tumor cells [27]. Accordingly, α3β1, α6β1 and α6β4 have all been implicated in the development and progression of cancer, but there is little evidence for such role of α7β1 in tumorigenesis and cancer progression [97–100]. In the limited amount of available literature its tumor suppressing function in a variety of tumor cell types, as well as in a reduction of metastatic potential in melanoma cells, is described (reviewed in [83]). Recently it was reported that α7β1 is upregulated in biopsies of hepatocellular carcinoma, while in vitro its downregulation caused decreased invasion and migration, indicating that α7β1 can also act as a tumor promoter [101]. Such dual role in cancer is not restricted to α7β1; α6β4 and especially α3β1 can have both, disease suppressive and promoting roles. This does not seem to be the case for α6β1, which has been predominantly characterized as a tumor promoter, contributing to the spreading and invasion of tumors as well as mediating dissemination and the formation of metastases in areas rich in laminin, such as bone matrix and the space surrounding the nerve of the prostate gland. The role of α6β1 in cancer progression has been recently reviewed by others [83,102].

In this review we will focus on laminin-binding integrins with both an inhibitory and a promoting role in cancer, i.e. α3β1 and α6β4, and try to elucidate the factors and mechanisms involved in these opposing effects. Cancer is a disease with several different stages of development, which can be correlated to specific processes that are essential for its progress and development, i.e. hallmarks of cancer [103]. During primary tumorigenesis cancer cells exhibit sustained proliferation and avoidance of apoptosis. With growing tumor mass, a switch of metabolism and angiogenesis become important for the further development of the disease. During later stages of tumor progression, cancer cells acquire invasive properties in order to spread to distant tissues and form metastases. Integrins represent an important link between tumors and their environment as well as between different tumor cells within the tumor mass. Their role in each stage of tumorigenesis therefore will depend on external influences, for example, the molecular composition of the microenvironment and juxtacrine signaling from neighboring cells. Also oncogenic insults can have an effect on the function of integrins in different stages of cancer progression.

Integrin α3β1 and cancer

The opposing role of laminin-binding integrins in cancer is especially evident when considering α3β1-mediated tumorigenesis events (Table 2). From a number of studies it is clear that different stages of cancer can be influenced by the presence or absence

of α3β1 and, conversely, that transformed cells can modulate the function of α3β1 by regulating its expression or post-translational modifications, through IAPs and via the induction of α3β1-mediated signaling. Furthermore, the expression of α3β1 in transformed cells can be influenced by oncogenic stimuli, such as the activation of K-Ras, and, vice versa, α3β1 can regulate the expression of a large number of genes in immortalized keratinocytes [104,105]. This suggests that transformed cells can be dependent on α3β1 for sustaining signaling pathways and cellular processes.

Tissue	Upregulated/ Cancer Promotor		Downregulated/Cancer Supressor	
	Primary Tumors	Metastasis/ Invasion	Primary Tumors	Metastasis/ Invasion
Skin	[106–108]	[106]	[109]	[108]
Brain	[110,111]	[112,113]		[114]
Oral cavity	[115]	[116–120]	[121]	[122]
Head and Neck	[123]	[124]		
Lung	[125,126]	[127,128]	[109,129]	[130]
Breast	[131–137]	[136,138]	[137]	[137,139–141]
Reproductive system	[142]			[143–149]
Stomach and Intestine	[150]	[151–153]		[154,155]
Pancreas	[156]			
Liver	[157]	[157]		
Bladder and Kidneys	[158,159]			[160]
Bone			[161]	

Table 2 *Summary of the studies on the role of α3β1 in cancer, including either biopsies of human diseased tissue or in vivo mammalian models. A role of α3β1 in both promoting and suppressing tumorigenesis and metastasis has been described.*

Role of α3β1 in supporting sustained proliferation and avoidance of apoptosis

One of the most fundamental traits of cancer is the ability of tumor cells to maintain sustained proliferation. Integrins regulate cell proliferation through adhesion to the ECM [162]. Although the adhesion-dependent control of cell proliferation is generally downregulated in tumors, several studies have shown that proliferation of transformed cells can still be affected by integrin-mediated adhesion. Two recent studies, investigating the role of α3β1 in tumorigenesis of the epithelium of the skin and mammary gland, showed that α3β1 is essential for the initiation of tumors and efficient proliferation of tumor cells [108,135]. The impaired proliferation in mammary epithelia was associated with the downregulation of activated FAK, resulting in a reduction of active Rac1 and its effector serine/threonine-protein kinase PAK1, and therefore in reduced activation of ERK1/2 and JNK [135]. This is consistent with the results of an earlier study, showing that the engagement of laminin-332 by α3β1 is essential for growth factor-stimulated cell proliferation, mediated through activation of the MAPK signaling pathway [163]. Both studies mentioned above observed that

transformed cells deposit laminin into the matrix; therefore, their proliferation may still be dependent on signals, derived from integrin-mediated cell adhesion. In line with this, both α3β1 and laminin are required for an efficient proliferation of various types of tumors and the upregulation of laminin-511 together with α3β1 was shown to be a marker of poor prognosis in breast cancer [136,164,165].

It is therefore evident that α3β1 can promote proliferation of tumors that are adherent to the pre-existing or newly deposited laminin matrix. However, in later stages of carcinogenesis, when tumors rely less on adherent-mediated proliferation, loss of α3β1 may destabilize E-cadherin-mediated cell-cell adhesion, resulting in epithelial to mesenchymal transition (EMT)-like events and consequently increased tumor progression and metastatic growth at distant sites [147,166]. Loss of α3β1 can also contribute to metastatic growth through interactions of tumors with the metastatic environment. It was shown that in vitro the adhesion and proliferation of α3-deficient prostate carcinoma cells on laminin-332 was impaired, but the growth of the tumor was increased when injected into mice. Increased growth of α3-depleted tumor cells was also observed in vitro when these cells were co- cultured with stromal cells or grown in fibroblast-conditioned medium [148]. These observations further suggest that the role of α3β1 in modulating cancer cell proliferation is dual, depending on the stage of tumor progression (Fig. 1).

Integrins also contribute to tumorigenesis by regulating cell survival; ligated integrins can prevent pro- apoptotic signaling cascades initiated by anoikis (cell death by loss of adhesion) and relay survival signals. Studies of transformed cells depleted of α3β1 showed increased activation of caspase-3/7, reduced cell survival and increased radiosensitivity [135,167,168]. In all cases, α3β1 supported survival through adhesion to laminin and initiation of the FAK/ERK signaling pathway, indicating the importance of this mechanism.

Role of α3β1 in tumor-associated angiogenesis

The potential role of α3β1 in regulating angiogenesis has received relatively little attention and is not yet fully understood. In Itga3 knockout mice the capillary loops in the kidneys are dilated and their number is reduced [53]. Furthermore, conditional deletion of Itga3 in the epidermis of mice caused impaired cutaneous wound healing, due to a defect in angiogenesis and failure of α3-negative keratinocytes to promote the expression of the pro-angiogenic factor MRP3 (mitogen-regulated protein 3) [169]. In cancer-induced angiogenesis, α3β1 seems to act as both a promoter and suppressor of angiogenesis, and it influences vascular formation when expressed by tumor or endothelial cells (Fig. 1). Studies investigating the effect of α3β1 on endothelial cells mostly reported its suppressive function in angiogenesis, due to the inhibition of cyclooxygenase-2 (COX-2)-dependent angiogenic signaling, the regulation of vascular endothelial growth factor (VEGF), or the inhibition of endothelial cell proliferation,

migration, and tubule formation [109,129,170,171]. In contrast, when expressed on tumor cells, α3β1 is predominantly associated with the promotion of angiogenesis. The expression of COX-2 and α3β1 is positively correlated in invasive ductal carcinoma, resulting in higher blood vessel density [134]. In MDA-MB-231 breast cancer cells, α3β1 controls the expression of COX-2 and influences endothelial cell function and invasion of tumor cells [131]. A possible explanation for the role of α3β1 in COX-2-mediated angiogenesis and stimulation of the tumor's microenvironment was recently provided by Subbram et al. [172], who showed that α3β1 can directly influence COX-2 expression by stabilizing its mRNA. Furthermore, there is data suggesting that the association of α3β1 with non-conventional ligands, such as the noncollagenous domain of the α3 chain of type IV collagen [α3(IV)NC1] [129], a tissue inhibitor of metalloproteinases (TIMP-2) [170,171,173] and thrombospondin-1 (TSP-1) [174,175], has an impact on tumor-associated angiogenesis.

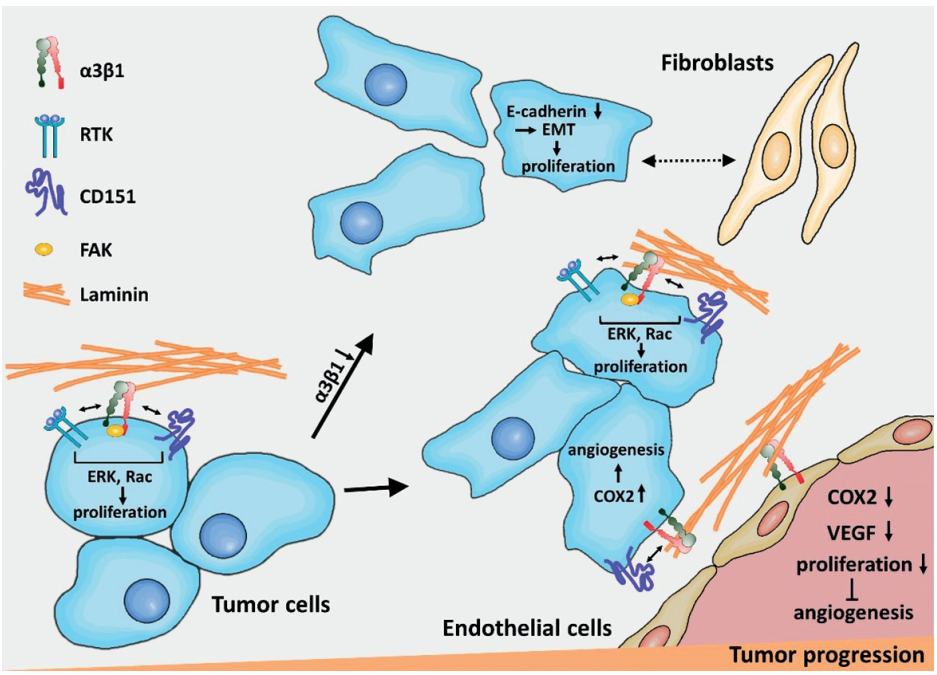


Figure 1 The role of α3β1 in tumor cell proliferation and angiogenesis. *Ligation of α3β1 by laminins can result in the initiation of survival and growth signals via activation of FAK signaling, leading to the activation of the MAPK signaling pathway, which can additionally be supported through crosstalk with GFRs or upon association of α3β1 with the tetraspanin CD151. In later stages of tumor progression, when tumor cells rely less on adherent-mediated survival signaling, loss of α3β1 can destabilize adherent junctions, promote EMT and proliferation. Loss of α3β1 can promote metastatic growth also through interaction of tumor cells with other cells (e.g. fibroblasts) in a metastatic environment. When expressed on tumor cells, α3β1 can promote angiogenesis through stabilization of COX2 and via its lateral association with CD151. However, when present on endothelial cells, α3β1 downregulates proliferation and tubule formation and inhibits angiogenesis via inhibition of COX2-mediated signaling and regulation of VEGF signaling. GFR, growth factor receptor; EMT, epithelial to mesenchymal transition.*

Integrin α3β1-dependent regulation of angiogenesis can be also mediated via its lateral association with tetraspanin CD151, an established IAP (see below). An increased expression of CD151 is correlated with increased vascularity in breast cancer, and in vitro experiments in three-dimensional (3D) extracellular matrices showed that CD151 modulates the response of endothelial cells to cancer cells through its association with both α3β1 and α6β4 [176].

Role of α3β1 in invasion and metastasis

The literature describing the role of α3β1 in later stages of tumor progression pays almost equal amount of attention to its cancer promoting and suppressing functions. While in numerous clinical studies a positive correlation between α3β1 expression and tumor invasiveness or poor prognosis has been observed, opposite findings have also been reported (Table 2). A similar trend was observed in in vitro studies investigating the invasive and migratory phenotype of cells from transformed cell lines. While an increased expression of α3β1 in a head and neck carcinoma cell line is correlated with a more invasive phenotype [124], a low expression of α3β1 has been associated with reduced migration and invasiveness of many different types of tumor cells [177–179]. On the contrary, loss or inhibition of α3β1 function can result in enhanced migration and invasion of tumor cells [108,143,154,180].

Different transcription factors have been reported that can either positively or negatively regulate the expression of α3β1 in tumor cells in order to acquire a more aggressive phenotype [161,181]. This suggests that the presence of α3β1 can have either beneficial or unfavorable effect on tumor cell invasion, and that it is important enough for tumor cells to develop mechanisms for regulating its expression. A remaining question is: what determines whether tumor cells will require either the presence or absence of α3β1 for successful invasion and the formation of metastases?

Laminin-dependent ligation of α3β1

Ligation of α3β1 by laminin-511 or -332 is one of the major events through which this integrin mediates cell adhesion and migration. This makes laminins of key importance for α3β1-mediated effects on cancer progression and invasion. Even more so since, as already discussed, laminins are implicated in the progression and spreading of cancer. In an early study it was shown that α3β1 is essential for the migration of keratinocytes on laminin-332 during wound healing [182]. A decade later Choma et al. [183] demonstrated that persistent keratinocyte migration is driven via the interaction of α3β1 with laminin-332, which induces FAK/Src kinase activity, thereby promoting Rac1 activation and polarized lamellipodium extension.

There is a strong positive correlation between the degree of invasiveness of glioma cells and α3β1-mediated migration. As it has been pointed out in a recent review, the attachment of the glioma cells to the ECM must be transient for them to be able

to invade [184]. Furthermore, ECM rich in laminin-332 and -511 contributes strongly to the migration of highly invasive gliomas [112,185,186], while downregulation of α3β1 in glioma cells led to decreased migration and invasiveness [93], which was correlated with decreased phosphorylation of ERK1/2 [113]. In invasive protrusions of glioblastomas, α3β1 was found to be co-localized with the Ephrin A2 (EphA2), a known promoter of cancer invasiveness, making it plausible that the cross-talk between EphA2 and α3β1 additionally contributes to the adhesion-dependent signaling that leads to a more invasive phenotype [187,188]. Integrin α3β1-mediated cell adhesion in areas of the brain that are rich in laminin not only drives the invasion of glioma cells, but also plays a role in the formation of brain metastases of non-small cell lung carcinoma [127].

A pattern is now emerging of how laminin-332 and -511 promote the spreading of cancer cells via α3β1. Firstly, they facilitate α3β1-mediated tumor cell migration and invasion from the primary tumor site, which, in addition to the cases mentioned above, was observed in numerous other types of cancers [98,189–195]. Secondly, ligation of α3β1 by laminin-332 can increase the secretion of matrix metalloproteinase-9 (MMP-9), which then further promotes the invasion and migration through the dense ECM [138,192,196]. Thirdly, laminins deposited by endothelial cells, can mediate α3β1-driven migration of tumor cells and stimulate trans-endothelial tumor cell invasion, thereby promoting tumor cell dissemination through the vasculature [197,198]. Fourthly, α3β1 expressed by endothelial cells may strengthen the adhesion of circulating tumor cells to the endothelium by stabilizing the binding of endothelium-expressed galectin-3 and cancer-associated carbohydrate Thomsen-Friedenreich antigen (TF-Ag) [199]. Lastly, α3β1 can mediate the initiation of new metastases in laminin-rich environment. Several studies have established a role of α3β1 in haptotatic migration and invasion toward laminin-511, suggesting that α3β1 plays an active role in the colonization of laminin-rich tissues by tumor cells [138,200–202]. This was confirmed in in vivo mouse studies, observing that α3β1 drives the formation of metastasis to the lung, lymph nodes and peritoneum [128,136,152,196,203].

Ligation of α3β1 by laminin, however, does not always clearly promote the spreading and invasion of cancer cells. For example, in highly invasive and metastatic prostate carcinoma cells the expression of α3β1 was decreased and they failed to spread when grown in vitro [143]. Furthermore, a recent study of patient samples of squamous cell carcinomas of the lower lip showed the absence of α3β1 at the invasive front, where the expression of laminin-332 was often detected [121]. One possible explanation for the anti-invasive-effect of ligated α3β1 is that the integrin suppresses the formation of invadopodia, actin-linked structures with putative adhesion properties, which are frequently observed to mediate BM degradation in epithelial tumors. It was proposed that a balance of focal contacts and invadopodia is necessary for cells to migrate and invade the BM [204]. In fact, Liu et al. [205] recently showed that depletion of

either laminin-332 or α3β1 resulted in an increased number of invadopodia in bladder carcinoma cells. They proposed a mechanism, by which laminin-332-α3β1 interaction acts as a potent upstream inhibitor of cell invasion via mediating focal contacts that in turn limit the availability of active Src, necessary for inducing the formation of invadopodia.

Association of α3β1 with tetraspanins

The ability of α3β1 to interact with several IAPs offers further explanation for its dual role in cancer invasion and its progression in general. Tetraspanins, multispanning membrane proteins that cluster into tetraspanin-enriched microdomains (TEMs) on the plasma membrane, are one of the most prominent proteins that can interact with laminin-binding integrins, thereby influencing their localization and function [83,206]. Several tetraspanins, including CD9, CD81 and CD63 have been suggested to associate with α3β1 and to influence the migration and invasiveness of tumor cells. With a few exceptions, these complexes are mainly associated with reduced migration and low metastatic potential, and thus a better prognosis [117,145,154,207–209]. Recently, the tetraspanin CO-029 was found to form a complex with α3β1 and rictor in malignant gliomas, and thus to mediate migration of glioma cells via mammalian target of rapamycin (mTOR) complex 2 (mTORC2), of which rictor is a key component [210]. However, a clear understanding of how these tetraspanins associate with α3β1 and regulate α3β1-mediated migration and invasiveness is still lacking.

A direct and stable association has only been shown for CD151 and α3β1 [211,212]. The interaction of CD151 and α3β1 influences the distribution of α3β1 and shifts it from FAs into TEMs [213]. Furthermore, it strengthens α3β1-mediated cell adhesion and promotes the proliferation and migration of different types of tumors cells on laminin-332 [214–216]. Two major mechanisms may account for the CD151-dependent regulation of tumor cell behavior by α3β1. Firstly, CD151, which contains a YXXϕ endocytosis motif in its C-terminal cytoplasmic domain, may stimulate cell migration by facilitating α3β1 recycling [217]. Secondly, CD151 may contribute to pro-migratory signaling of α3β1 by suppressing RhoA activity and formation of stress fibers [83,183,218,219]. Additionally, the signaling properties of α3β1 may be influenced by phosphatidylinositol 4-kinase (PI4K) that is associated with CD151 [212] (Fig. 2).

Recent data suggests that CD151 can also control cell migration independently of its association with α3β1, and that the balance between integrin-free CD151 and CD151-α3β1 complexes is important with regard to tumor invasion [119,149,220]. Scales et al. [219] demonstrated that the ligation of α3β1 by laminin promoted the association between α3β1 and CD151 and that cells lacking α3β1 exhibited increased formation of CD151 homodimers. This suggests that α3β1-mediated cell adhesion to laminin skews the balance from CD151-CD151 homodimers towards CD151-α3β1 complexes. The balance can also be altered by changes in the expression of either α3β1 or CD151,

which is not uncommon in cancer [83]. Alternatively, association of α3β1 with CD151 could be regulated via α3 or β1 glycosylation, as it was shown in highly metastatic melanoma cells [221].

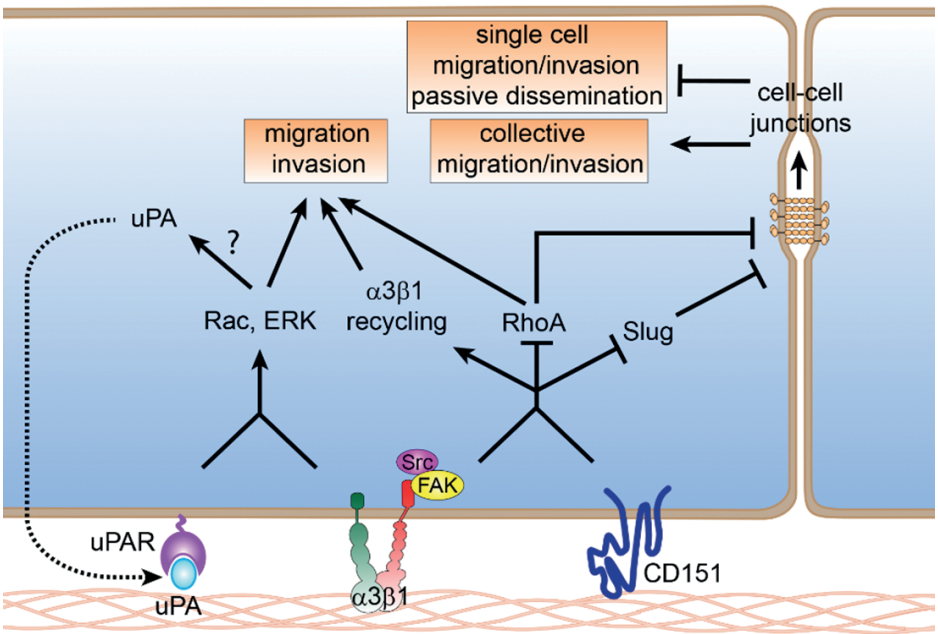


Figure 2 Effects of α3β1 association with CD151 or uPAR on tumor cell migration and invasion. *Integrin α3β1 forms complexes with several IAPs, which can have an impact on the progression and development of tumors. The association of CD151 with α3β1 might be required to prevent the integrin from becoming linked to the actomyosin cytoskeleton and to support high RhoA activity. Suppression of RhoA activity leads to a shift of the Rho/Rac balance in favor of Rac1 and hence to the formation of smaller focal adhesion complexes. Additionally, the association of CD151 promotes tumor cell migration and invasion by facilitating the recycling of α3β1. Furthermore, α3β1-CD151-mediated suppression of RhoA activity and Slug expression may stabilize cell-cell junctions, thereby inhibiting tumor cell migration, invasion and passive shedding and dissemination of tumor cells. However, it may favor tumor spreading via collective cell migration. The association of α3β1 with CD151 may also skew the balance of CD151 homodimers in cells, which can also have an impact on their migratory and invasive properties. The α3β1-uPAR complex promotes migration and invasion of tumor cells via activating the Src/ERK signaling pathway and/or upregulating uPA expression.*

In breast cancer, it was shown that the complex of CD151 and α3β1 mediates malignancy through interaction with ErbB-2 (HER2) [222,223]. In invasive ductal carcinomas, the CD151-α3β1 complex is a marker of poor outcome, and experiments with ErbB-2 overexpressing breast cancer cells indicated that CD151-α3β1 complexes promote dimerization of ErbB-2 by keeping Rho activity low [114]. In contrary to invasive ductal carcinomas, in invasive lobular carcinomas poor patient survival is connected to the lack of correlation between CD151 and α3β1 [137]. The biological differences between the two diseases were related to differences in cell-cell and cell-matrix interactions, the loss of E-cadherin being the most prominent characteristic of

invasive lobular carcinoma [224]. As previously mentioned, α3β1 does not only mediate adhesion and migration on laminin substrates, but also plays a role in regulating the stability of cell-cell contacts. Although a precise mechanism has not yet been defined, it has been suggested that the CD151-α3β1 complex controls the stability of E-cadherin mediated cell-cell adhesion by regulating the expression of the membrane protein tyrosine phosphatase PTPμ and its association with the E-cadherin-catenin complex in embryonic kidney cells [225]. Stipp and colleagues [226,227], however, showed that this mechanism is not operative in A431 epidermoid carcinoma cells. In these cells, CD151 promotes the stability of cell-cell junctions by reducing α3β1-dependent activation of RhoA. In the absence of CD151, Rho activation is increased, which resulted in reduced collective migration in two-dimensional (2D) in vitro assay. However, in 3D matrices, the level of Rho activation, although being disruptive for junctional stability, did not prevent their formation and cells still invaded in a collective manner. Furthermore, there is evidence that the CD151-α3β1 complex plays a role in maintaining the integrity of ovarian carcinomas by repressing Slug-mediated EMT and canonical Wnt signaling [147]. Integrin α3β1-mediated cell-cell cohesion could hinder metastasis also in the context of passive tumor cell shedding to the blood stream. In line with this, tumor cells that have been released into the circulation of mice that contained induced (primary) renal tumors exhibited reduced levels of α3β1 [160]. Thus, the role of the CD151-α3β1 complex in carcinoma progression appears to be context-dependent and to depend on the mode of invasion and the phenotype of the tumor.

Association of α3β1 with uPAR

uPAR, the receptor for urokinase (uPA), is a glycosylphosphatidyl inositol-anchored protein expressed by many cell types. It forms complexes with several integrins, including α3β1, and has been implicated in tumor progression (Fig. 2). In oral squamous cell carcinomas, increased expression of α3β1 and uPAR correlates with a poor prognosis. In vitro and in vivo studies have shown that α3β1 clustering induces the recruitment of uPAR and the formation of α3β1-uPAR complexes that promote invasive cell behavior via Src and ERK1/2 signaling as well as via enhanced uPA expression [115,228]. In an independent study, it was shown that p130Cas is phosphorylated by Src in response to uPAR-α3β1-laminin-332 engagement, and that this led to enhanced cell motility through activation of Cdc42 and actin reorganization [229]. Complexes of uPAR and α3β1 have also been implicated in the fibroblast associated protein α (FAPα)- stimulated migration of ovarian cancer cells via activation of Rac1 [230]. In fact, an early study had already described that this complex can mediate binding to vitronectin [231]. Recently, an interesting novel mechanism was reported by Ferraris et al. [232], who showed that uPAR-mediated cell adhesion to vitronectin triggers integrin signaling independently of integrin-matrix engagement, by increasing the membrane tension. The same group also proposed that, in integrin ligand-independent conditions, the frictional membrane resistance participates in establishing adequate lamellipodial tension, which predominantly depends on coupling of the C-terminal

talin-actin binding site to actomyosin-driven retrograde actin flow force [233]. This mechanism could provide an explanation for the role of uPAR-binding integrins, such as α3β1, in migration in an environment lacking conventional integrin ligands.

Altered glycosylation of α3β1

The α3 and β1 subunits contain 14 and 12 potential N-glycosylation sites, respectively, and it has become increasingly clear that malignant transformation is associated with aberrant glycosylation of α3β1, which can modulate its function, signaling and lateral associations with IAPs [234,235]. In bladder carcinomas, the overexpression of aberrantly glycosylated α3β1 is correlated with poor clinical outcome, and a monoclonal antibody that recognizes the aberrantly glycosylated epitope on α3β1 has potent anti-tumor activity in bladder cancer in vivo. In vitro experiments revealed that aberrant glycosylation of α3β1, conferred by the glycosyltransferase GALNT1, initiates FAK signaling, resulting in c-Jun phosphorylation and increased cell cycle progression, and proliferation through upregulation of cyclin D1 and activation of CDK4 [158]. Aberrant N-glycosylation of α3β1 also influences the motility and invasiveness of cancer cells [236,237]. The presence of high mannose and sialylated tri- or tetra-antennary complex type N-glycans on α3β1 is associated with a reduced adhesion to laminin and an increased invasive behavior of bladder cancer cells [238,239]. Baldwin et al. [240] has shown that α3β1-mediated cell migration can also be influenced by N-glycosylation of α3β1 without a detectable loss of cell adhesion to laminin-332. They found that the changes to N-glycosylation of α3β1, induced by its binding to CD151 during biosynthesis, influenced tumor cell migration toward laminin-332 [240]. The exact mechanism underlying the effect of α3β1 glycosylation on cell migration is unknown, but may involve galectin-3-mediated clustering of α3β1 and subsequent activation of Rac1 signaling [241]. Recently, a study was published proposing a role of N-glycosylation modifications in the efficient translocation of α3β1 to the plasma membrane [242]. Glycosylation of α3β1 was observed to be suppressed by hypoxia, resulting in decreased levels of α3β1 at the plasma membrane, which facilitated the invasion of epidermoid carcinoma cell line A431. There is therefore strong evidence that glycosylation of α3β1 can play a role in promoting cell migration and invasiveness, as well as contribute to increased tumorigenesis.

Role of α3β1 in gene regulation

As already mentioned, in transformed cells the expression of α3β1 is often regulated to modulate its function. One of the examples of such regulation is mediated by transforming growth factor β (TGF-β). In invasive hepatocellular carcinoma cells, TGF-β stimulates the expression of α3β1 by transcriptional upregulation via Ets transcription factors, resulting in a pro-invasive phenotype on laminin-332 [157,181]. Similar observations were reported in bladder cancer and in oral squamous carcinoma cells [243,244]. However, there is also an increasing amount of evidence for the role of α3β1 in gene regulation and for the consequences this brings to the development and

progression of tumors. α3β1-mediated gene regulation in the context of metastasis has been recently reviewed [98], and will therefore only be briefly mentioned here. The most striking examples of the gene regulatory role of α3β1 were observed in immortalized keratinocytes. In these cells α3β1 was shown to induce expression of MMP-9 upon transformation of cells [245], which was mediated via α3β1-dependent stabilization of MMP-9 mRNA transcripts [246]. Furthermore, the production of MMP-9 in immortalized keratinocytes was potentiated by α3β1 in response to TGF-β stimulation [247]. The mechanism behind mRNA stabilization was recently proposed by Missan et al. [248], who observed that a shorter, more stable mRNA was preferentially generated in immortalized keratinocytes expressing α3β1. The presence of this short transcript was dependent on active ERK/MAPK signaling. Integrin α3β1 was also shown to influence the stability of COX-2 mRNA (discussed in chapter on proliferation and angiogenesis) and to regulate the expression of fibulin-2, a matrix-associated protein that binds laminin-332 and serves as a mediator of matrix remodeling and invasion [104]. In line with the latter, a recent study reported that α3β1 mediates the stability of the BM through fibulin-2 induction, indicating the importance of α3β1-mediated gene regulation not only in cancer progression, but also in maintenance of healthy tissue [249].

Integrin α6β4 and cancer

Early studies have identified the integrin α6β4 as a tumor antigen [250,251], whose expression is increased in squamous cell carcinomas, as well as in other types of solid cancers (reviewed in [252]). Some controversy exists regarding the stage of cancer development and progression at which β4 overexpression becomes apparent, but most studies agree that it increases with tumor grade [253,254]. In addition to increased expression of β4, changes in its distribution have also been linked to the grade of tumors [255,256]. In normal epithelial tissues, β4 is concentrated at the basal membrane of basal epithelial cells, while in many tumors, it is diffusely expressed in multiple cell layers [257–259]. Although abnormal and high expression of α6β4 in cancer is generally associated with poor patient outcome and overall survival, in certain settings α6β4 suppresses tumorigenesis. Furthermore, in tumors such as prostate carcinomas and basal cell carcinomas, the expression of α6β4 is downregulated [260–264].

Different roles of α6β4 in tumor development and progression might derive in part from its ability to assemble HDs. Additionally, α6β4 may modulate oncogenic signaling by binding to its ligand laminin-332 in the ECM or through cooperative signaling with RTKs that stimulate or suppress proliferation. Finally, the role of α6β4 depends on the ability of tumor cells to remodel the ECM and on their oncogene mutational profile.

Role of α6β4 in tumor initiation

Although α6β4 cannot induce tumorigenesis on its own [265], it has been implicated in human papilloma virus (HPV)-mediated tumor initiation. HPV plays a role in the initiation of several cancers such as anal, cervical and oropharyngeal cancer. Once the virus enters a cell, its proteins interfere with the normal cell machinery, which results in uncontrolled cell growth and avoidance of cell death. In HPV16 infected cervical cancer cells, α6 expression levels were correlated to the binding of the virus particles to the cells [266]. And in fact, it recently became clear that β4 expression and α6 processing are important for HPV entry into the basal cells [267,268]. However, viral DNA replication occurs primarily in the differentiating suprabasal cells of the epidermis. The HPV E2 protein may trigger this differentiation step by downregulating β4 expression [269,270]. These data strongly suggest that α6β4 has a dual role in different stages of tumor initiation by HPV.

Role of α6β4 in sustained proliferation and avoidance of apoptosis

The unique function of α6β4 in potentiating growth factor receptor signaling is evident from its role in supporting sustained proliferation and avoidance of apoptosis during tumor development and progression. Integrin α6β4 has been implicated in the modulation of signal transduction pathways downstream of several RTKs, including the epidermal growth factor receptor (EGFR) family members EGFR [85] and ErbB-2 [271,272], the macrophage stimulating protein (MSP) receptor (also known as Ron) [273], the hepatocyte growth factor (HGF) receptor (also called c-Met) [91,274] and the insulin-like growth factor-1 receptor [275].

Many studies attribute the synergy between α6β4 and RTK-mediated signaling to the phosphorylation of specific tyrosine residues in the cytoplasmic domain of β4 and subsequent recruitment and activation of signaling intermediates to the phosphorylated subunit (reviewed in [276]). The C-terminal segment of the β4 cytoplasmic domain that harbors the tyrosine residues is also known as the β4 signaling domain. Tyrosine phosphorylation of β4 is typically mediated by the Src family of kinases (SFKs) downstream of RTKs [273,277,278], although direct tyrosine phosphorylation of β4 by the HGF receptor c-Met has also been demonstrated [279]. Additionally, clustering of the α6β4 molecules by itself can lead to tyrosine phosphorylation of the β4 subunit [280]. Recent data suggests that members of the syndecan family of cell-surface proteoglycans may play an important role in the phosphorylation of the β4 cytoplasmic domain by positioning this domain near the plasma membrane to be phosphorylated by the SFK member Fyn downstream of EGFR and ErbB-2. Syndecans can bind directly to the cytoplasmic domain of the β4 subunit [281,282].

The signaling intermediates that are recruited by tyrosine phosphorylated β4 include the adapter proteins Shc [279,283] and IRS-1/2 [90], and the protein-

tyrosine phosphatase Shp2 (also known as PTPN11) [91]. Binding of Shc by tyrosine phosphorylated β4 has been shown in squamous carcinoma cells expressing the EGFR at high levels [279], but also upon EGF treatment of cells that express normal levels of the EGFR [277]. Shc links α6β4 to the MAPK signaling pathway, which is essential for inducing cellular proliferation and transformation [283]. On the other hand, c-Met-mediated tyrosine phosphorylation of β4 has been shown to recruit Shp2, which enhances the activation of Src. Subsequently, Src induces the phosphorylation of the multi-adapter Gab1, which leads to activation of the MAPK and phosphatidylinositol 3-kinase (PI3K) signaling pathways [91,274]. Activation of MAPK signaling can also be mediated by a fraction of α6β4 that is localized in lipid rafts and associated with palmitoylated SFKs [284]. Binding of IRS-1/2 by tyrosine-phosphorylated β4 has also been implicated in the activation of PI3K downstream of α6β4 clustering [90]. Furthermore, there is data suggesting that FAK can be recruited by tyrosine phosphorylated β4 and that the subsequent activation of FAK promotes malignancy by increasing the activity of p38MAPK and Akt [278]. Tyrosine phosphorylation of β4 and subsequent activation of PI3K signaling can also promote the survival of breast cancer cells through enhanced VEGF translation and stimulation of VEGFR-mediated autocrine signaling [285] (reviewed in [286]).

Most studies agree that α6β4 supports PI3K activation by different RTKs, although the details of the mechanisms may differ between cell types. Activation of PI3K by α6β4-mediated cell adhesion was first shown by Shaw et al. [287], and since then, numerous other studies have reported an association between α6β4-mediated adhesion and the requirement of PI3K activation for cell survival. In breast cancer cells, blocking α6β4 function with an antibody against β4 caused a reduction in PI3K signaling, which led to increased apoptosis [288]. The induced apoptosis could be rescued by the expression of constitutively active Akt, the downstream target of PI3K [288,289]. Integrin α6β4-mediated PI3K signaling can also support cell survival through activation of the transcription factors STAT3 and c-Jun, as was shown in an ErbB-2-driven breast cancer mouse model [271].

Intriguingly, it has been reported that α6β4 can also promote cell death. In vitro, treatment of cells with chemical or pharmacological agents induced apoptosis via elevating the levels of β4, while the depletion of β4 promoted survival [290–294]. Furthermore, in an immunocompromised SCID mouse model of human gastric cancer, the expression of β4 at high levels promoted apoptosis [295]. Bachelder et al. [296] suggested that and the ability of α6β4 to either promote or suppress apoptosis depends on the p53 status of the cells. Integrin α6β4 stimulates p53-transactivating function promotes p53-dependent apoptosis in carcinoma cells that express wild-type p53, but not in p53-deficient carcinoma cells, in which it promotes survival in a PI3K/Akt dependent manner. Interestingly, in the same colon carcinoma cells depletion of p53 associates also with enhanced β4 transcription through the p53 family members p63

and p73, thereby further augmenting the survival function of α6β4 [297]. Although the inhibition of α6β4-mediated survival signaling by p53 activation has so far been only conclusively shown in RKO colon carcinoma cells [298], the fact that p53 mutations and overexpression of α6β4 are positively correlated in a number of human malignancies [252] suggests a general mechanism by which the activity of p53 in carcinoma cells is regulated by the signaling function of this integrin. Interestingly, p53 has also been suggested to regulate adhesion of cancer cells via α6β4 [299].

In addition to supporting cell proliferation and survival by providing an additional platform for RTK signaling, α6β4 may be needed to secure attachment during oncogenic transformation when the adhesive function of integrins that are linked to the actin cytoskeleton is compromised, while oncogenic signaling is still dependent on the structural integrity of the actin cytoskeleton. This might be responsible for the requirement of the presence of α6β4 and its ligand laminin-332 in squamous cell carcinomas, induced by oncogenic Ras and IκBα expression [300]. Similarly, the promotion of cell growth by α6β4, which was reported to be anchorage-independent [91,301], could still have been dependent on α6β4-mediated adhesion to autocrine-produced laminin. In support of this notion, in 3D culture of mammary spheroids, α6β4-mediated cell adhesion to autocrine produced laminin-332 conferred resistance to apoptosis by stimulating Rac1-Pak signaling and activation of NF-κB [302,303]. Moreover, Bertotti et al. [274] showed that the removal of the extracellular domain of β4 reduced anchorage-independent colony formation in soft agar.

Additionally, it has been suggested that suprabasal α6β4 contributes to cancer progression by enhancing proliferation of basal keratinocytes by relieving the growth inhibition of TGF-β [265]. TGF-β negatively regulates keratinocyte proliferation in the early stages of epidermal tumor promotion [304,305]. This growth inhibitory effect of TGF-β is dependent on cadherin-mediated cell-cell adhesion and PI3K, but not MAPK activity. Suprabasal α6β4 appears to perturb TGF-β signaling by blocking nuclear translocation of activated Smad2/3, resulting in increased cell proliferation and formation of skin papillomas and SCCs [265]. Surprisingly, tumorigenesis was further increased when mice expressed a mutant β4 subunit that lacked the cytoplasmic domain in the suprabasal layers of the epidermis, suggesting that α6, rather than β4 cytoplasmic domain might play a role in TGF-β signaling [265].

Role of α6β4 in angiogenesis

Within the vasculature, the expression of α6β4 in endothelial cells is dynamically regulated during angiogenesis and vessel maturation [306]. α6β4 is predominantly detected in small arterial vessels, where it may mediate strong endothelial cell adhesion, necessary to withstand the high shear rates in these vessels [44]. Contrary to β4, α6 is expressed in all vasculature, which suggests the presence of α6β1 in the absence of β4 [44,307]. Integrin α6β1 is known to promote angiogenesis; inhibition

of α6 prevented endothelial cell migration and tube formation [308]. However, the role of β4 in angiogenesis is less clear. The exclusive presence of β4 in mature vessels suggests that it negatively regulates angiogenesis [306], and several studies report that α6β4 does not promote endothelial cell proliferation or growth of new vessels [44,306,309]. Furthermore, it has been recently suggested that downregulation of α6β4 is necessary for endothelial proliferation and tube formation during early stages of angiogenesis [307]. In line with this notion it was proposed that α6β4 can block angiogenesis by inducing endothelial cell death [310] (reviewed in [311]). On the other hand, experiments in mice in which the C-terminal domain of β4 was deleted, showed that α6β4 signaling is important for vascular remodeling and for a proficient angiogenic response to VEGF and basic fibroblast growth factor (bFGF) [309]. Furthermore, in mice lacking β4 in endothelial cells, hypoxia-induced arteriolar remodeling was defective, which was suggested to result from changed TGF-β signaling [44]. Alternatively, β4 can regulate angiogenesis via stimulation of translation and signaling of VEGF [285,312] (Fig. 3).

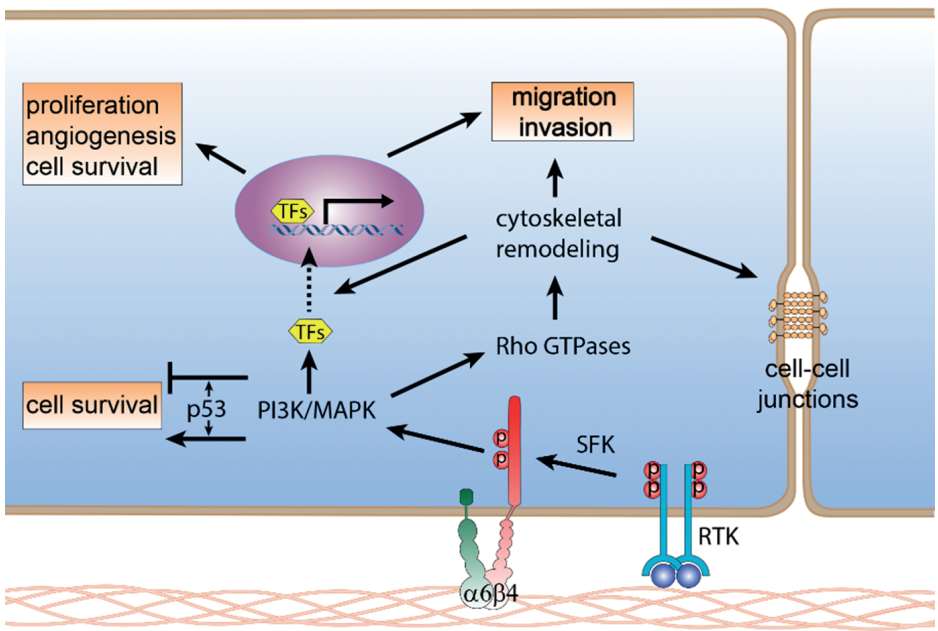


Figure 3 Integrin α6β4 regulates cell behavior by cooperating with RTKs. *Integrin α6β4 amplifies intracellular signaling of RTKs. SFK that are activated by RTKs phosphorylate several tyrosine residues (red dots) in the signaling domain of the β4 cytoplasmic tail. These tyrosine residues act as docking site for adaptor proteins to activate the PI3K and MAPK signaling pathways. Activated PI3K and MAPK further regulate cell migration, proliferation, angiogenesis, apoptosis, invasion and survival via activation of downstream effectors, by regulating cell-cell adhesion or via regulation of gene expression by transcription factors. RTK, receptor tyrosine kinase; SFK, Src family kinase; PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinases; TFs, transcription factors.*

Although α6β4 seems to play a general regulatory role in angiogenesis, there is very little known about its role in tumor angiogenesis. In mice, carrying a deletion of the C-terminal domain of β4, vascularization in subcutaneously implanted tumors was impaired, suggesting that α6β4 promotes tumor angiogenesis [309]. However, the levels of tumor vascularization in a mammary gland tumor model were the same in mice carrying a similar β4 deletion as in β4 wild-type mice [271].

Role of α6β4 in invasion and metastasis

Unlike that of α3β1, the expression of α6β4 is positively correlated with tumor grade in most instances, indicating that this integrin promotes tumor progression and metastatic spread. The role of α6β4 in invasion and metastasis has been reviewed extensively over the last two decades, in articles primarily focusing on the mechanisms responsible for its tumor-promoting function [87,88,252,313,314]. However, there are certain cases in which α6β4 is negatively correlated with tumor invasion and formation of metastases. In order to elucidate the circumstances that determine the function of α6β4 in the final stages of cancer, it is important to understand under which conditions it contributes to cellular migration and invasion, as well as to understand the differences between tumor settings, in which the role of α6β4 has been implicated.

Migration and invasion

It is generally accepted that HDs have to be disassembled before cells can migrate (Fig. 4). Indeed, studies using primary keratinocytes have revealed that classical HDs (type I HDs), which contain α6β4, CD151, plectin, BP180 and BP230, prevent cell migration [315,316]. However, α6β4 does not necessarily impede cell migration as part of type II HDs, a less complex type of HDs that only contains α6β4 and plectin [317]. Type II HDs are found in simple epithelia and many cultured epithelial cells, and in contrast to type I HDs, their components have a turnover rate that is fast enough not to limit the migration of cells [70,72,318].

In many transformed cells the number of HDs is reduced and they are completely and/or partially disassembled. In these cells, α6β4 facilitates growth-factor stimulated migration and invasion via its cytoplasmic domain (reviewed in [6]). Induction of carcinoma cell migration by EGF is associated with a redistribution of α6β4 from HDs to the leading edge of the cell (reviewed in [87]), where it colocalizes with actin in lamellipodia and filopodia [73,74]. How α6β4 associates with actin and how this contributes to migration is not clear. Like α3β1, α6β4 has been implicated in actin cytoskeletal dynamics during migration through the regulation of the Rho family of GTPases [319,320]. Although RhoA plays a crucial role in the retraction of the tail of the cells, both Rac1 and RhoA are required for stimulating cell migration on 2D substrates by inducing the formation of actin-based protrusive structures [321]. Rac1, which is activated by α6β4-mediated cell adhesion, promotes the formation of lamellipodia and, interestingly, the localization of α3β1 in these structures [322]. Integrin α6β4

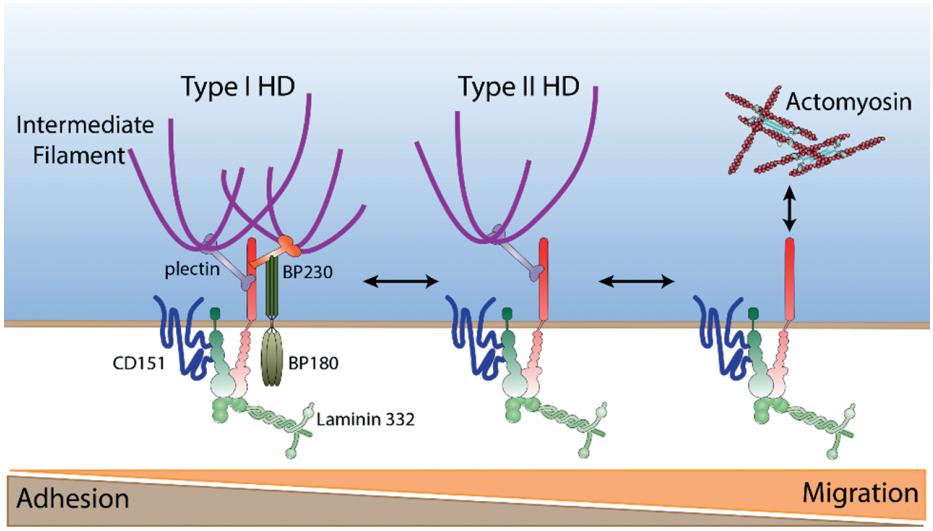


Figure 4 Integrin α6β4 regulates adhesion and migration by the formation of HDs. *Type I HDs contribute to stable adhesion of basal epithelial cells to laminin-332 in the basement membrane and therefore inhibit cell migration. Although type II HDs contribute to adhesion as well, they are more dynamic and do not impede migration. In migrating cells, HDs are disassembled and α6β4 is suggested to be associated with the actin cytoskeleton. HD, hemidesmosome.*

supports PI3K-Rac1 signaling [287] downstream of several pro-migratory factors (e.g. EGF [323], HGF [324], PTHrP (parathyroid hormone-related protein) [325] and LPA (lysophosphatidic acid) [326]) (Fig. 3). EGF-induced activation of Rac1 requires both the extracellular and the cytoplasmic domain of the β4 subunit [323]. On the other hand, when it is part of HDs, ligation of α6β4 by laminin activates Rac1 independently of the signaling domain of β4 [327,328]. In addition to stimulating migration by increasing the activity of Rac1 in tumor cells, α6β4 has also been implicated in augmenting the activity of RhoA by a mechanism that involves suppression of the intracellular cAMP concentration by activating a cAMP specific phosphodiesterase [280,329].

Several studies have shown that α6β4 influences migration and invasion of tumor cells through the NFAT (nuclear factor of activated T-cells) transcription factors [330] (Fig. 3), which are activated downstream of Src and PI3K/Akt signaling [331] (reviewed in [87]). α6β4-mediated activation of NFAT1 induces the transcription of autotaxin [332], which promotes LPA-induced cell motility and invasiveness [333]. In line with this, α6β4-mediated cell motility was decreased in breast carcinoma cells that were depleted of autotaxin [332]. NFAT5, a transcription factor responsible for the upregulation of the calcium-binding protein S100A4, can also be activated by α6β4-mediated cell adhesion [334]. S100A4 is a metastasis-promoting protein implicated in the invasion of a number of tumor types including colon and breast carcinomas (reviewed in [335,336]). Another protein that plays a role in the calcium-dependent regulation of migration is the transient receptor potential vanilloid channel (TRPV1).

In the absence of TRPV1, both directional migration and β4 expression are reduced [337].

Several studies suggested that α6β4 can influence ovarian and breast cancer cell migration and invasion through the activation of FAK [278,338]. α6β4-mediated FAK activation was observed upon ligation of α6β4 to laminin-332, but also to alternative ligands, such as CLCA1 and MUC5Ac [339,340]. Recent findings have shown that FAK can directly bind to β4 and suggest that this association is regulated by tyrosine phosphorylation of the β4 subunit [278].

During invasion, the ECM is often remodeled, enabling tumor cells to efficiently migrate and disseminate. α6β4 has been implicated in ECM remodeling through its ability to contribute to the activation of PI3K and RhoA, and subsequent induction of traction forces generated by the actomyosin cytoskeleton [341,342]. Additionally, α6β4 plays a role in remodeling of the ECM by supporting signals that lead to the production of MMP1 and MMP2 [343,344]. MMP levels can also be induced by several of its interactors, such as CD151 (reviewed in [345–347]).

Metastasis – intravasation, extravasation and niche preparation
Many studies have demonstrated a critical role of α6β4 in promoting the formation of metastases (reviewed in [97,252,348]). Moreover, α6β4 serves as a marker to detect distant metastases in the early stages of specific malignancies [120,349,350]. However, the mechanism underlying its pro-metastatic role has received little attention. Metastases occur when cancer cells invade into the blood or lymph vessels, travel through these systems and subsequently extravasate into the stroma of the target organ. α6β4 contributes to intravasation and extravasation of tumor cells by upregulating VEGF expression (Fig. 3), which enhances transendothelial permeability and migration of malignant cells [351–354]. The effect of α6β4 on VEGF expression appeared to be dependent on the signaling domain of β4 [354].

Following extravasation, tumor cells need to adhere, proliferate and grow in the new environment (i.e. the metastatic niche) in order to form a metastasis. Cells from specific tumors tend to form metastasis in certain organs. Recently, Hoshino et al. [355] suggested that exosomes can contribute to the formation of a metastatic niche in specific organs. They showed that expression of α6β4 and α6β1 on exosomes was associated with lung metastases in mice, and that blocking of exosomal α6β4 decreased the formation of such metastases.

Crosstalk with growth factors and hormones
The crosstalk between α6β4 and growth factor receptors not only plays a role in tumor growth (see previous chapters), but also strongly contributes to the α6β4-mediated tumor invasion, metastasis formation and cell migration. The synergy

between α6β4 and several RTKs (e.g. c-Met [279,287], Ron [273,356], ErbB-2 [357], EGFR [277] and ErbB-3 [358]) primarily promotes tumor spreading and invasion. This tumor promoting function of α6β4, like its promotion of tumor growth, often involves the disassembly of HDs and subsequent β4-mediated signaling (reviewed in [6,88,99,359,360]). Additionally, α6β4 can have an impact on invasion and metastasis through regulating cell-cell junctions [271] (Fig. 3). In ErbB-2 transformed mammary epithelial cells, β4 promotes invasion and metastasis by disorganizing cell-cell junctions via SFK-dependent ErbB-2 activation of STAT3. Accordingly, in these cells the loss of β4 signaling restores cell-cell adhesion, and as a consequence cell invasion is compromised. However, this negative effect of β4 on cell-cell contacts is only seen in the presence of receptors of the EGFR family, and blocking these receptors promotes reassembly of cell-cell junctions [271]. Like α3β1, α6β4 was reported to promote the formation of cadherin dependent cell-cell junctions, resulting in restrained cell migration [361,362]. Furthermore, upon induction of EMT by TGF-β a decrease in E-cadherin expression has been correlated with a reduction of the levels of β4 [363]. Such a reduction has also been shown in carcinoma cells in which EMT was induced by overexpression of Snail [364]. In contrast, suppression of the EMT regulator Slug resulted in a reduction of the α6β4 levels [365]. In addition to the reduced levels of β4 in tumor cells, which have undergone an EMT, there is evidence that β4 is less sialylated during EMT, which may further decrease the tumor promoting capacity of this integrin [366,367].

Besides a signaling cooperation between α6β4 and growth factor receptors, there is also crosstalk between α6β4 and hormones during tumor progression. Estrogen and PTHrP signaling was shown to enhance the pro-tumorigenic effects of α6β4 [368,369]. On the other hand, the expression of β4 in prostate cancer is reduced by the androgen receptor and the absence of β4 in the androgen-sensitive tumors reduces their invasiveness [370,371].

Targeted disruption of the crosstalk between α6β4 and growth factor receptors or between α6β4 and hormone receptors has been recognized as a potential form of therapy in the treatment of cancer [271,358,372]. However, because of its dual role in tumor development and promotion, targeting β4 can only be beneficial under well-defined and specific circumstances. In certain cancers, such as gastric carcinomas, the degree of β4 expression is inversely correlated with invasive potential and blocking β4 function would likely increase the tumor burden [295]. Furthermore, the analysis of transformed keratinocytes suggests that blocking the function of β4 in the early-stages of tumorigenesis might promote the disease [373].

Regulation of β4 expression
As previously discussed, the expression of β4 is frequently upregulated in cancer. For example, hypoxia increases the surface expression of α6β4 by promoting Rab11-

dependent trafficking, resulting in increased breast tumor cell invasion [64]. Other factors and proteins implicated in the expression of β4 in tumors include Rac1 [374], TR3/Nur77 [375], PTHrP [369,376,377], IL24 [378] and H-Ras [379], ARRDC3 [380] and ZEB1 [381]. Expression of α6β4 is also regulated by palmitoylation of the α6 and β4 subunits and ablation of the protein acyl transferase, responsible for their palmitoylation results in accelerated degradation of α6β4 [382]. miRNAs have emerged in the past decade as key regulators of gene expression. They repress gene expression by blocking mRNA translation or promoting mRNA degradation [383]. Several miRNAs have been reported to regulate expression of β4. Overexpression of miR-221 and miR-222 resulted in reduced β4 expression, which was partially responsible for impaired invasion of breast cancer cells [384], while in the absence of miR-21 a higher expression level of β4 was correlated with a reduction in the rate of colorectal cancer cell migration [383]. Furthermore, loss of miR-205 in prostate cancer resulted in reduced secretion of laminin-332 and β4 in vitro [385]. Therefore, the different expression patterns of β4 in cancer might be explained, at least to some extent, by specific miRNAs present in the different tissues.

Role of CD151 and α6 in α6β4-regulated tumorigenesis

Most studies have focused on the role of α6β4 in cancer without considering the fact that two isoforms of the α6 cytoplasmic domain (i.e. A and B) can be generated by alternative splicing [38,41]. In colorectal carcinomas, in which both a relative decrease and increase of β4 expression was observed during malignant transformation [386,387], the tumorigenic outcome of β4 positive cancers was dependent on which isoform of the α6 subunit was associated with β4 [388]. A relationship between the expression of specific α6 isoforms and tumor malignancy has also been observed in skin tumorigenesis [389]. As well as forming dimers with the β4 subunit, the α6 subunit can also dimerize with β1 to form the α6β1 heterodimer, which has a tumor promoting role and is correlated with a poor prognosis (discussed in introduction) [390]. Similar to α6β4, the role of α6β1 in cancer can be dependent on α6 isoform expression [391,392]. α6β1 and α6β4 are co-expressed in numerous cell lines. However, the ratio of these complexes varies considerably from one cell line to another [46]. Since α6 preferentially binds to the β4 subunit [250,393], the absence of β4 might lead to a switch from α6β4 to α6β1, and thus to promoting carcinogenesis in an α6β1-dependent manner. A switch from α6β4 to α6β1 has been observed during prostate cancer progression, in which expression of the androgen receptor suppresses α6β4 expression [260].

We have already discussed the impact of CD151-α3β1 on tumor progression. However, CD151 can also bind α6 and the presence of CD151-α6β4 in breast cancer was associated with tumor progression [223,394]. CD151 may affect the function of α6β4 through its association with PI4K or by regulating α6β4 trafficking, its incorporation into TEMs and/or its ligand binding activity [3,212,214]. Recently, it has been shown that CD151 is also involved in the crosstalk between α6β4 and RTKs [223,395] and that deletion of

CD151 reduces β4 phosphorylation at specific serine residues [394,396]. These serine residues have been previously implicated in the regulation of the interaction between α6β4 and plectin [70,397]. While the interaction between α6β4 and CD151 is well-established, there is data supporting that α6β4 can also form complexes with two other tetraspanins (D6.1A and CD9) and thus might also influence tumorigenesis by interacting with these molecules [398,399].

Conclusions

The importance of laminin-binding integrins in the development and progression of tumors has been demonstrated in many studies. However, the role they play during these processes is complex, and possibly determined by several intra- and extracellular factors. Among the factors that have been shown to impact the outcome of tumor cells expressing laminin-binding integrins are the oncogenic profile and the nature of the tumor cells (e.g. secretion of hormones, presence of different membrane receptors, hypoxia, etc.), the presence of different ECM components (especially laminins), and the regulation of posttranslational modifications of the integrins.

Integrins influence multiple aspects of tumorigenesis through their impact on adhesion and their ability to enhance signaling pathways downstream of RTKs. One of the features of laminin-binding integrins that strongly influences tumorigenesis and tumor progression is their ability to mediate bi-directional signaling with small GTPases and to promote cytoskeletal remodeling. Several decades ago it was already known that tension-dependent changes in cell shape are necessary for progression of the cell cycle [400], and since then numerous studies have shown that integrin-mediated adhesion and spreading of cells is essential for nuclear translocation of transcription factors [328,401,402]. Therefore, the activation of GTPases by either α3β1- or α6β4-mediated cell adhesion not only influences tumor cell migration and invasion, but may also enable nuclear translocation of transcription factors to promote proliferation and survival of tumor cells that have not yet acquired the ability to grow anchorage independent. Furthermore, adhesion mediated by laminin-binding integrins, especially α6β4, might be essential for tumor development and progression simply by promoting pro-oncogenic signaling pathways.

The pro-migratory role of laminin-binding integrins is often connected to their ability to support PI3K-Rac1 signaling, which might be due to a lack of association of these integrins with the actomyosin cytoskeleton. Integrin α6β4 is connected with the intermediate filament system in type I and type II HDs, while α3β1, when associated with CD151, is incorporated into TEMs. Activation of Rac1 by either α3β1- or α6β4-mediated cell adhesion most likely occurs through synergy with aberrantly expressed and activated RTKs. Additionally, two tyrosine phosphorylation sites in the third fibronectin type III repeat of the β4 cytoplasmic domain (i.e. Y1494 and Y1526)

have been reported to be sufficient for the activation of Rac [89,403]. Although these residues are located in NXXpY motifs, their structural environments are not compatible with binding to the PTB and SH2 binding domains of Shp2 and Shc, respectively [404]. The relevance of this mode of signaling therefore remains debatable.

Finally, the crosstalk between laminin-binding integrins and RTKs, and their association with IAPs, are the key determinants of the role of integrins in modulating cell behavior. As discussed in length above, α3β1 and α6β4 can interact with several different proteins. Therefore, it is very likely that a crosstalk between integrins and several different RTKs occurs in individual cells, and that several different α3β1 or α6β4 integrin-containing complexes exist simultaneously. However, little is known about the localization of these complexes, or how these different complexes interact. Therefore, a better understanding of how the balance between these complexes is established and how they influence oncogenic signaling pathways in cells will be necessary to fully understand the role of laminin-binding integrins in the development and progression of tumors.

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CHAPTER 3

PKD2 and RSK1 regulate integrin β4 phosphorylation at threonine 1736

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Abstract

The integrin α6β4, a major component of hemidesmosomes (HDs), stabilizes keratinocyte cell adhesion to the epidermal basement membrane through binding to the cytoskeletal linker protein plectin and association with keratin filaments. Disruption of the α6β4-plectin interaction through phosphorylation of the β4 subunit results in a reduction in adhesive strength of keratinocytes to laminin-332 and the dissolution of HDs. Previously, we have demonstrated that phosphorylation of T1736 in the C-terminal end of the β4 cytoplasmic domain disrupts the interaction of β4 with the plakin domain of plectin. Furthermore, we showed that β4-T1736 can be phosphorylated by PKD1 in vitro, and although both PMA and EGF induced T1736 phosphorylation, only PMA was able to activate PKD1. Here, we show that depletion of [Ca2+]i augments PMA- and EGF-induced phosphorylation of β4-T1736 and that this is caused by inhibition of the calcium-sensitive protein phosphatase calcineurin and augmentation of ERK1/2 activation. We also show that in keratinocytes the PMA-stimulated phosphorylation of β4-T1736 primarily is mediated by PKD2 activation downstream of PKCδ. On the other hand, both the EGF-stimulated phosphorylation of T1736 and the EGF-induced dissolution of HDs are dependent on a functional MAPK signaling pathway, and treatment with the RSK inhibitor BI-D1870 prevented EGF-stimulated phosphorylation of β4-T1736. Moreover, phosphorylation of β4-T1736 is enhanced by overexpression of wild-type RSK1, while it is reduced by the expression of kinase-inactive RSK1 or by siRNA-mediated depletion of RSK1. In summary, our data indicate that different stimuli can lead to the phosphorylation of β4-T1736 by either PKD2 or RSK1.

Introduction

Hemidesmosomes (HDs) are junctional protein complexes that are crucial for maintaining firm adhesion of keratinocytes to the underlying basement membrane and thus for epithelial tissue integrity [1,62]. The integrin α6β4 is an important component of HDs. Together with Bullous Pemphigoid 180 (BP180), a transmembrane collagen, it binds to laminin-332 in the epidermal basement membrane [1,19,20]. At the cytoplasmic face, these proteins are connected via plectin and BP230, two members of the plakin family of cytoskeletal linker proteins, to keratin intermediate filaments [11,17,18].

Studies with cultured keratinocytes revealed that the formation of HDs is critically dependent on the binding of plectin to the cytoplasmic domain of β4 [9,13,405]. Mutations that prevent this interaction lead to a loss of HDs, similarly to that seen for mutations that cause the complete absence of α6β4 or plectin [49,406–408]. Subsequent studies therefore have focused on the regulation of this interaction by phosphorylation as a means to promote HD disassembly in migrating keratinocytes during wound healing. It was thus demonstrated that the β4 cytoplasmic domain harbors several residues that are phosphorylated in response to stimulation of keratinocytes with agents known to cause HD disassembly, e.g. phorbol 12-myristate 13-acetate (PMA) and Epidermal Growth factor (EGF) [68,69,72,397]. Two of these residues (S1356 and S1364) are present in the connecting segment that separates the two pairs of type III fibronectin (FnIII) domains in the cytoplasmic domain of β4, while a third one (T1736) is located in the C-tail that follows the last FnIII domain. Phosphorylation of S1356 and S1364 prevents interaction of β4 with the actin-binding domain (ABD) of plectin, possibly by a mechanism of phosphorylation-dependent auto-inhibition, while T1736 phosphorylation results in the disruption of the binding site for the plakin domain of plectin that follows the ABD [68,69,397]. The phosphorylation of a fourth site (S1424) on β4, which is enriched in the trailing edge of migrating keratinocytes, has been associated with the dissociation of BP180 from HDs [70].

Both PMA and EGF activate the mitogen-activated protein kinase (MAPK) signaling pathway and evidence has been presented that S1356 and S1364 are phosphorylated by two components of this signaling pathway, ERK-1/2 and their downstream effector kinases RSK-1/2, respectively [68]. Recently, we have shown that PKD1, a downstream effector kinase of PKC, phosphorylates β4 at T1736 in vitro and that this kinase is activated in keratinocytes upon PMA stimulation [69]. However, evidence that this occurs in keratinocytes treated with PMA has not yet been reported. Furthermore, because PKD1 is not activated in keratinocytes stimulated with EGF, it is not clear which kinase is responsible for the phosphorylation of this residue downstream of EGFR activation.

Here we set out to identify the kinases that phosphorylate β4 at T1736 upon PMA and EGF treatment of keratinocytes. We show that PKD2 is robustly activated by PMA and that PKD2 phosphorylates T1736, while RSK1 is the kinase that phosphorylates this residue downstream of the EGF receptor in keratinocytes. Furthermore, we show that phosphorylation of T1736 is influenced by cytoplasmic calcium levels, which is in part regulated by the serine-threonine phosphatase calcineurin.

Results

In previous studies, we empirically found that keratinocytes form HDs more efficiently when we switched the keratinocyte growth medium from one with a low calcium concentration (KGM) to one with a high calcium concentration (DMEM) 24h before analysis [13]. Here we confirm these findings and show that HDs in the low calcium medium, occur in patches at the cell-substrate contact sites, which often are organized in a typical "cauliflower-like" pattern. In high calcium medium, HDs are more abundant and occupy a large part of the basal surface of cells (Fig. 1A and 1B). As the cytosolic calcium levels are affected by the extracellular calcium concentration, and phosphorylation of β4 at S1356 has been associated with a reduced number of HDs [409,410], we studied the effect of the intracellular calcium [Ca2+]i chelator BAPTA-AM and the calcium ionophore A23187 on the phosphorylation of T1736 and S1364. Treatment of PA-JEB/β4 keratinocytes, grown in complete KGM, with BAPTA-AM resulted in a 2–3 fold increase in the phosphorylation of β4 at T1736. BAPTA-AM did not increase the already high basal level of S1364 phosphorylation, probably because this phosphorylation was already maximal (Fig. 1C). In contrast to the BAPTA-AM treatment, A23187 decreased the basal level of phosphorylation of S1364 and T1736. It did not reduce the phosphorylation of these two residues in the presence of BAPTA-AM, likely because the concentration of BAPTA-AM was sufficient to completely prevent the A23187-induced increases in cytosolic calcium ions.

The changes in phosphorylation of T1736 in PA-JEB/β4 keratinocytes treated with BAPTA-AM or A23187 paralleled to some extent those by ERK1/2, which may point to a possible role of the MAPK signaling pathway in the phosphorylation of this residue. However, compared to the phosphorylation of S1364, which is known to be controlled by the MAPK signaling pathway [68], phosphorylation of T1736 may only occur when this pathway is strongly activated.

Because the level of phosphorylation of proteins reflects a balance between protein kinases and phosphatases, we treated PA-JEB/β4 keratinocytes with the calcineurin inhibitor FK506. Consistent with the reported role of calcineurin in the regulation of β4-S1356 phosphorylation [411], we found that the PMA-induced phosphorylation of this residue, as well as that of S1364 was increased by FK506 (Fig. 1D). FK506 had no effect on the PMA-induced activation of ERK1/2. A small, but consistent, increase in

the PMA-stimulated phosphorylation of β4 was also observed with FK506 at T1736; however, the effect was always less than that seen with BAPTA-AM (Fig. 1D). Notably, BAPTA-AM alone did not increase basal phosphorylation of β4 at T1736, S1356 or S1364 (Fig. 1D). A small increase in T1736 phosphorylation was also observed when PA-JEB/β4 keratinocytes were treated with the calcineurin inhibitor Cyclosporin A (CsA) (Fig. 1E). These effects were not restricted to PA-JEB/β4 keratinocytes, as both FK506 and CsA also slightly increased the PMA-stimulated phosphorylation of T1736 in A431 epidermoid carcinoma cells (Fig. 1E).

Together these data indicate that HD formation is regulated by the extracellular calcium concentration, and that phosphorylation of β4 at T1736 is increased by reducing the cytosolic calcium concentration, which may in part be due to the inhibition of calcineurin.

PMA- and EGF-stimulated phosphorylation of β4 at S1364 and T1736

Previously, we have shown that PKD1, a downstream effector kinase of PKC, phosphorylates β4 at T1736 in vitro, and that this kinase is activated in PA-JEB/β4 keratinocytes upon stimulation with PMA, but not with EGF [69]. However, the finding that the basal phosphorylation of T1736 is increased by lowering the cytosolic calcium concentrations and is accompanied by an increase in the activity of ERK1/2, suggested that T1736 phosphorylation may also be regulated through MAPK signaling. To gain further insight in the role of PKD and MAPK signaling, we tested the effects of inhibitors of PKC (Gő6983) and MEK1/2 (UO126) on the PMA- and EGF-induced phosphorylation of T1736. Additionally, we investigated the effects of the two inhibitors on the PMA- and EGF-induced phosphorylation of S1364. As shown in Fig. 2, while UO126 inhibited both the PMA- and EGF-induced phosphorylation of β4-S1364, Gő6983 only blocked the PMA-stimulated phosphorylation of S1364. These findings are consistent with RSK1/2 being the two kinases that phosphorylate β4-S1364 downstream of PKC and EGFR activation. In contrast, phosphorylation of β4-T1736 by PMA is not sensitive to inhibition by UO126; it is only inhibited by Gő6983 (Fig. 2). Since Gő6983, but not UO126, also prevented the PMA-induced phosphorylation of PKD1 at S744/748 (two activation loop sites) by PKC and of S916 (autophosphorylation site), which is correlated with catalytic activity of PKD1, this kinase may indeed be responsible for the phosphorylation of β4-T1736 by PMA. In line with previous results, we found that PKD1 is not obviously activated downstream of the EGFR, and therefore, it is unlikely that this kinase participates in the phosphorylation of T1736 downstream of the EGFR activation [69]. Because the phospho-PKD1-S916 and phospho-PKD1-S744/748 antibodies, used to detect the activation state of PKD1, also recognize the autophosphorylation site in PKD2 and the activation loop sites in PKD2 and PKD3, respectively, a role of these kinases in the EGF-mediated phosphorylation of β4-T1736 can also be excluded.

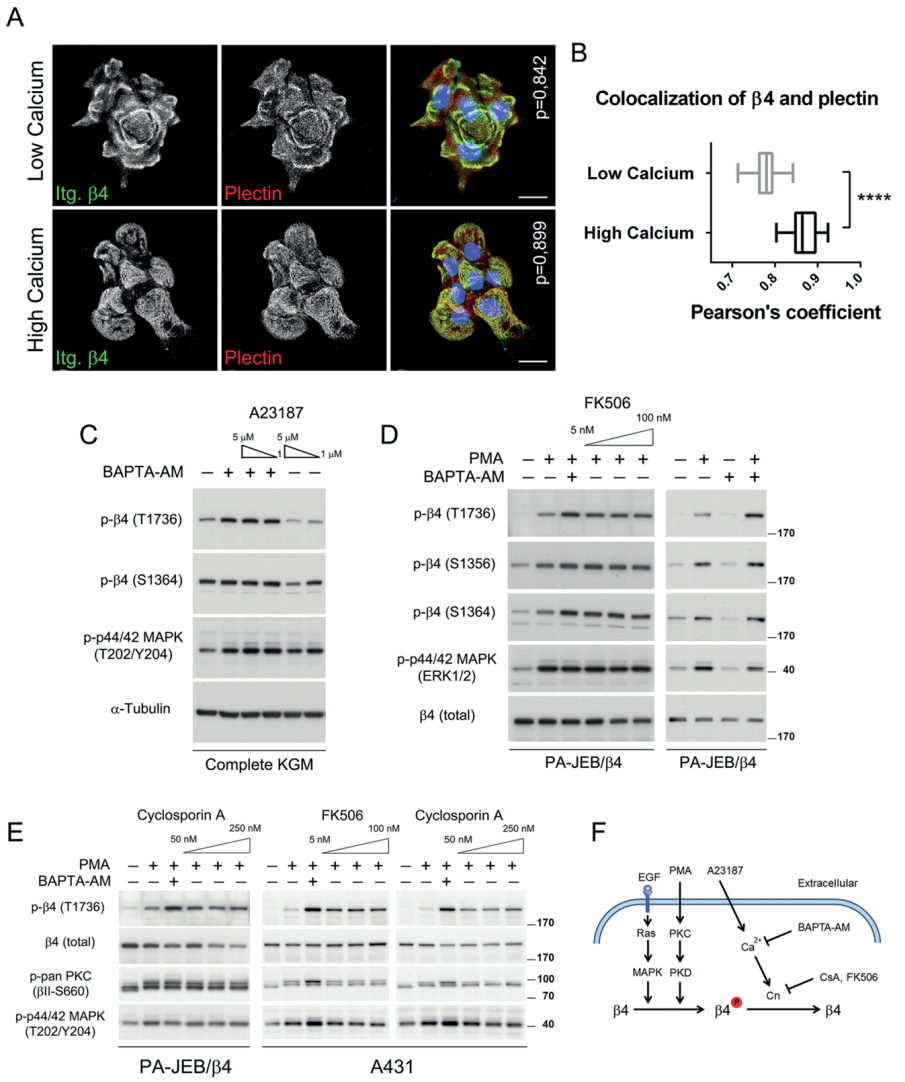


Figure 1 Regulation of hemidesmosome formation and β4-T1736 phosphorylation by extracellular calcium. *(A)Confocal microscopic images show the effect of extracellular calcium on the formation and organization of HDs in PA-JEB/β4 keratinocytes. After 24 hours of growth in either low (KGM; left picture) or high calcium (DMEM; right picture) medium, PA-JEB/β4 keratinocytes were stained with mAbs 439-9B against β4 (green) and 31 against plectin (red). Cells were counterstained with DAPI (blue) to identify nuclei. HDs are identified as dots containing both β4 and plectin and appear in yellow. Scale bars: 20 μm. (B) Co-localization between β4 and plectin was quantified for each cell using Pearson's correlation coefficient. Box plots show median (solid line) and 25th and 75th percentiles (boxes) of the distribution for each condition (n = 3, ~10 images per experiment). **** <0,0001. (C) PA-JEB/β4 keratinocytes were pre-treated with the intracellular calcium chelator, BAPTA-AM and/or different concentrations of the calcium ionophore A23187 (1–5 μM), or left untreated. Cell lysates were analyzed by immunoblotting with antibodies as indicated. (D) PA-JEB/β4 keratinocytes were deprived of growth factors overnight, pre-treated with BAPTA-AM, different concentrations (5–100 nM) of FK506 or left untreated before stimulation with PMA. Cell lysates were analyzed by immunoblotting with the indicated antibodies. (E) PA-JEB/β4 keratinocytes and A431 epidermoid carcinoma cells were*

serum-starved overnight, pre-treated with BAPTA-AM or different concentrations of FK506 (5–100 nM) or CsA (50–250 nM) and stimulated with PMA or left unstimulated. Cell lysates were analyzed by immunoblotting with the indicated antibodies. (F) Schematic diagram of the different stimuli and inhibitors along with the signaling pathways that control β4 phosphorylation. Cn, calcineurin; CsA, Cyclosporin A; FK506, tacrolimus; BAPTA-AM, 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester).

We again could establish an association between EGF-stimulated phosphorylation of T1736 and MAPK signaling, i.e. the EGF-stimulated phosphorylation of T1736 was reduced by UO126, albeit not completely abolished. We also observed a reduction in the EGF-stimulated phosphorylation of β4-T1736 with Gő6983, but because phosphorylation of PKD1 (PKD2 or PKD3) by PKC could not be demonstrated, the significance of this reduction is not clear. A similar reduction was observed for the phosphorylation of β4 at S1364 and of RSK at T359/S363.

It is perhaps noteworthy that S380 of RSK1/2 is homologous to S660 of PKCβII (both are present in the conserved bulky ring motif) and that the phosphorylation of this residue therefore may also be detected by the phospho-pan PKC antibody. Indeed, the phospho-pan PKC antibody detected phosphorylated proteins that migrated in gel electrophoresis at a position, corresponding to that of phosphorylated RSKs (approximately 90 kDa). Furthermore, the patterns of phosphorylation and sensitivity of these proteins to Gő6983 and UO126 exactly matched those of the phosphorylation of RSK at S380.

In conclusion, the results further suggest a role of RSK1/2 in the phosphorylation of β4 at S1364 and provide further evidence for the role of PKD1 in the phosphorylation of β4 at T1736 downstream of PKC stimulation and of the MAPK signaling downstream of EGFR stimulation.

PMA-stimulated phosphorylation of β4-T1736 is primarily mediated by PKD2

To confirm the role of PKD isoforms in PMA-stimulated phosphorylation of T1736, we silenced the expression of endogenous PKD1 and PKD2 in PA-JEB/β4 keratinocytes by transfection with isoform-specific PKD siRNAs. The transfected cells were then treated with PMA to evaluate the contributions of the two PKD isoforms in the phosphorylation of T1736. Fig. 3A shows that depletion of PKD2, but not PKD1, strongly reduced the PMA-stimulated phosphorylation of β4 at T1736. The residual phosphorylation of T1736, remaining after depletion of PKD2, could not be attributed to PKD1, because the combined depletion of PKD1 and PKD2 did not further decrease T1736 phosphorylation. The efficiency of the siRNA-mediated depletion of both PKD1 and PKD2 was confirmed, not only by the failure of the anti-PKD1/2 antibody to react with unstimulated cells, but also by the absence of reactivity of the phospho-PKD1-S916 antibody with the PMA stimulated cells. Noticeably, because the anti-PKD1/2 antibody has been generated against the synthetic peptide corresponding to residues surrounding S916, it does not recognize these two kinases when phosphorylated at S916.

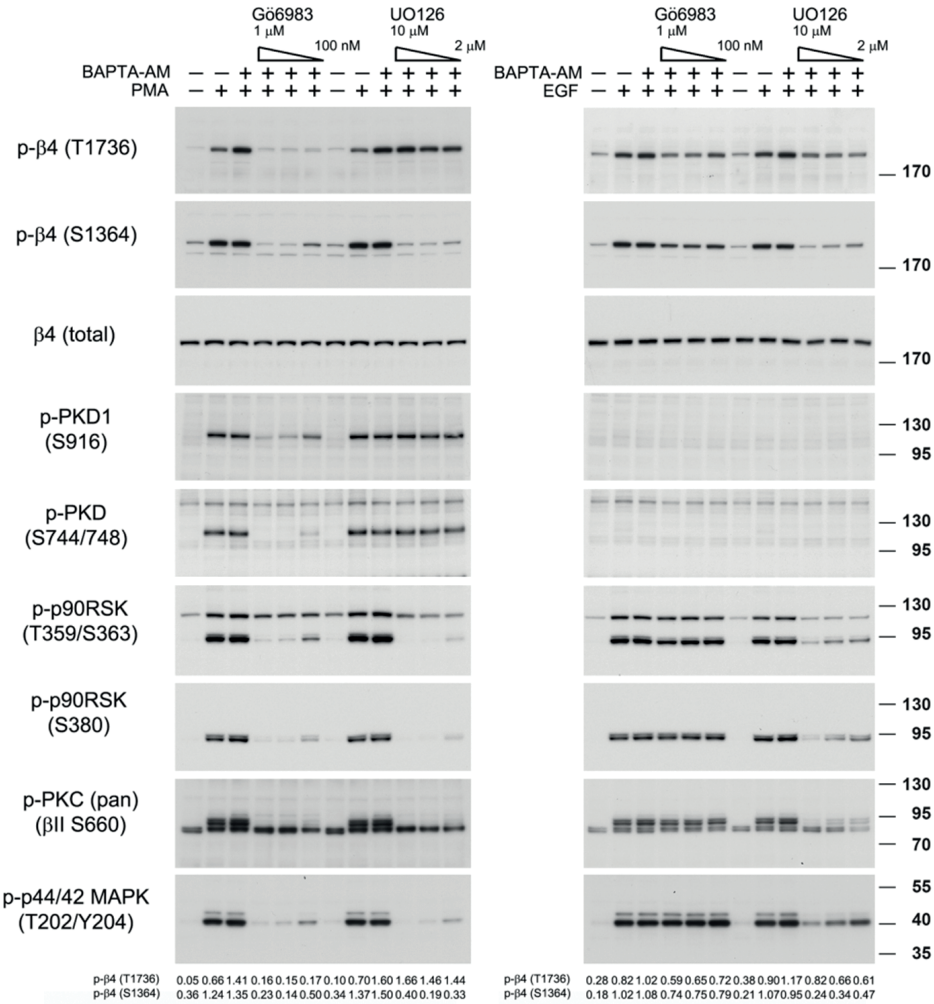


Figure 2 PMA- and EGF-stimulated phosphorylation of β4-T1736 is dependent on PKC and MAPK, respectively. *PA-JEB/β4 keratinocytes were deprived of growth factors overnight, pre-treated with BAPTA-AM for 30 min and then treated for 30 min with different concentrations of Gö6983 (100 nM-1 μM) or UO126 (2–10 μM). Subsequently the cells were stimulated with EGF or PMA for 10 min. Cell lysates were analyzed by immunoblotting with antibodies as indicated. The values below the blots indicate the signal intensities for the phosphorylated β4-T1736 and β4-S1364 proteins after normalization to the level of β4.*

Furthermore, a role of the MAPK signaling cascade in the remaining phosphorylation of T1736 could be excluded. No further reduction in the PMA-stimulated phosphorylation of T1736 was observed in UO126-treated cells depleted of PKD1, PKD2 or both proteins. Next, we considered the possibility that the residual phosphorylation of β4 at T1736 is caused by the activation of PKD3. RT-qPCR showed that PKD3 is indeed expressed by PA-JEB/β4 keratinocytes (Fig. 3B). In fact, its expression is much stronger than that of PKD1 and PKD2. The PKD3 protein could also be detected by antibodies

on immunoblots and showed a mobility shift upon PMA stimulation, indicative of phosphorylation and activation of the kinase. Consistent with the activation of PKD3 by PMA, depletion of both PKD1 and PKD2 did not completely abolish the reactivity of the phospho-PKD1-S744/748 antibody. Some residual reactivity remained detectable.

Together these results indicate that the PMA induced phosphorylation of β4 at T1736 is primarily mediated by PKD2 with a possible small contribution by PKD3.

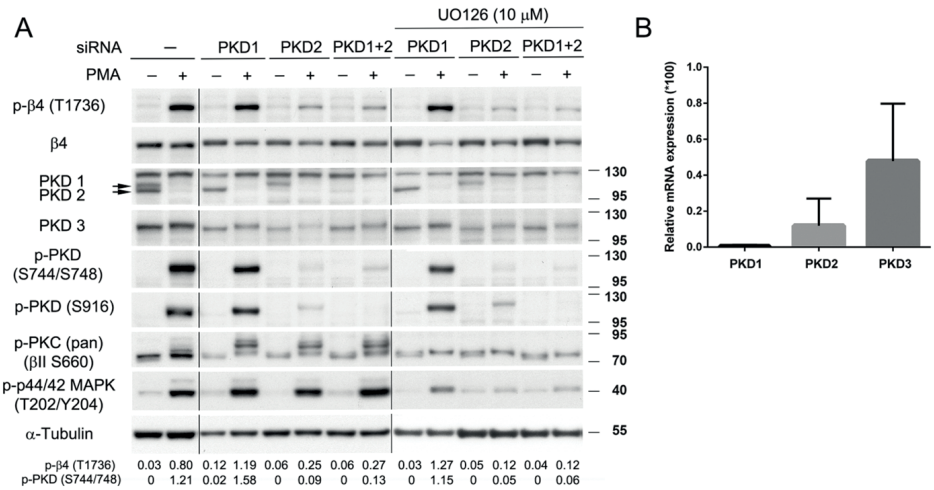


Figure 3 PMA-stimulated phosphorylation of β4-T1736 is primarily mediated by PKD2. *(A) PA-JEB/β4 keratinocytes, not transfected (-) or transfected with siRNAs against PKD1 or PKD2, or a combination of PKD1 and PKD2 siRNAs, were deprived of growth factors overnight, pre-treated with UO126 (10 μM) or left untreated, and then stimulated with PMA for 10 min. The cell lysates were analyzed by immunoblotting with the indicated antibodies. The values below the blots indicate the signal intensities for the phosphorylated β4-T1736 and PKD (S744/748) proteins after normalization to the level of α-tubulin. (B) Expression levels of the mRNAs for the three PKD family members were determined by RT-qPCR relative to cyclophilin A mRNA levels in PA-JEB/β4 cells. Values are means ± SD from four independent experiments performed in duplo.*

PMA-induced PKD activation is mediated by PKCδ

Previous studies have implicated Ca2+-independent, "novel" PKCs as major upstream kinases in the phosphorylation of the PKD activation loop [25]. PA-JEB/β4 keratinocytes express three of the four novel PKCs, including PKCε, PKCδ and PKCη, as judged by RT-qPCR (Fig. 4A). The expression of PKCε is relatively low compared to that of PKCδ and PKCη. To test which of these PKCs mediates phosphorylation of the PKD activation loop, we used specific PKC siRNAs to suppress their expression individually and in any of the possible combinations of the proteins, two-and-two and all three together. The knockdown efficiency for each PKC isoform was confirmed by RT-qPCR and/or immunoblot analysis (Fig. 4B and 4C).

Both the PMA-induced PKD activation loop phosphorylation at S744/748 and the phosphorylation of β4 at T1736 are attenuated when PKCδ expression is suppressed (Fig. 4C). However, depletion of PKCδ seems to have only little effect on the activity of PKD1/2, as measured by the phospho-PKD-S916 antibody. On the contrary, phosphorylation of either β4 or PKD was not obviously reduced following depletion of PKCε or PKCη (Fig. 4C), or when these two PKC isoforms were depleted together (Fig. 4D). Furthermore, combining siRNA-mediated depletion of PKCδ with either PKCε or PKCη, or with both PKCε and PKCη did not result in a further reduction of the phosphorylation of β4-T1736 or of the activation loop phosphorylation of PKD other than that already seen after depletion of PKCδ alone (Fig. 4D).

Although depletion of PKCδ substantially reduced the PMA-induced β4-T1736 phosphorylation, it did not completely abrogate it. This may be due to incomplete siRNA-mediated depletion of PKCδ, or to the existence of an alternative kinase that phosphorylates PKD-S744/748 and β4-T1736. The possibility that PKCδ cooperates with "classical" Ca2+-dependent PKCs in mediating PMA-induced phosphorylation of PKD-S744/748 and β4-T1736 could be excluded, because the combination of siRNA-mediated depletion of PKCδ with BAPTA-AM did not reduce the PMA-induced phosphorylation of β4-T1736 further than that already caused by siRNA-mediated depletion of PKCδ alone (Fig. 4E). Even after depleting all three PKC isoforms, BAPTA-AM treatment did not completely abrogate the PMA-stimulated phosphorylation of β4-T1736. The fact that PKCδ depletion reduced the PMA-induced phosphorylation of β4-T1736 to a similar extent in both BAPTA-AM treated and untreated cells indicates that the effects of BAPTA-AM on the PMA-induced phosphorylation of β4-T1736 are mediated through PKCδ.

We conclude that efficient PMA-induced phosphorylation of β4-T1736 is dependent on PKCδ but not PKCε or PKCη, or the "classical" PKCs. The remaining phosphorylation of PKD (S744/748 and S916) and β4-T1736 seen after combined treatment with PKCδ siRNA and BAPTA-AM treatment may indicate that some phosphorylation of PKD and β4 is PKCδ independent.

EGF-dependent phosphorylation of β4 depends on RSK1/2

The results obtained so far showed that EGF-stimulated phosphorylation of β4-T1736 is not mediated by PKD, but is dependent on ERK1/2 signaling. To investigate whether RSK, a known downstream effector kinase of ERK1/2, is involved in EGF-stimulated phosphorylation of β4 at T1736, we pre-treated PA-JEB/β4 keratinocytes with the RSK inhibitor BI-D1870 and subsequently stimulated the cells with EGF or PMA in the presence or absence of BAPTA-AM. BI-D1870 acts as an ATP-competitive inhibitor of the N-terminal AGC kinase domain of RSK, but does not inhibit the C-terminal kinase, whose activity is regulated by ERK1/2 [412]. As shown in Fig. 5, inhibition of RSK by BI-D1870 reduced the EGF-stimulated phosphorylation of β4-T1736 to basal levels.

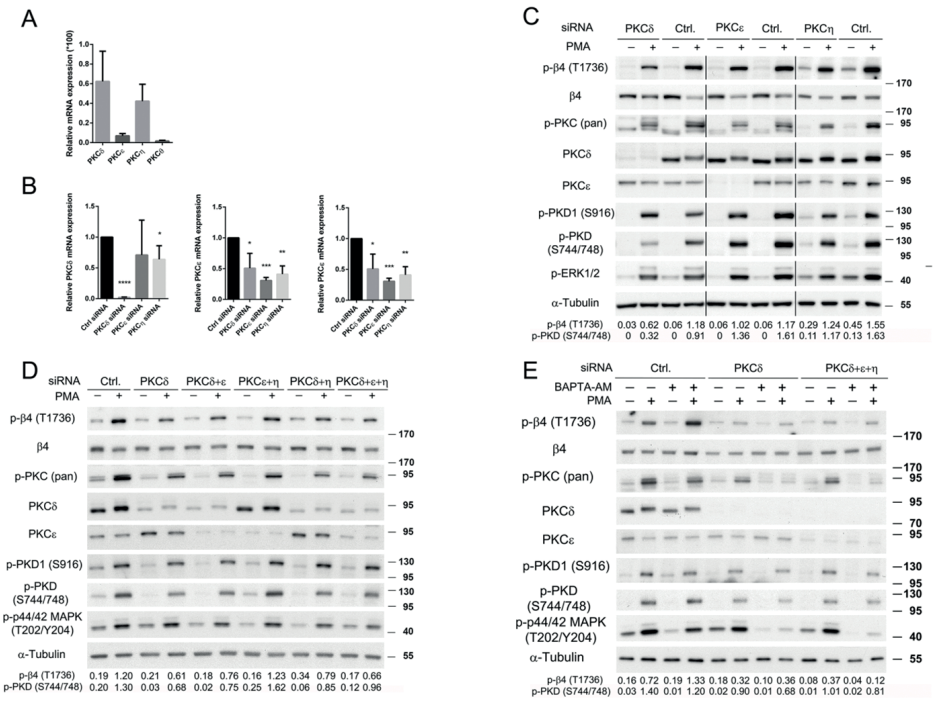


Figure 4 PKCδ mediates PMA-induced PKD activation. *(A) Expression levels of the indicated mRNAs for the four novel PKCs were determined by RT-qPCR and normalized to cyclophilin A mRNA levels in PA-JEB/β4 cells. Values are means ± SD from four independent experiments performed in duplo. (B) Relative mRNA levels of PKCδ, PKCε and PKCη were determined by RT-qPCR in PA-JEB/β4 cells, transfected with control (Ctrl) siRNA or siRNAs targeting PKCε, PKCδ, or PKCη. The values were normalized to cyclophilin A mRNA and compared to the mRNA levels in the cells treated with control siRNA. They are the means ± SD from four independent experiments performed in duplo. ****P, <0.0001; ***P, <0.001; **P, <0,01; *P, <0,05. (C, D) PA-JEB/β4 keratinocytes were transfected with single siRNAs or combinations of siRNAs as indicated. After 15 h, the cells were deprived of growth factors for 24h and subsequently stimulated with PMA for 10 min or left unstimulated. Cell lysates were analyzed by immunoblotting with antibodies as indicated. (E) PA-JEB/β4 keratinocytes, transfected with control, PKCδ or PKCδ siRNA in combination with PKCε and PKCη siRNAs, were deprived of growth factors for 24h, pre-treated with BAPTA-AM for 1h and subsequently treated with PMA for 10 min or left untreated and stimulated. Cell lysates were analyzed by immunoblotting with antibodies as indicated. The values below the blots shown in panels C, D and E indicate the signal intensities for the phosphorylated β4-T1736 and PKD (S744/748) proteins after normalization to the level of α-tubulin.*

BI-D1870 also slightly inhibited the PMA-induced (PKD2-mediated) phosphorylation of T1736, while it had no inhibitory effect on the activity of PKD, as judged by the phosphorylation of PKD2 at S916 and S744/748. Pre-treatment with BAPTA-AM did not alter the inhibitory effect of BI-D1870 on the PMA- and EGF-stimulated phosphorylation of T1736. Consistent with our previous results, BI-D1870 completely abolished the phosphorylation of S1364 induced by PMA or EGF [68]. Unexpectedly, we found that BI-D1870 exerted a small, but consistent, inhibitory effect on the ERK1/2-dependent phosphorylation of RSK at T359/S363, while it had no effect on

the activity of ERK1/2, or on the autophosphorylation of RSK at S380 (mediated by its C-terminal kinase domain). Together these data suggest that downstream of ERK1/2, RSK plays an important role in the EGF-stimulated phosphorylation of β4 at T1736.

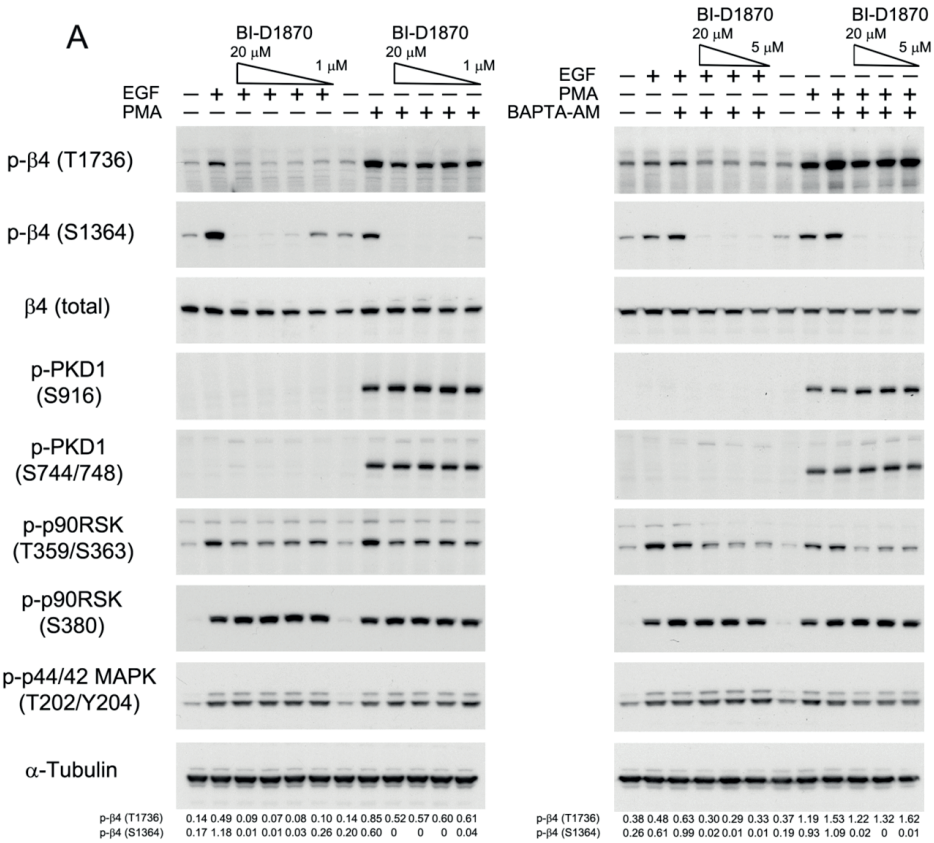


Figure 5 EGF- but not PMA-stimulated phosphorylation of β4 at T1736 and S1364 depends on RSK1/2. *PA-JEB/β4 keratinocytes were deprived of growth factors overnight, pre-treated with BAPTA-AM for 30 min and/or BI-D1870 for 30 min at the indicated concentrations or left untreated and then stimulated with EGF or PMA. The cell lysates were analysed by immunoblotting with the indicated antibodies. The values below the blots indicate the signal intensities for the phosphorylated β4-T1736 and β4-S1364 proteins after normalization to the level of α-tubulin.*

Kinase-inactive, but not wild-type, RSK1 or RSK2 reduces β4-T1736 phosphorylation

Next, we overexpressed wild-type RSK1 and RSK2, and kinase-inactive mutants thereof in PA-JEB/β4 keratinocytes, and compared the phosphorylation of β4 at T1736 with that at S1364. Overexpression of wild-type RSK1 or RSK2 increased the phosphorylation of β4-T1736 and β4-S1364 in unstimulated keratinocytes, but not (S1364) or only slightly (T1736) in keratinocytes stimulated with EGF, probably because these residues are already (almost) maximally phosphorylated by the endogenous kinases (Fig. 6A). The increased phosphorylation of β4-T1736 and β4-S1364 is correlated

with elevated levels of phosphorylation of RSK at S380, T359 and S363, which is in accordance with results of the inhibition studies with BI-D1870 that phosphorylation of β4 at T1736 and S1364 requires active RSK. Overexpression of kinase-inactive RSK1 and RSK2, on the other hand, did not lower the basal level of phosphorylation of β4 at T1736 or S1364 in unstimulated cells, but reduced the EGF-stimulated phosphorylation of these residues, albeit that of S1364 more substantially than that of T1736 (Fig. 6A). Interestingly, we found that the two RSK isoforms have a feedback inhibitory effect on the activity of ERK1/2. The level of both phosphorylated ERK1/2 (T202/Y204) and phosphorylated RSK (T359/S363) diminished in the cells overexpressing wild-type RSK1 or RSK2, as compared to in those overexpressing the kinase-inactive mutants of RSK1 or RSK2 under basal conditions. Although we anticipated that phosphorylation of the two kinase-inactive RSK mutants at S380 would be completely absent, low levels of phosphorylation could still be observed. This particularly in the case of the RSK2 mutant, suggesting that this mutant is not completely kinase-dead. The remaining activity of this "kinase-inactive" mutant, however, is not sufficient for inducing phosphorylation of β4-T1736 and β4-S1364 in unstimulated keratinocytes.

In summary, the results lend further support to the contention that like β4-S1364, T1736 is a target for RSK-mediated phosphorylation.

Depletion of RSK1, but not RSK2, reduces β4 T1736 phosphorylation

We next investigated the effects of depletion of RSK1 and RSK2 on the phosphorylation of β4-T1736 by siRNA-mediated knockdown (Fig. 6B). A small but reproducible reduction in the basal level of phosphorylation of β4-T1736 was observed when PA-JEB/β4 keratinocytes were transfected with a siRNA against RSK1, but not with one against RSK2. Moreover, simultaneous depletion of RSK1 and RSK2 did not reduce the basal level of β4-T1736 phosphorylation to a greater extent than depletion of RSK1 alone. Comparable effects of RSK1 and RSK2 depletion were obtained for β4-S1364 phosphorylation, except for a small reduction in β4-S1364 phosphorylation by RSK2 depletion. Overall, the effects of RSK1 and RSK2 depletion on the phosphorylation of the two β4 residues closely correspond to the activity of these kinases as measured by the levels of phosphorylated RSK at T359/S363 and S380. Interestingly, depletion of RSK1 either alone or together with RSK2 caused a reduction in the basal activity of ERK1/2. Since a similar reduction was seen after overexpression of wild-type RSK1 or RSK2 in PA-JEB/β4 keratinocytes (Fig. 6A), it further supports the notion that the expression of RSK and the activity of ERK1/2 are intimately connected.

While the treatment of cells with the lipid-based transfection reagent had no effect on the basal levels of β4-T1736 phosphorylation, it caused a substantial reduction in the EGF-stimulated phosphorylation of β4-T1736. However, a further reduction was seen when also RSK1 expression was depleted. In contrast, depletion of RSK2 had no or only little effect on the EGF-stimulated phosphorylation of β4-T1736, when compared

with that of the transfection reagent control. Furthermore, simultaneous depletion of RSK1 and RSK2 did not result in a greater reduction in the phosphorylation of β4-T1736 than is seen after depletion of RSK1 alone, indicating that the RSK2 activity remaining after RSK1 depletion was insufficient to induce phosphorylation of β4-T1736. In contrast, depletion of RSK2 caused a substantial reduction in the EGF-stimulated phosphorylation of β4-S1364 comparable to that of RSK1 depletion. Depletion of both RSK1 and RSK2 reduced the phosphorylation of β4-S1364 to almost basal levels. Thus, while the EGF-stimulated activation of RSK2 is insufficient to induce phosphorylation of T1736, it induces that of S1364. We conclude that both RSK1 and RSK2 are required for the basal and EGF-stimulated phosphorylation of β4-S1364, while RSK1 is mainly responsible for the phosphorylation of β4 at T1736.

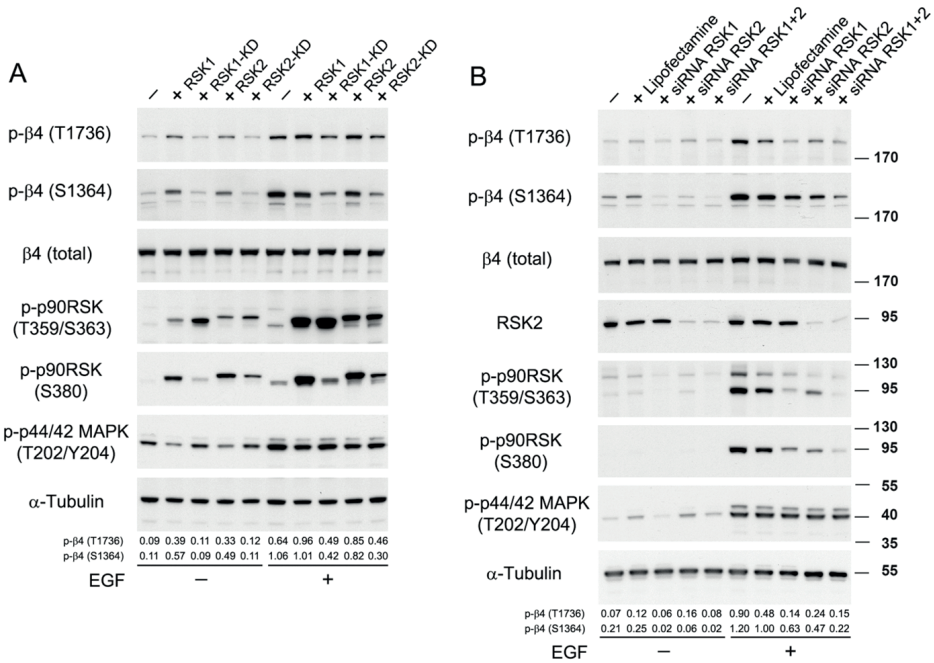


Figure 6 EGF-stimulated phosphorylation of β4 at T1736 is mediated by RSK1. *(A) PA-JEB/β4 keratinocytes, transiently overexpressing RSK1, RSK2, kinase-dead RSK1 or kinase-dead RSK2, were deprived of growth factors overnight and stimulated with EGF or left unstimulated. The cell lysates were analysed by immunoblotting with antibodies as indicated. (B) PA-JEB/β4 keratinocytes transfected with RSK1 and/or RSK2 siRNAs were deprived of growth factors overnight and stimulated with EGF or left unstimulated. The cell lysates were analysed by immunoblotting with the indicated antibodies. The values below the blots indicate the signal intensities for the phosphorylated β4-T1736 and β4-S1364 proteins after normalization to the level of α-tubulin.*

PI3K/Akt signaling pathway is not required for EGF-stimulated phosphorylation of β4

Although our findings clearly show that the MAPK signaling pathway is involved in the EGF-stimulated phosphorylation of β4 at T1736, they do not exclude the possibility that additional (EGFR activated) pathways also play a role in this phosphorylation event.

One major signaling pathway that relies on EGFR is the PI3K/Akt signaling pathway. This pathway plays a crucial role in cell growth and survival. We found that the PI3K/Akt signaling cascade is constitutively activated in PA-JEB/β4 keratinocytes, as indicated by the phosphorylation of Akt on S473 and T308 (Fig. 7, not shown). To investigate whether phosphorylation of β4 is dependent on sustained Akt activation, PA-JEB/β4 keratinocytes were treated with the PI3K inhibitor (GDC-0941) and the mTOR kinase inhibitor (AZD 8055). As shown in Fig. 7, stimulation of the PA-JEB/β4 keratinocytes with EGF led to an increase in the phosphorylation of β4 at T1736, which occurred irrespective of whether the cells had been treated with GDC-0941 or AZD 8055. Likewise, the two inhibitors did not antagonize the EGF-stimulated phosphorylation of T1736, which is further increased by the exposure of the keratinocytes to BAPTA-AM. These results indicate that sustained activation of the PI3K/Akt signaling pathway in PA-JEB/β4 is not required for phosphorylation of β4 at T1736.

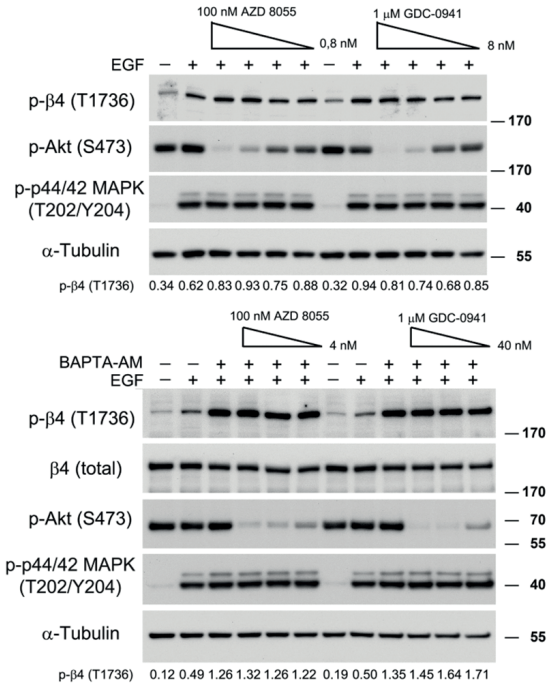


Figure 7 PI3K and mTOR are not required for EGF-mediated β4 phosphorylation. *PA-JEB/β4 keratinocytes were deprived of growth factors overnight, pre-treated with BAPTA-AM and/or different concentrations of the mTOR kinase inhibitor AZD 8055 (0,8–100 nM) or the PI3K inhibitor GDC-0941 (8 nM- 1 μM) and subsequently stimulated with EGF or left unstimulated. The cell lysates were analysed by immunoblotting with the indicated antibodies. The values below the blots indicate the signal intensities for the phosphorylated β4-T1736 after normalization to the level of α-tubulin.*

MAPK inhibition prevents EGF-induced HD dissolution

We next sought to establish the importance of the EGF-stimulated MAPK signaling pathway in the dissolution of HDs by determining the co-localization between plectin and integrin β4 in PA-JEB/β4 keratinocytes treated with EGF in the presence or absence of the MEK inhibitor UO126. As shown in Fig. 8, pre-treatment of PA-JEB/β4 keratinocytes with UO126 prevented the EGF-induced disassembly of HDs. Treated and untreated PA-JEB/β4 keratinocytes showed similar "cauliflower-like" patterns of HDs, while in the EGF-treated cells, not pre-treated with UO126, HDs appeared

more diffuse and their number was reduced. We conclude that inhibition of the MAPK signaling pathway prevents EGF-induced HD dissociation.

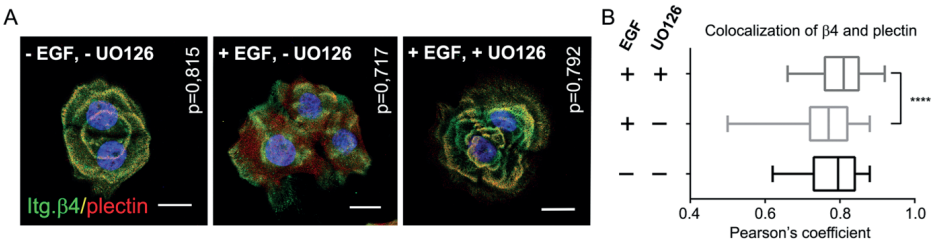


Figure 8 EGF-induced HD dissolution is dependent on MAPK signaling. *PA-JEB/β4 keratinocytes, seeded on coverslips, were deprived of growth factors overnight, pre-treated with the MEK inhibitor UO126 and/or stimulated with EGF. (A) PA-JEB/β4 keratinocytes were stained with mAbs 439-9B against β4 (green) and 31 against plectin (red). Cells were counterstained with DAPI (blue). Co-localization of β4 and plectin in HDs appears in yellow. Scale bars: 20 µm. (B) Co-localization between β4 and plectin was quantified using Pearson's correlation coefficient for each image. Box plots show median (solid line) and 25th and 75th percentiles (boxes) of the distribution for each condition (n = 4, ~20 images per experiment). ****P, <0.0001.*

Discussion

The interaction between integrin α6β4 and plectin, which is critical for the formation of HDs is dynamically regulated by phosphorylation of β4 at several residues. Two of these residues, S1356 and S1364, are located in the connecting segment of the β4 cytoplasmic domain and their phosphorylation by ERK1/2 and RSK1/2 prevents interaction of the ABD of plectin with β4 [68]. The phosphorylation of a third residue, T1736, located in the C-terminal end of the β4 cytoplasmic domain causes dissociation of β4 from the plakin domain, which is located next to the ABD of plectin [69]. In a previous study, we presented evidence suggesting that T1736 is directly phosphorylated by PKD1 in vitro [69]. In this study, we show that, although PA-JEB/β4 keratinocytes express all three PKD isoforms, PMA-induced phosphorylation of β4-T1736 is primarily mediated by PKD2. RT-qPCR analysis revealed that in PA-JEB/β4 keratinocytes the RNA levels of the three PKD isoforms differ greatly, with PKD3 being expressed at a much higher level than PKD2, while PKD1 is expressed at a relatively low level. The low level of PKD1 expression is not obvious from the reactivity of the anti-PKD antibody on Western blots. On these blots, the two PKD isoforms appear to be expressed at comparable levels. The discrepancy between the RNA and protein data may be explained by a different affinity of the antibody for the two PKD isoforms, i.e. the anti-PKD antibody may react more strongly with PKD1 than with PKD2. Interestingly, we found that despite the strong expression of PKD3 at the transcriptional level, the involvement of PKD3 in the phosphorylation of β4-T1736 is negligible. Why PKD3 is unable to efficiently phosphorylate β4-T1736 is not known. It may be due to a different subcellular localization of PKD3 and β4.

PKD activation by PMA in cells is mediated by "novel" Ca2+-independent PKCs through phosphorylation of S744/748 in the activation loop [410]. PA-JEB/β4 keratinocytes express three of the four novel PKCs, PKCε, PKCη and PKCδ, but only the latter seems to participate in the PKD-mediated phosphorylation of β4-T1736. PKC isoform-specific activation of PKD has been previously shown by Storz et al. [413]. These authors showed that in response to oxidative stress, PKCδ but not PKCε mediates PKD activation. Importantly, PKCε-mediated phosphorylation of PKD has been demonstrated in co-transfection experiments of PKD with an active form of PKCε [413–415]. Thus, the inability of PKCε to activate PKD cannot be simply attributed to an inability of the kinase to recognize its substrate. Rather, it seems that PKCε does not contribute significantly to PKD activation because of its low expression in PA-JEB/β4 keratinocytes.

There is evidence that PKCδ, but not PKCε or PKCη can directly phosphorylate β4 and downregulate its expression [416]. However, PKCδ does not mediate this effect through direct phosphorylation of β4-T1736, because depletion of PKD2, one of the downstream effector kinases of PKCδ, almost completely prevented the PMA-induced phosphorylation of β4-T1736.

In contrast to the PKD2-mediated phosphorylation of β4-T1736 after stimulation with PMA, the EGF-induced phosphorylation of this residue is not mediated by PKD2, but is under the control of the Ras-MAPK signaling pathway. We found that pharmacological inhibition of MEK1/2 or RSK using the inhibitor UO126 or the pan RSK inhibitor BI-D1870, respectively, reduced the EGF-induced phosphorylation of β4-T1736. Moreover, overexpression of RSK1 or RSK2 enhances phosphorylation of β4-T1736, whereas expression of kinase inactive RSK1 and RSK2 antagonizes this phosphorylation. Although these data suggest that both RSK1 and RSK2 can mediate phosphorylation of β4-T1736, studies with RSK1 and RSK2 RNA interference revealed that in PA-JEB/β4 keratinocytes phosphorylation of β4-T1736 is largely dependent on RSK1. This is in contrast to the phosphorylation of β4-S1364 that is dependent on both RSK1 and RSK2. Since no other effector kinases downstream of RSKs have been identified [417], we assume that RSK1 and RSK2 are the kinases that also directly phosphorylate β4-S1364, while RSK1 phosphorylates β4-T1736. In support of this assumption, all four RSKs have been identified as major candidate kinases that can phosphorylate S1364 and T1736 using the Kinexus PhosphoNet kinase predictor (http://www.phosphonet.ca/kinasepredictor). MNK1/2, like the RSKs, are downstream effector kinases of ERK1/2 and have not been identified as candidate protein kinases for the phosphorylation of β4-T1736 by the predictor. Correspondingly, we have not observed a reduction in EGF-stimulated phosphorylation of β4-T1736 with the MNK1/2 inhibitor CPG 57380 (unpublished data). In addition, we demonstrated that the PI3K pathway does not contribute to the phosphorylation of β4-T1736.

Although the RSK1 depletion and inhibition studies with BI-D1870 clearly implicate RSK1 as the sole kinase downstream of EGFR activation that phosphorylates T1736, the finding that phosphorylation of β4-T1736 is not completely inhibited by UO126 suggests that (an)other kinase(s) acting upstream of MEK1/2 may also play a role. Raf kinases which act upstream of MEK1/2 can be excluded as a candidate kinase because BI-D1870, the pan RSK inhibitor, that acts downstream MEK1/2 in the MAPK pathway, completely prevented EGF-stimulated phosphorylation of β4-T1736. It has been reported that UO126 may have other pharmacological effects independently of MEK inhibition, including the activation of protein kinases [418,419]. It is therefore possible that the phosphorylation of β4-T1736, remaining after MEK inhibition, is due to the activation of other kinases and is not part of the signaling pathways downstream of EGFR. We also observed a small reduction in the EGF-stimulated phosphorylation of β4-T1736 with Gő6983. However, because a similar reduction was seen of β4-S1364, we assume that this is due to non-specific inhibition of RSK by the PKC inhibitor Gő6983. A diagram summarizing the signaling pathways leading to β4 phosphorylation downstream of PKC and EGFR stimulation is presented in Fig. 9.

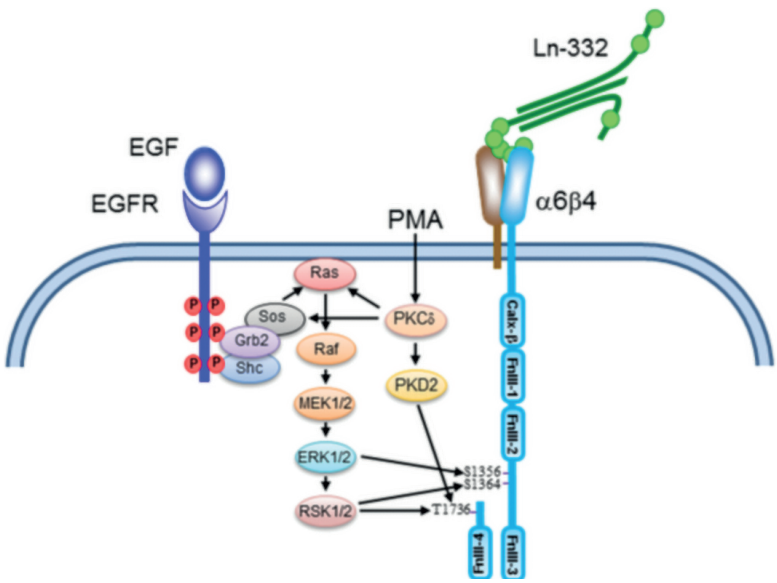


Figure 9 Signaling pathways controling β4 phosphorylation at serine and threonine residues.

Calcineurin is a serine/threonine phosphatase that is activated by the calcium/calmodulin complex [420]. Recent studies have implicated this phosphatase in the regulation of phosphorylation of β4-S1356 through a MAPK-dependent pathway [410]. In line with this study, we show that in the presence of the calcineurin inhibitors CsA and FK506 the PMA-stimulated phosphorylation of β4 was increased not only on S1356, but also on S1364 and T1736. However, the effects were relatively small and neither CsA nor FK506 increased the PMA-induced activation of ERK1/2. On the contrary, BAPTA-AM

markedly increased the PMA-stimulated phosphorylation of β4-T1736. In A431 cells, but not in PA-JEB/β4-keratinocytes, this increased phosphorylation was paralleled by an increase in ERK1/2 activation (Fig. 1D). In a previous study, it has been shown that chelation of [Ca2+]i with BAPTA-AM enhances and prolongs EGF-induced ERK1/2 activation in mouse embryo fibroblasts [421]. However, exposure of cells to BAPTA-AM in the absence of EGF had no effect on the activity of ERK1/2 [421]. We confirm these results in PA-JEB/β4 cells and additionally found that FK506 and CsA are also unable to induce ERK1/2 activation in the absence of EGF or PMA (unpublished data). As it has been suggested that the negative role of [Ca2+]i on the activation of ERK1/2 is caused by the induction of Ca2+-dependent expression of MAP kinase phosphatases [421], the applied period of EGF stimulation (10–15 min) might simply be too short to induce the expression of these phosphatases in PA-JEB/β4 keratinocytes. How calcineurin decreases β4 phosphorylation at T1736 is not known, but it may involve the activation of one or more phosphatases that directly dephosphorylate this residue. In this regard, it is perhaps worth mentioning that we have previously shown that treatment of cells with calyculin A, a potent protein phosphatase 2A (PP2A) inhibitor, strongly augments β4-T1736 phosphorylation [69,422].

Our finding, that RSK regulates the interaction between β4 and plectin via phosphorylation of T1736, combined with previous observations that ERK1/2 and RSK regulate the interaction between β4 and the ABD of plectin, implicate the ERK/MAPK signaling pathway as a crucial signaling cascade in keratinocyte migration through controlling HD stability. Indeed, inhibition of the MAPK pathway by UO126 prevents EGF-induced HD dissolution. Recent evidence suggests that HD stability may also be controlled by the ERK-MNK2 pathway through the phosphorylation of plectin at S4642 [71]. This phosphorylation attenuates the interaction of plectin with keratin filaments, and may lead to the destabilization of HDs, as supported by a study showing that in keratinocytes devoid of all keratins plectin is not localized in HDs [423]. In addition to a role of MAPK signaling as an upstream regulator of HD stability and keratinocyte migration, there is data suggesting that MAPK signaling can also be activated downstream of α6β4 and promote keratinocyte migration [85]. However, this activity of α6β4 has been linked to the binding of Shc to tyrosine phosphorylated β4 and may occur only in transformed keratinocytes, not in normal immortalized keratinocytes [6,89]. Indeed, we have found no evidence that β4 is tyrosine phosphorylated in PA-JEB/β4 keratinocytes upon EGF stimulation (unpublished data).

In conclusion, we provide evidence, that depending on the stimulus, PKD2 or RSK1 mediates phosphorylation of β4-T1736, a site that regulates the interaction of β4 with the plakin domain of plectin.

Materials and Methods

Antibodies

The generation of the rabbit polyclonal antibodies specific for phosphorylated β4 (T1736, S1364 or S1356) and the rabbit polyclonal antibodies recognizing the first pair of FNIII domains (residues 1115–1355) of β4 have been previously described [3,68,69]. The rabbit polyclonal antibodies against PKD1 (also reacting with PKD2), PKD3 (D57E6), phospho-PKD1 (S916), which crossreacts with phospho-PKD2, phospho-PKD (S744/748) recognizing the phosphorylated activation loop in PKD1/2/3, phospho-pan PKC (βII S660), RSK2 (D21B2), phospho-p90RSK (T359/S363), phospho-p90RSK (S380) and phospho-Akt (S473) and the rabbit monoclonal antibody (mAb) against phospho-p44/42 ERK1/2 (T202/Y204) were purchased from Cell Signaling. Antibodies against PKCδ (sc-937) and PKCε (sc-214) were from Santa Cruz Biotechnology. The rat mAb against integrin β4 (439-9B) and the mouse mAb against plectin (31/plectin) were from BD Bioscience. The mouse mAb against α-tubulin (clone B-5-1-2) was obtained from Sigma-Aldrich. Anti-mouse and anti-rabbit HRP-conjugated secondary antibodies were purchased from GE Healthcare, and TexasRed-conjugated goat anti-mouse and FITC-conjugated goat anti-rat were from Invitrogen.

Cell culture

PA-JEB immortalized keratinocytes were isolated from a patient with Pyloric Atresia associated with Junctional Epidermolysis Bullosa (PA-JEB) [13]. Since the derivation of the cell line was done for diagnostic purposes, the research using these cells was exempt of the requirement for ethical approval. PA-JEB/β4 keratinocytes stably expressing wild-type β4 were generated by retroviral transduction and maintained in serum-free keratinocyte medium (KGM; Invitrogen) supplemented with 50 μg/ml bovine pituitary gland extract, 5 ng/ml EGF, and antibiotics (100 units/ml streptomycin and 100 units/ml penicillin), as described previously [20]. A431 epidermoid carcinoma cells, obtained from the American Type Culture Collection (Manassas, VA), were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS), and antibiotics [424].

Immunofluorescence

PA-JEB/β4 cells were seeded on glass coverslips and cultured in either low (KGM; 0.09 mM Ca^{2+}) or high calcium (DMEM; 1.8 mM Ca^{2+}) medium for 24h, or cultured overnight in KGM medium deprived of growth factors, pre-treated with UO126 (10 μM; Calbiochem) for 1h and stimulated with 50 ng/ml EGF (Sigma-Aldrich) for 30 min. The cells were fixed with 1% paraformaldehyde and permeabilized with 0.2% Triton-X100 for 5 min. Cells were blocked with PBS containing 2.5% bovine serum albumin (Sigma-Aldrich) and incubated with the primary antibodies (mAb 31/Plectin (dilution 1:100; final concentration 2.5 μg/ml) and mAb 439-9B against β4 (dilution 1:100, final concentration 2 μg/ml)) for 1h. Cells were washed three times before

incubation with the secondary antibodies for 1h. After three wash-steps with PBS, the coverslips were mounted onto glass slides in Mowiol-DAPCO and studied by using a Leica TCS SP5 confocal microscope.

The degree of co-localization between β4 and plectin was quantified using the Pearson's correlation coefficient generated with the JaCoP macro, Image-J software [425]. Statistical significance was analysed using unpaired t-tests (corrected for multiple testing with the Bonferroni method) in SPSS and the graphs were made in GraphPad Prism 6.

cDNA constructs, siRNAs and transfection
The plasmids encoding human RSK1, mouse RSK2 and their kinase inactive mutants, RSK1 (K94/447R) and RSK2 (K100/541R), were kindly provided by Dr. J. Blenis (Harvard Medical School, Boston, MA). The RSK1 (SI02223060), RSK2 (SI00288190), PKD1 (SI00301350), PKD2 (SI02224768), PKCδ (SI02660539) and PKCη (SI02224082) siRNAs were purchased from QIAGEN and the PKCε siRNA (J-004653-08) was obtained from GE Healthcare. PA-JEB/β4 keratinocytes were transiently transfected with cDNAs or siRNAs using lipofectamine® 2000 (Invitrogen). Lipofectamine (14 µl/ml) and cDNA (10 µg/ml) or siRNA (0,5 µM) solutions in Opti-MEM were mixed (1:1) and incubated for 30 min at room temperature. Cells were incubated with the transfection solution for approximately 15h.

Western blotting
For western blot analysis, (transfected) PA-JEB/β4 keratinocytes and A431 cells were deprived of growth factors overnight in growth factor-free KGM and in DMEM without FCS, respectively. Cells were pre-treated with the calcium chelator BAPTA-AM (20 µM) and/or the phosphatase inhibitors FK506 (5–100 nM; Cell Signaling) or cyclosporin A (50–250 nM; Cell Signaling), the kinase inhibitor Gö6983 (100 nM—1 µM; Calbiochem), UO126 (2–10 µM; Calbiochem), BI-D1870 (1–20 µM; Enzo Life Sciences), GDC-0941 (8 nM-1 µM; Selleckchem), or AZD 8055 (0.8–100 nM; Selleckchem) for 30–60 min, before they were stimulated with 50 ng/ml EGF (Sigma-Aldrich) or 100 ng/ml PMA (Sigma-Aldrich) for 10 min. For studies with the calcium ionophore A23187, PA-JEB/β4 keratinocytes were grown in complete KGM (with pituitary gland extract and EGF) and pre-treated with BAPTA-AM (20 µM) and/or A23187 (1–5 µM; Sigma-Aldrich). Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with 1.5 mM Na_3VO_4, 15 mM NaF, 50 nM calyculin A (Cell Signaling) and a protease inhibitor cocktail (Sigma-Aldrich) and the lysates were cleared by centrifugation at 14.000 x g for 60 min at 4°C. Proteins were separated using NuPAGE or Bolt Novex 4–12% gradient Bis-Tris gels (Invitrogen), transferred to Immobilon-P transfer membranes (Millipore Corp), incubated with antibodies and visualized by chemiluminescence (GE Healthcare). Signal intensities were quantified using Image J and normalized to the level of α-tubulin or β4.

Reverse transcriptase-quantitative polymerase chain reaction
Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) analysis was performed in quadruplicate for determination of the levels of PKD and PKC mRNAs in PA-JEB/β4 keratinocytes. Cells were grown in complete KGM medium, or transfected and serum-starved as described above, and lysed in RNA-Bee (Tel-test Inc.) or Trizol® reagent (Invitrogen). Total RNA was separated from DNA and proteins by the addition of chloroform (Sigma-Aldrich) and subsequent centrifugation at 12.000 x g for 15 min at 4°C. The RNA was precipitated with isopropanol and washed with 70% ethanol. Integrity of the isolated RNA was assessed by agarose gel electrophoresis.

First strand cDNA synthesis was performed with 3 µg of total RNA using the first strand cDNA synthesis kit K1612 (Thermo Fisher Scientific) following manufacturer's directions. The PCR reactions were run using SYBR® Advantage® qPCR premix (Clontech) on a 7500 Fast Real-Time PCR system (Applied Biosystems) and the following primers were used: PKD1: forward 5'-GCATCTCGTTCCATCTGCAG-3', reverse 5'-AGGTAGGGTCATGGCGAAAA-3'; PKD2: forward 5'-AAGTTCCCTGAGTGTGGCTT-3', reverse 5'-GCAGTGATCACAGAAGGCAG-3'; PKD3: forward 5'-CGCCATGACATGAACTCAGA-3', reverse 5'-TCCTTGACGTACCAATCCCC-3'; PKCδ: forward 5'-AGCCGACCATGTATCCTGAG-3', reverse 5'-ACTGTTTGCAATCCACGTCC-3'; PKCε: forward 5'- CATCGATCTCTCAGGGTCGT-3', reverse 5'- CCGAAGATAGGTGGCCATGA-3'; PKCη: forward 5'-ACGAGGAGTTTTGCGCTAAC-3', reverse 5'-ATACTTTCCCCTCTGGCTCG-3'; PKCθ: forward 5'-TTGTCCAACTTTGACTGCGG-3', reverse 5'-GCATCAAAAGTGCTGTCCCA-3' and cyclophilin A: forward 5'-CATCTGCACTGCCAAGACTGA-3', reverse 5'-TTGCCAAACACCACATGCTT-3'.

In the presence or absence of a treatment control, relative mRNA quantities were obtained using the 2-ΔΔCt or 2-ΔCt method, respectively. The ΔCt is the obtained Ct value of PKD or PKC minus the obtained Ct value of cyclophilin A (endogenous control). Graphs were made, and statistical significance was analysed using paired t-tests, in GraphPad Prism 6.

Acknowledgments

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CHAPTER 4

EGFR-dependent tyrosine phosphorylation of integrin β4 is not required for downstream signaling events in cancer cell lines

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Manuscript in revision

Abstract

In epithelial cancers, the epidermal growth factor receptor (EGFR) and integrin α6β4 are frequently overexpressed and found to synergistically activate intracellular signaling pathways that promote cell proliferation and migration. In cancer cells, the β4 subunit is phosphorylated at tyrosine residues not normally recognized as kinase substrates; however, the function of these phosphotyrosine residues in cancer cells is a subject of much debate. In EGFR-overexpressing carcinoma cells, we found that the Src family kinase (SFK) inhibitor PP2 reduces β4 tyrosine phosphorylation following the activation of EGFR. However, siRNA mediated knockdown of the SFKs Src, Fyn, Yes and Lyn, individually or in combination, did not affect the EGF-induced phosphorylation of β4. Using phospho-peptide affinity chromatography and mass spectrometry, we found that PLCγ1 binds β4 at the phosphorylated residues Y1422/Y1440, but were unable to verify this interaction in A431 carcinoma cells that overexpress the EGFR. Furthermore, using A431 cells devoid of β4 or reconstituted with phenylalanine specific mutants of β4, the activation of several downstream signaling pathways, including PLCγ/PKC, MAPK and PI3K/Akt, were not substantially affected. We conclude that tyrosine-phosphorylated β4 does not enhance EGFR-mediated signaling in EGFR-overexpressing cells, despite the fact that this integrin subunit is highly tyrosine phosphorylated in these cells.

Introduction

Cellular behavior is regulated by a multitude of intracellular signaling pathways. These signaling pathways, which are activated by environmental cues such as hormones or growth factors (GFs), regulate proliferation, survival and migration of the cells [103]. In tumor cells, normal signaling is frequently impaired due to increased expression of or mutations in receptor-tyrosine kinases (RTKs), such as the epidermal growth factor receptor (EGFR) (reviewed in [426]). Additionally, the expression of integrins, which are cell surface receptors that mediate interaction between cells and the extracellular matrix, is frequently deregulated in tumor cells (reviewed in [427]).

Integrin α6β4, which is a receptor for laminin, is expressed by a variety of epithelial tissues and cell types [428]. In stratified and pseudostratified epithelia, the integrin is a major component of hemidesmosomes [429], in which it mediates anchorage of cytoskeletal intermediate filaments to the plasma membrane through interaction with the plakin family members BP230 and plectin [1,2]. Overexpression and mislocalization of this integrin has been correlated with cancer progression and poor prognosis of multiple tumor types (reviewed in [8-10]). Evidence suggests that integrin α6β4 contributes to cancer progression and cell migration by promoting PI3K/Akt and Ras/MAPK signaling in cells [283,284,287,433–437]. However, how β4 contributes to the activation of intracellular signaling pathways is still a point of discussion. Upon binding to its ligand, clustering of β4 and cross-phosphorylation of β4 by non-receptor tyrosine kinases has been shown to contribute to downstream signaling in carcinomas [438]. Alternatively, part of β4's contribution to signaling could occur upon RTK activation, such as the EGFR [272,277].

The EGFR is a RTK that is activated by binding of its ligand EGF. It activates multiple signaling pathways, including the PI3K/Akt and MAPK signaling pathways [439]. Furthermore, in some cell types, EGF also activates STAT3 and PLCγ/PKC signaling pathways [440–442]. Some of the kinases activated downstream of the EGFR, are involved in the phosphorylation of residues in the cytoplasmic domain of β4. The bulk of these residues reside in the connecting segment (CS) between the two pairs of fibronectin type III (FnIII) domains (FnIII-1,2 and FnIII-3,4), although some sites in FnIII-3,4 and in the C-terminal tail of β4 have been reported to be phosphorylated as well [443].

Phosphorylation of β4 on serines 1356, 1360, 1364 and 1424 in the CS, and threonine 1736 in the C-tail promotes HD disassembly by interfering with β4-plectin and β4-BP180/BP230 binding [68–70,72,397]. On the contrary, phosphorylation of β4 on tyrosines,Y1422 and Y1440 in the CS, Y1257 in FnIII-2 and Y1494, Y1526 and Y1642 in FnIII-3,4, has been suggested to play a role in the activation of the PI3K/Akt and MAPK signaling pathways, via binding and activation of adaptor proteins, like Shc and IRS1

[85,91,277,432,433,437,444]. However, the importance of β4 tyrosine phosphorylation for cancer progression is unclear which is primarily due to the use of various cell types and assays (2D, 3D or *in vivo*) [445].

In this paper we investigated the crosstalk between integrin β4 and the EGFR in the activation of cancer promoting signaling pathways in a variety of cell lines. Special attention was paid to the role of the tyrosine residues in the β4 CS, since their role in growth factor-stimulated signaling pathways has remained controversial. Our studies confirm previous findings that β4 is tyrosine phosphorylated in A431 cells overexpressing the EGFR, but excluded a role of Src family kinases (SFKs) in the EGFR-stimulated phosphorylation of β4. Furthermore, we demonstrate that the phosphorylated residues Y1422 and 1440 in β4 can function as binding sites for PLCγ1 *in vitro*, but that they do not contribute substantially to signaling from the EGFR to the PLCγ1/PKC, PI3K/Akt and MAPK pathways in cells.

Results

EGF-mediated tyrosine phosphorylation of β4 is correlated with EGFR overexpression

To investigate possible crosstalk of integrin β4 with the EGFR in the promotion of cancer signaling, we first characterized EGFR-mediated β4 tyrosine phosphorylation in multiple benign and malignant epithelial cell lines derived from breast, colon and skin tissues. By analyzing whole cell lysates of the different cell lines by western blot, we observed that the total β4 and EGFR expression levels varied strongly among the different cell lines. Furthermore, the expression levels of β4 and EGFR do not appear to be related to each other or to their tissue of origin. However, we did observe a clear relationship between tyrosine phosphorylation of β4 and the levels of EGFR in cells treated with EGF (Fig. 1A). This effect was independent of the tissue of origin, since the two cell lines which had the highest levels of EGFR expression and β4 tyrosine phosphorylation, Difi and A431, were derived from colon and skin tissue, respectively (Fig. 1A,B). Furthermore, these assays showed that β4 tyrosine phosphorylation occurred specifically in the presence of EGF, since tyrosine phosphorylation of β4 was not observed in serum-starved cells (Fig. 1A; unpublished data).

In conclusion, the amount of β4 that is tyrosine phosphorylated is independent of tissue origin and the total amount of β4 in the cells, but is dependent on the presence of EGF and correlated with the amount of EGFR in the cells.

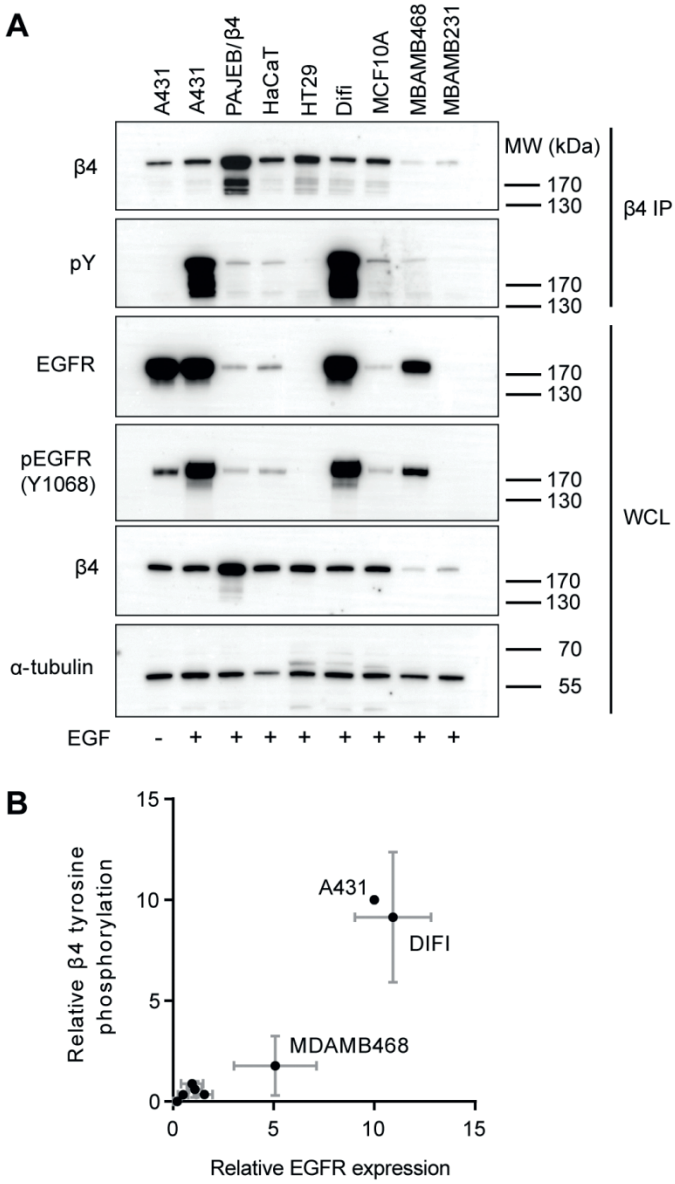


Figure 1 EGF-mediated tyrosine phosphorylation of β4 occurs in EGFR overexpressing cell lines. *(A) WCL and β4 IP samples of multiple transformed and untransformed cell lines, expressing β4 and EGFR at different levels, treated with EGF for 1,5 min after 20h of serum starvation, were analyzed by WB for total β4 levels, tyrosine phosphorylation of β4, total EGFR levels, tyrosine phosphorylation of EGFR and α-tubulin levels (loading control). (B) The protein and tyrosine abundances were quantified, normalized to the levels of A431 (at 10), and visualized in a graph. β4 tyrosine phosphorylation (IP) is normalized to total β4 levels (IP) on the Y-axis, and total EGFR levels (WCL) were normalized to α-tubulin levels (WCL) on the X-axis. The graph shows the mean + standard deviation (SD) of 3 independent experiments.*

β4 tyrosine phosphorylation occurs abundantly on the β4 connecting segment
Multiple tyrosine phosphorylation sites have been identified in the CS of β4 [443]. To study which of these sites are phosphorylated downstream of EGFR activation and whether they play a role in β4 signaling in conjunction with the EGFR, we generated constructs in which specific tyrosines in the β4 CS were mutated to phenylalanines (Fig. 2A). Firstly, the 2Y14F and the 4Y-F β4 mutants were transiently expressed in COS7 cells and β4 tyrosine phosphorylation induced by EGF was analyzed (Fig. 2B). In the 2Y14F mutant, in which two well-studied tyrosine residues, Y1422 and Y1440, were mutated, tyrosine phosphorylation of β4 was clearly reduced. But in the 4Y-F mutant in which Y1343 and Y1349 were mutated in addition to Y1422 and Y1440, β4 tyrosine phosphorylation was almost undetectable (Fig. 2B). This suggests that in addition to Y1422 and Y1440, these other CS tyrosines are phosphorylated upon EGFR activation. The Y1343 and Y1349 sites were identified as tyrosine phosphorylation sites on β4 in several mass spectrometry data sets [443], but have not yet been studied using cell biological assays.

To examine the extent and contribution of the different tyrosine phosphorylation sites in the CS of β4 at endogenous levels, we depleted β4 in A431 cells using CRISPR-Cas9 and reintroduced wild-type β4 or the different β4 tyrosine mutants, including the 2Y13F mutant in which only the Y1343 and Y1349 have been mutated, in these knockout cells. FACS analysis showed that the surface levels of β4 in the stably transformed cells expressing wild-type or mutant β4 proteins were comparable to one another and to the endogenous surface levels of β4 on A431 cells (Fig. 2C). Furthermore, using phospho-site specific antibodies, we confirmed the absence of tyrosine phosphorylation on Y1440 and Y1422 in the 2Y14F and 4Y-F mutants, but not in the 2Y13F mutant (Fig. 2D). All β4 mutants co-localized with plectin in hemidesmosomal-like adhesion structures similarly to wild type β4. By contrast, β4 did not obviously co-localize with the EGFR at these adhesion structures, although the two proteins were found in close proximity of each other. Intriguingly, co-localization between β4 and the EGFR was occasionally observed at the lateral membranes of A431 cells (Fig. 2E). After treatment of the stably transformed cell lines with EGF, the levels of total tyrosine phosphorylation of β4 were assessed by western blot with anti-phosphotyrosine antibodies (Fig. 2F). Quantification of the relative amount of β4 tyrosine phosphorylation confirmed that mutation of all four tyrosines drastically reduced the total amount of tyrosine phosphorylation of β4 and that in addition to Y1422 and Y1440, the Y1343 and Y1349 sites also contributed to β4 tyrosine phosphorylation (Fig. 2F, G). However, the data also shows that some residual tyrosine phosphorylation of β4 remains in the 4Y-F mutant, which suggests that additional sites such as Y1399, the only tyrosine left untouched in the β4-CS, are phosphorylated as well.

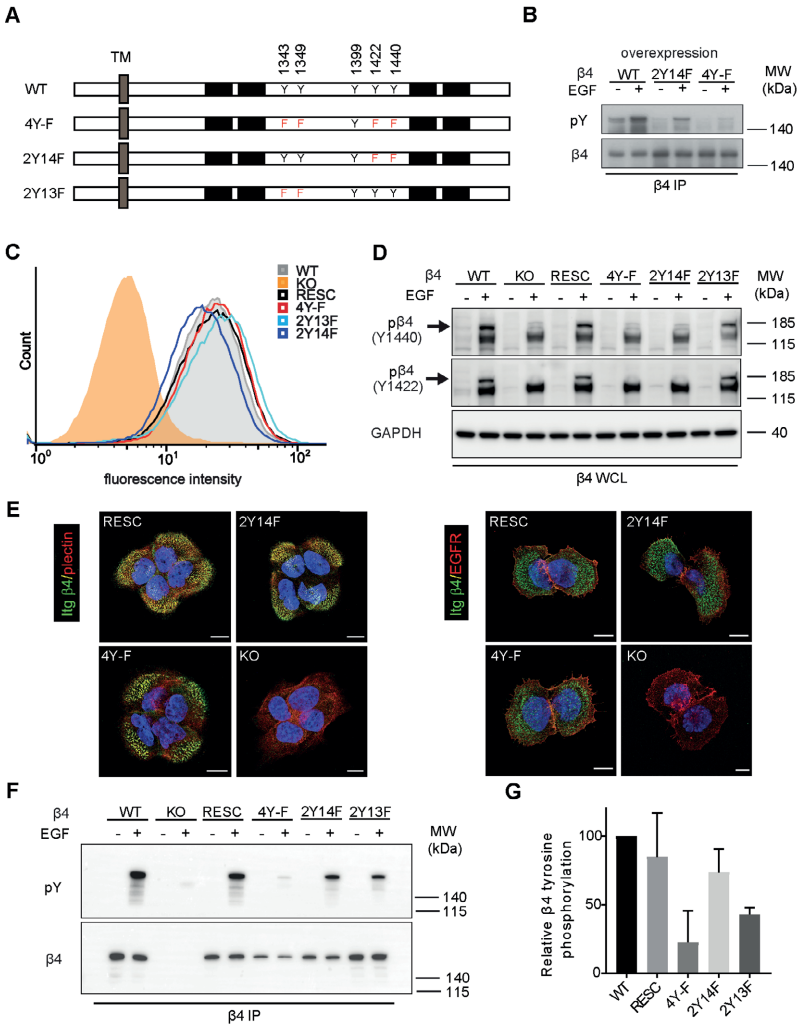


Figure 2 β4 phosphorylation occurs abundantly on the β4 CS. *(A) Representation of the tyrosines (Y) present and mutated to phenylalanines (F) in the CS of the various β4 mutants (4Y-F, 2Y14F and 2Y13F) and wild type (WT) β4. (B) EGF-induced β4 tyrosine phosphorylation in β4 tyrosine mutants shown by WB analyses of β4 IP samples of EGF-treated and untreated COS7 cell overexpressing WT and mutant β4. Blots were probed for β4 and phospho-tyrosine (pY). (C) Comparable β4 surface levels in A431 cell lines analyzed by FACS. The A431 cell lines analyzed are WT; expressing endogenous β4, KO; depleted from endogenous β4 by CRISPR-Cas9, RESC; β4KO cells reconstituted with WT β4 and 4Y-F, 2Y13F and 2Y14F β4 mutants; β4KO cells reconstituted with mutated β4. (D) Representative WB analyses of WCLs of the various A431 cells for phosphorylation of β4 at Y1440 and Y1422 using phosphosite-specific antibodies. Cells were untreated or treated with EGF for 1,5 min after 20h serum starvation. (E) Double immunofluorescence staining for β4 with plectin or the EGFR in β4KO A431 cells and β4KO cells reconstituted with WT β4, 4Y-F or 2YF14 β4. Scale bars: 10 µm. (F) Representative WB analyses of β4 tyrosine phosphorylation and total β4 levels in β4 IP samples obtained from the various A431 cells untreated or treated with EGF for 1,5 min after 20h serum starvation. (G) Quantification of β4 tyrosine phosphorylation levels in the various A431 cell lines, per sample normalized to total β4 levels and per experiment to the pY/β4 WT levels. Graphs shows the mean + SD from 3 independent experiments.*

Src family kinases do not contribute to β4 tyrosine phosphorylation

To examine which tyrosine kinase phosphorylates β4 upon EGFR activation in A431 cells, we focused our investigations on the Src family kinases (SFKs), which previously have been shown to be involved in tyrosine phosphorylation of β4 [277,278]. Treatment of A431 cells with the SFK inhibitor PP2 showed a concentration dependent inhibition of EGF-induced tyrosine phosphorylation of β4. Such a reduction was not observed with the PP3 control (Fig. 3A). Since the PP2 inhibitor inhibits multiple SFKs, we performed siRNA-mediated knock-down of Src, Fyn and Yes, the most common SFKs [446], and tested whether their knock-down has an effect on β4 tyrosine phosphorylation. WB analysis showed a strong reduction of Src, Fyn or Yes after transfection with their respective siRNAs, but total tyrosine phosphorylation of β4 was not reduced. Furthermore, although a small reduction in the phosphorylation of Y1422 and Y1440 was observed in the absence of Fyn or Yes (Fig. 3B), a decrease in the phosphorylation of these residues was not observed when expression of both Fyn and Yes were simultaneously reduced in the same cell (Fig. 3D).

Expression analysis by quantitative RT-PCR showed that, in addition to Src, Fyn and Yes, A431 cells express the SFK Lyn but not Lck, Fgr, Hck and Blk (Fig. 3C). A common feature of α6β4, EGFR and the SFKs Fyn, Yes and Lyn is that these proteins are all palmitoylated and thus could be together in cholesterol- and sphingolipid rich, liquid ordered membrane domains [382,447–450]. Therefore, it was of interest to also study the contribution of Lyn to β4 tyrosine phosphorylation. siRNA mediated silencing of Lyn alone or in combination with Fyn, or Fyn and Yes, however, did not reduce the phosphorylation on β4 at Y1422 or Y1440 (Fig. 3D).

In conclusion, our data show that although the PP2 inhibitor reduces tyrosine phosphorylation of β4 upon EGFR activation, Src and the palmitoylated SFKs, Fyn, Yes and Lyn are not essential for tyrosine phosphorylation of β4 (at Y1422 or Y1440).

β4 Y1422 and Y1440 provide a docking platform for PLCγ1

In order for β4 to use tyrosine phosphorylation for intracellular signaling, it must bind proteins that contain a phospho-tyrosine binding domain, such as an SH2 or PTB domain. The Y1422 and Y1440 sites have been reported to bind the intracellular signaling adapter protein Shc [279,283]. To investigate the interactions of the phosphorylated Y1422 and Y1440 residues in keratinocytes, we performed peptide pull down experiments from lysates of PA-JEB/β4 keratinocytes with phosphorylated and unphosphorylated peptides containing Y1422, Y1440, or Y1494, a non-CS-tyrosine residue which previously has been shown to regulate multiple signaling pathways important for tumor development and progression [91,403,437,451]. By SDS gel electrophoresis, we observed a prominent 135 kDa protein in the pulldowns with phosphorylated Y1440 (pY1440). A band of similar molecular weight is also observed in the pulldown with the phosphorylated Y1422 (pY1422) peptide, but not

with phosphorylated Y1494 (pY1494) or the corresponding unphosphorylated peptides (Fig. 4A). Mass spectrophotometry analysis revealed this band as PLCγ1.

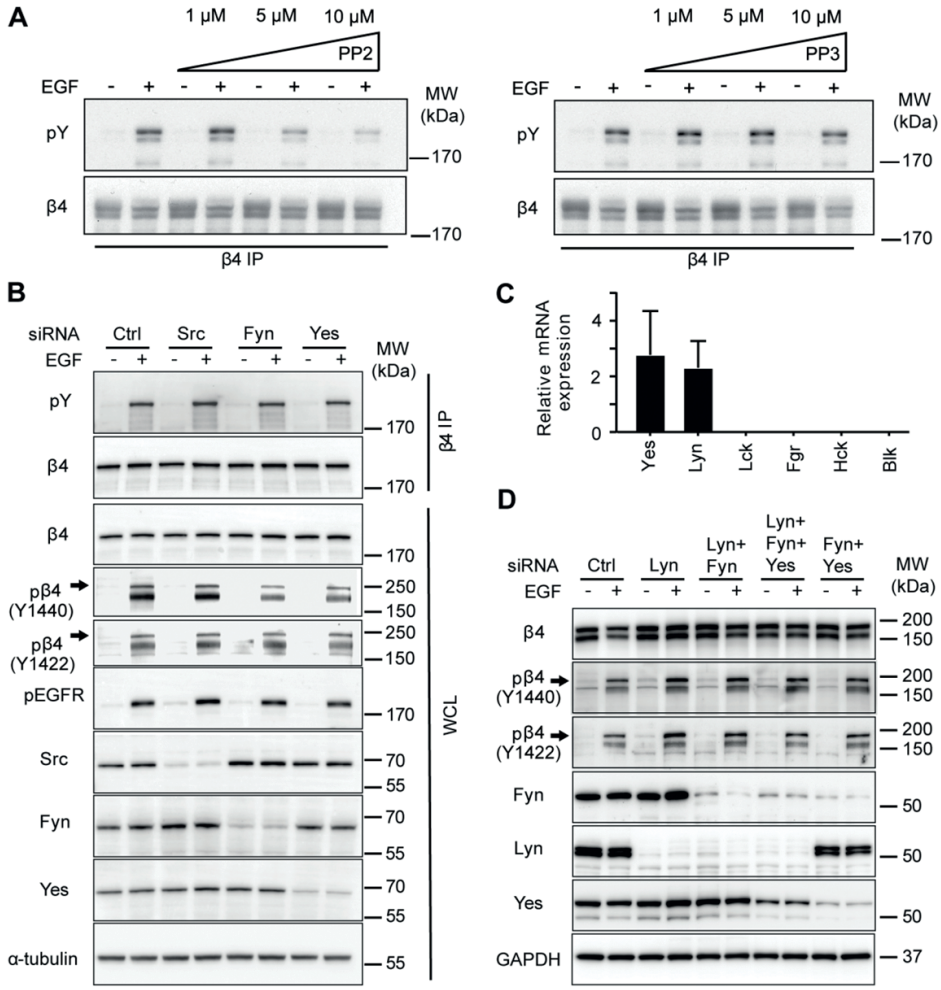


Figure 3 Effects of PP2 and siRNA-mediated knockdown of SFKs on the tyrosine phosphorylation of β4. *(A) WB analyses of β4 tyrosine phosphorylation and total β4 levels in β4 IP samples of A431-β4-GFP (expressing both endogenous β4 and β4GFP) cells untreated or treated for 5 min with EGF, after treatment with 0, 1, 5 or 10 μM PP2 or PP3. (B) WB analyses of β4 IP and WCL samples of WT A431 cells transfected with siRNAs for Src, Fyn and Yes and Ctrl siRNAs, untreated or treated for 5 min with EGF. The blots were proped for pY, total β4, pβ4 (Y1440), pβ4 (Y1422), pEGFR (Y1068), Src, Fyn, Yes and α-tubulin (loading control). (C) Relative mRNA expression of Yes, Lyn, Frk, Lck, Fgr, Hck and Blk SFKs in WT A431 cells analyzed by RT-PCR. Graph shows the mean + SD of 3 independent experiments. (D) WB analyses of WCL samples of WT A431 cells transfected with siRNAs for Fyn, Lyn and/or Yes or Ctrl siRNAs, untreated or treated for 5 min with EGF. The blots were proped for β4, pβ4 (Y1440), pβ4 (Y1422), Fyn, Yes, Lyn and GAPDH (loading control).*

PLCγ1 contains two tandem SH2 domains, which likely are responsible for the binding of this phospholipase to the two phosphorylated tyrosine residues in β4. By analyzing similar pulldown experiments using western blot, we confirmed the interaction of PLCγ1, but not Shc, with pY1422 and pY1440 (Fig. 4B). Additionally, we showed that both Shc and PLCγ1 bind the phosphorylated peptide Y1526 (pY1526), which resides in FnIII-3 of β4. However, the crystal structure of the FnIII-3 domain revealed that the structural environment of Y1526 is incompatible with binding SH2 or PTB domains [404]. Hence, it is not likely that either Shc or PLCγ1 can bind this phosphorylated tyrosine residue in the context of a competently folded FnIII-3 domain. To further study the interaction of PLCγ1 (and Shc) with integrin β4 under more physiologically relevant conditions, we used untreated and EGF-treated A431 cells and performed β4 immunoprecipitations (Fig. 4C). Under these conditions, as well as in cells exogenously overexpressing integrin β4 (unpublished data), we were unable to confirm its interaction with either Shc or PLCγ1. In contrast, we could confirm the interaction of Shc and PLCγ1 with the phosphorylated EGFR.

In conclusion, we show that phospho-peptides based on the β4-sequence containing residues Y1440 and Y1422 associate with PLCγ1, but not Shc, in pull down assays. However, we were unable to detect an association between PLCγ1 and β4 in A431 cells [452].

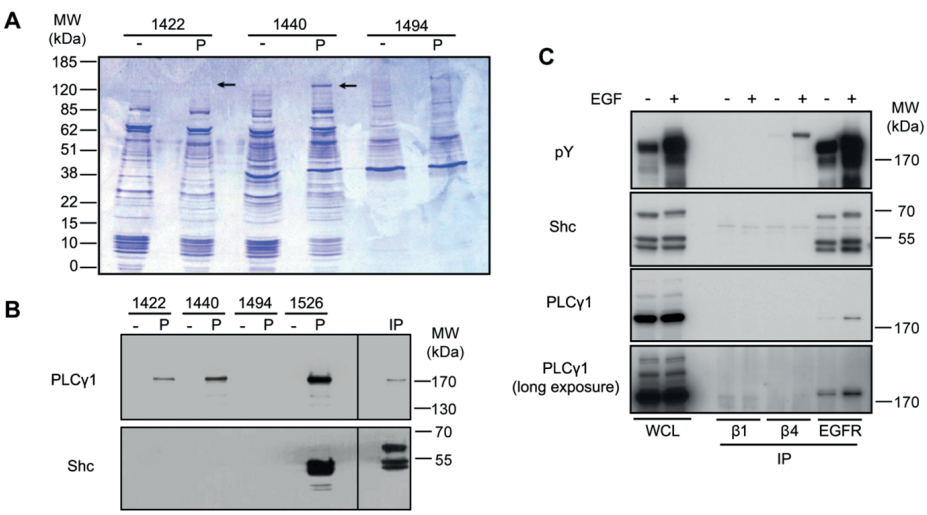


Figure 4 β4 tyrosine phosphorylation might provide a docking platform for PLCγ1 *(A) A431 cells were lysed and different synthetic phosphorylated (P) and unphosphorylated (-) peptides derived from β4 were used for IP. IPs were separated on gel and stained with Coomassie blue. The arrows indicate the protein band identified by mass spectrometry as PLCγ1. (B) A431 cells were lysed and different phosphorylated (P) and unphosphorylated (-) synthetic peptides derived from β4 were used for IP. IP samples were analyzed by WB for Shc and PLCγ1. (C) A431 cells were serum starved and subsequently treated with EGF for 10 min. A431 WCL and β1, β4 and EGFR IP samples were analyzed by WB for phospho tyrosine, Shc and PLCγ1. Molecular weight (MW) markers are indicated.*

β4 and its tyrosine phosphorylation does not alter downstream signaling of the EGFR

To investigate whether tyrosine phosphorylation of the β4-CS contributes to EGF-induced signal transduction, we compared the activation of signaling molecules in pathways downstream from the EGFR in β4 knockout A431 cells reconstituted with wild-type or mutant β4 proteins. By western blot analysis, we observed no differences in the level of EGFR phosphorylation between β4 knockout cells and reconstituted cells, suggesting that β4 does not modulate EGFR kinase activity. ERK1/2 and Akt, two well-known downstream signaling molecules of the EGFR, were also equally activated among the different cell lines (Fig. 5A). The same results were observed when β4 proficient and deficient cells were treated for different periods of time with EGF (Fig. 5B). Furthermore, although we identified PLCγ1 as a potential binding partner of tyrosine phosphorylated β4, PLCγ1 and PKC (downstream kinase of PLCγ1) activation downstream of the EGFR receptor were not dependent on tyrosine phosphorylation of β4 (Fig. 5A,B). These results show that neither β4 nor its tyrosine phosphorylation substantially affect EGFR activity or downstream signaling events in A431 cells.

To investigate whether β4 affects EGFR-mediated signaling pathways in other cell types, we first characterized the signaling pathways that are induced by EGF in multiple benign and malignant cell lines. All cell lines, except MDA-MB-231 cells and DiFi rectal carcinoma, responded robustly to EGF stimulation by phosphorylating ERK1/2 (Fig. 5C). In contrast, activation of the PI3K/Akt and PLCγ1 signaling pathways varied considerably among the cell lines. While some cell lines already had a high basal PI3K/Akt activity, which cannot be further induced by EGF stimulation, others displayed a clear increase in the levels of pAkt in response to EGF stimulation. Strong PLCγ1 phosphorylation was observed in EGF-stimulated A431 and Difi cells, which both overexpress the EGFR (Figs. 1A and 5C). To assess the contribution of β4 to EGFR signaling, we selected two non-cancerous cell lines PA-JEB/β4 and HaCat keratinocytes, which displayed only moderate levels of PLCγ1 phosphorylation upon EGF stimulation, but differed in the degree of constitutive phosphorylation of Akt. Comparison of the effects of β4 expression on the phosphorylation of PLCγ/PKC signaling pathway components, as well as those of the MAPK/ERK and PI3K/Akt signaling pathways, did not reveal any detectable difference in their phosphorylation status irrespective of EGF treatment (Fig. 5D).

In conclusion, we show that signaling downstream of the EGFR differs between cell lines, but β4 or its tyrosine phosphorylation does not affect the signaling of the EGFR, neither in cancer cells nor in non-cancerous epithelial cells.

EGFR activation in A431 cells promotes growth-arrest rather than proliferation

Fetal calf serum (FCS)-containing medium is used to grow and maintain A431 cells in the laboratory setting and endogenous bovine EGF within the serum may contribute

to the EGFR-β4 signaling axis. Therefore, we next investigated the effects of serum on the activation of the EGFR and phosphorylation of the integrin β4 subunit in A431 cells. Unlike EGF, serum treatment of serum-starved A431-β4 WT cells (A431 cells reconstituted with wild-type β4) did not result in phosphorylation of the

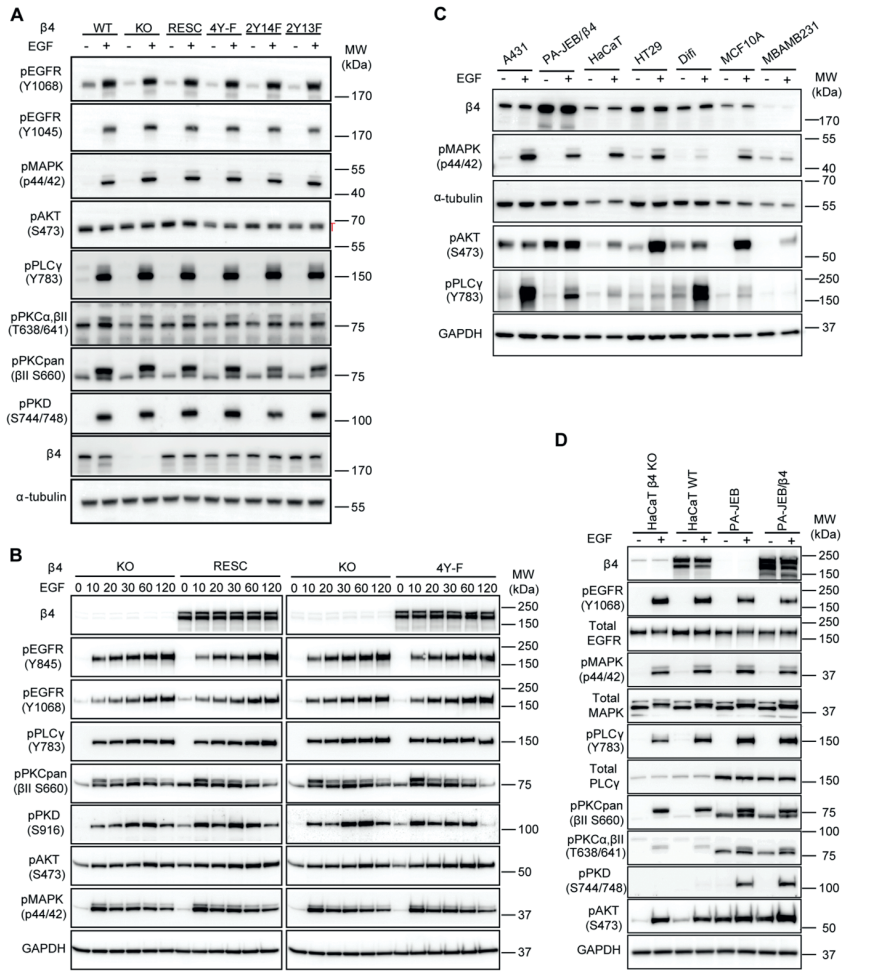


Figure 5 β4 and its tyrosine phosphorylation at the CS does not alter signaling downstream of the EGFR. *(A) WB analyses of various WT and β4 mutant A431 cell line WCLs, untreated or treated for5 min with EGF after 20h serum starvation, for pEGFR (Y1068 and Y1045), pMAPK, pAKT, pPLCγ1, pPKCα,β II (T638/641), pPKCpan (βII S660), pPKD (S744/748), β4 and α-tubulin (loading control). (B) WB analyses of A431 WT, β4 KO and β4 4Y-F cells untreated or treated for different timepoints (0, 10, 20, 30, 60 and 120 min) with EGF after 20h serum starvation, for β4, pEGFR, pPLCγ1, pPKCpan (βII S660), pPKD (S916), pAKT, pMAPK and GAPDH (loading control). (C) WB analyses of A431, PA-JEB/β4, HaCaT, HT29, Difi, MCF10A, MBAMB231 cells, untreated and treated with EGF for 1,5 min after 20h serum starvation, for β4, pMAPK, pAKT, pPLCγ1, α-tubulin and GAPDH (loading controls). (D) WB analyses of PA-JEB/β4 and HaCaT cells, β4 proficient and deficient, treated with or without EGF for 1,5 min after 20h serum starvation, for β4, pEGFR, total EGFR, pMAPK, total MAPK, pPLCγ1, total pPLCγ1, pPKCpan (βII S660), pPKCα,β II (T638/641), pPKD (S744/748), pAKT and GAPDH (loading control).*

EGFR and the integrin β4 subunit at Y1422 and Y1440 (Fig. 6A, B). By contrast, PKC and PKD phosphorylation was strongly induced after serum stimulation which indicates that serum factors, other than EGF, are sufficient for their induction. Serum treatment also increased the levels of phosphorylated ERK and Akt. However, while EGF stimulated the phosphorylation of ERK more strongly than serum, the opposite effect was observed for Akt phosphorylation. Intriguingly, phosphorylation of PLCγ and Shc was not increased following serum addition, indicating that serum-induced activation of PKC and PKD is not dependent on PLCγ and Shc. Besides serum, we also tested the contribution of HGF and PDGF at intracellular signaling in A431 cells. Treatment with HGF resulted in an increase in the phosphorylation of Akt and ERK1/2, but did not induce the phosphorylation of β4 at Y1422 and Y1440. Consistent with A431 cells lacking the PDGF receptor, these cells also did not respond to the treatment with PDGF.

We also examined the phosphorylation of β4 in A431-β4 WT cells grown in regular growth medium (DMEM supplemented with 10% FCS) both before and after EGF addition. Similar to our findings after serum treatment of serum-starved cells, tyrosine phosphorylation of the EGFR and β4 was not detected in A431-β4 WT cells grown in regular growth medium in the absence of exogenous EGF. However, similar to the serum-starved cells, EGF treatment induces the robust phosphorylation of the EGFR and β4 subunit and increases activation of downstream signaling pathways found downstream of the EGFR. Only, phosphorylation of Akt, a critical effector of the EGFR signaling cascade, was not enhanced, probably because this phosphorylation was already maximal in A431-β4 WT cells grown in regular growth medium. Together these findings indicate that serum factors do not induce EGFR activation or phosphorylation of β4 at Y1422 and Y1440. However, serum factors are responsible for the increase in ERK, Akt, PKC and PKD phosphorylation observed in A431 cells treated with serum.

Next, we investigated the effect of EGF on the proliferation of the A431-β4 WT cells. In these analyses we also included A431 cells reconstituted with β4-4YF and -2Y14F to assess possible effects of EGF-induced tyrosine phosphorylation of β4 on cell proliferation. Previous studies have reported a growth inhibitory effect of EGF for A431 cells at doses that are mitogenic in many other cells [453–455]. Consistent with these findings, the addition of EGF to the growth medium strongly decreased the proliferation of A431 cells (Fig. 6C). Because the growth inhibitory effects by EGF were similar in the three cell lines, and the cell lines exhibited comparable growth in the absence of EGF, we conclude that β4 phosphorylation by EGFR activation has no role in the proliferation of A431 cells in serum-containing medium.

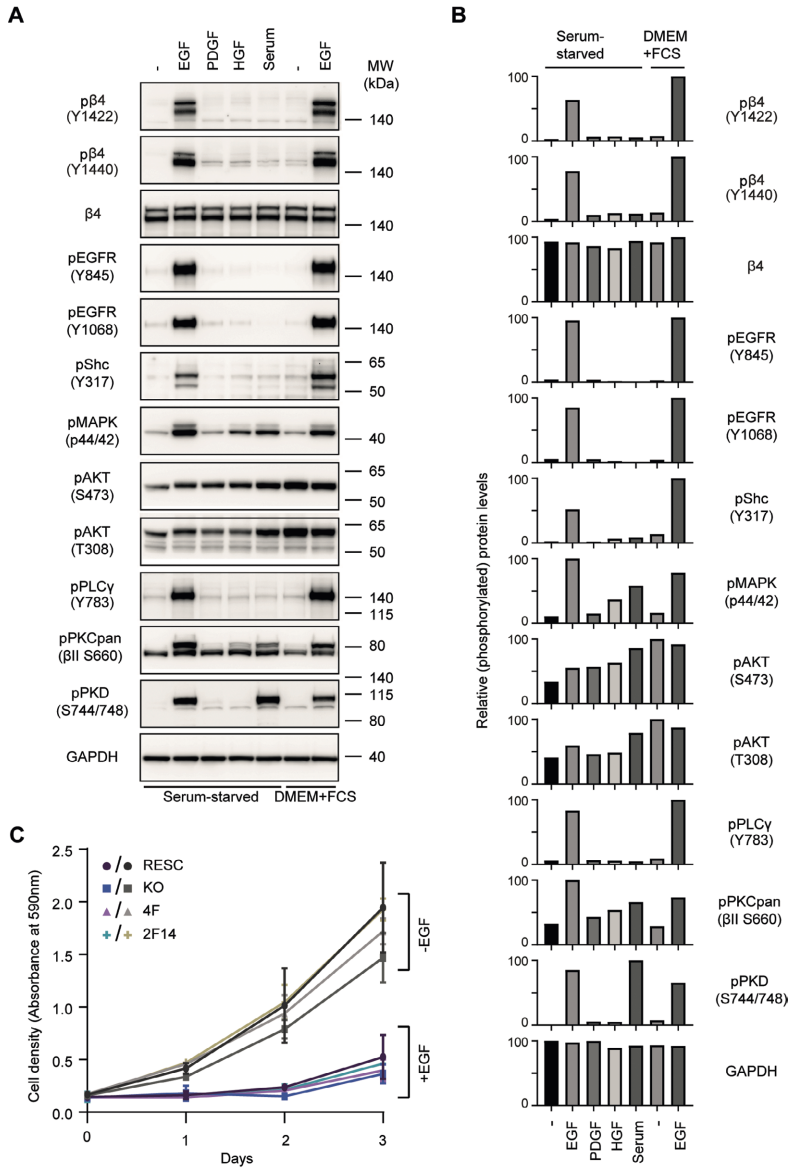


Figure 6 Inhibition of A431 cell proliferation by EGF. *(A) A431-β4 KO cells reconstituted with WT β4 (RESC) grown in DMEM/FCS and serum-starved for 20 hrs were left untreated or treated with 50 ng ml⁻¹ EGF, 50 ng ml⁻¹ HGF or 50 ng ml⁻¹ EGF PDGF for 10 min. Additionally, serum-starved cells were treated with 2% FCS. Cell lysates were immunoblotted for pβ4 (Y1422), pβ4 (Y1440), total β4, pEGFR (Y845), pEGFR (Y1068), pShc (Y317), pMAPK (p44/42), pAKT (S473), pAKT (T308), pPLCγ (Y783), pPKC βII (S660), pPKD (S744/748) and GAPDH (loading control). Note, that the lower band of β4 (the precursor) run at the same height as the phosphorylated EGFR. (B) Quantification of A. (C) Growth inhibition of A431-β4 KO and A431-β4 WT cells and the β4 mutant A431 cell lines A431-β4 2Y14F and A431-β4 4Y-F by EGF. Cells were seeded in triplicate at 5.000 cells per well in a microtiter plate in DMEM/FCS in the presence or absence of 50 ng ml-1 EGF. After adhesion, cell proliferation was assessed by crystal violet staining on four consecutive days. Each point is the mean ± SD from two independent experiments.*

Discussion

Both EGFR and β4 are frequently overexpressed in epithelial cancers, and cooperation between EGFR and β4 signaling has been reported to promote tumorigenesis. The ability of integrin α6β4 to amplify and potentiate signals from growth factor receptors is believed to be dependent on specific tyrosine phosphorylation sites in the β4 cytoplasmic domain, which could serve as docking sites for adaptor proteins that link the integrin to PI3K/Akt and Ras/MAPK signaling pathways [433,436,437]. In this paper we investigated the contribution of β4 and four tyrosines in the CS, Y1343, Y1349, Y1422 and Y1440, to EGFR-mediated intracellular signaling. We confirmed the involvement of EGFR overexpression in β4 tyrosine phosphorylation and identified PLCγ1, but not Shc, as a possible binding partner of pY1422 and pY1440. However, in contrast to previous reports [283,435], we found that SFKs are not involved in β4 tyrosine phosphorylation, and neither β4 nor its tyrosine phosphorylation contribute significantly to EGFR signalling output in cells.

Several studies have shown that β4 can enhance PI3K/Akt signaling downstream of RTKs. However, in A431 cells, in which Akt phosphorylation is already constitutively high and EGF stimulation only slightly increased Akt phosphorylation, a β4-dependent effect on Akt phosphorylation could not be demonstrated. It is possible that the high level of Akt phosphorylation in A431 cells precluded detection of a subtle effect of β4 on Akt phosphorylation. In line with this reasoning, previous reports have shown that β4 does influence PI3K signaling, but that the effect of β4 on PI3K signaling is diminished by the presence of constitutively active Akt [288,289]. However, in HaCaT keratinocytes that do not exhibit high constitutive levels of phosphorylated Akt and in which the EGFR is not overexpressed, an effect of β4 on the EGFR-mediated phosphorylation of Akt could not be demonstrated. The absence of such a β4 effect is not entirely surprising considering the fact that EGF caused only a very modest increase in the tyrosine phosphorylation of β4 in HaCaT keratinocytes. Recently, we showed that β4 through its ability to modulate cellular tension can influence Akt phosphorylation [456]. Differences in Akt phosphorylation have also been observed in cells growing in 2D versus 3D [457]. Whether these differences can be related to alterations in cellular tension between the two culture conditions is not known, but they clearly show that mechanisms other than growth factor-induced phosphorylation of β4 can activate Akt.

In addition to PI3K/Akt signaling, β4 tyrosine phosphorylation has been shown to stimulate the Ras/MAPK signaling pathway. In HUVEC cells which transiently co-express α6 together with wild-type or phospho-defective mutants of β4 (Y1526, Y1422 and Y1440), MAPK signaling was shown to be dependent on the phosphorylation of these residues [284,433]. However, using A431 cells, we were unable to demonstrate a role of Y1422 and Y1440 phosphorylation in the EGF-stimulated activation of ERK1/2.

The absence of an effect of β4 on MAPK signaling is in agreement with our finding that Shc, which has been shown to be the first step in the activation of the MAPK signaling pathway, does not bind phosphorylated β4 at Y1422 and Y1440 [283,433]. Although a role of Y1526 in the activation of MAPK signaling in this study has not been investigated, we consider it unlikely that this residue contributes to EGF-stimulated activation of MAPK since (1) almost all of β4 tyrosine phosphorylation in A431 cells occurs on Y1343, 1349, 1422 and 1440 and (2) the structural environment of Y1526 in FnIII-3 is not compatible with recognition by the SH2 or PTB domain of Shc [404].

Like many others, we used phosphorylated ERK1/2 as a readout of MAPK pathway activation. Although we found no evidence that tyrosine-phosphorylated β4 plays a role in the activation of ERK1/2, alternative readouts of MAPK signaling could provide additional information. For example, previous work *in vivo* has shown that the phosphorylation of ERK1/2 does not change, but instead the translocation of ERK1/2 to the nucleus differed between cells expressing wild-type β4 and a β4 mutant lacking its signaling domain (truncated after 1355) [458]. Since nuclear translocation of ERK1/2 is required for regulation of the cell cycle and proliferation, but not for activation of other downstream kinases such as RSKs, MSKs and MNKs or phosphorylation of other cytoplasmic targets (e.g. β4 itself [459]), our data does not exclude a contribution of β4 downstream of ERK1/2 activation to gene expression via the MAPK pathway.

In addition to the MAPK/ERK1/2 and PI3K/Akt signaling pathways, EGF stimulates the PLCγ1/PKC pathway. We identified PLCγ1 as a possible binding partner of tyrosine phosphorylated β4 by peptide pulldowns but were unable to confirm this interaction within a cellular environment by co-immunoprecipitation. Moreover, the absence of β4 or its tyrosine phosphorylation in A431 cells also did not affect the activation of PLCγ1 or its downstream effectors PKC and PKD. These results indicate that although the phosphorylated residues Y1422 and Y1440 in β4 can provide docking sites for PLCγ1, binding of PLCγ1 to β4 is not needed for efficient PLCγ1/PKC signaling downstream of the EGFR. PKC and PKD are activated not only by PLCγ downstream of RTK activation, but also by GPCR-mediated PLCβ activation. Because we were unable to detect EGFR and PLCγ activation by serum stimulation of A431 cells, it is possible that activation of PKC/PKD activation in these cells occurs downstream of GPCR activation. Similarly, GPCR activation might be responsible for the increase in Akt phosphorylation upon serum stimulation, while the increase in ERK1/2 activation is mediated by PKC downstream of PLCβ activation.

Our finding that β4 tyrosine phosphorylation does not significantly contribute to growth factor mediated signaling is consistent with findings reported by Merdek *et al.* [451]. These authors expressed a chimeric receptor containing the β4 cytoplasmic domain in MDA-MB-435 breast cancer cells and showed that the β4 chimera was tyrosine phosphorylated upon stimulation with hepatocyte growth factor (HGF), but

did not enhance HGF-induced Akt and ERK1/2 phosphorylation [451]. Furthermore, several studies showed that α6β4 ligation with antibodies or ligand results in the activation of SFKs and subsequent Akt and ERK1/2 signaling [283,436,437] and that β4 signaling is only relevant when growth factors and hormones are limited by low availability [289,328]. However, because β4 is minimally tyrosine phosphorylated in cells that do not overexpress the EGFR and stimulation of these cells with suboptimal concentration of EGF will only further decrease its phosphorylation level, we do not consider it likely that β4 would make a significant contribution to EGFR signaling in such conditions. Moreover, in A431 cells that overexpress the EGFR and exhibit high levels of tyrosine phosphorylated β4, EGF stimulation causes growth arrest of the cells. This phenomenon has been previously observed and suggests that the low level of ligand-independent activation of the overexpressed EGFR in A431 cells is sufficient to support their growth [453–455,460]. Thus, the EGF-induced tyrosine phosphorylation of β4 in A431 cells might be the result of the abnormal high levels of RTK activation in these cells and have no functional significance.

Previously the involvement of SFKs in tyrosine phosphorylation of β4 was shown by others [277,278]. Our data shows that, in contrast to results from the Giancotti lab [277], tyrosine phosphorylation of β4 is not dependent on any one of the highly expressed SFKs Src, Fyn, Yes or Lyn. Even when the expression of both Fyn, Yes and Lyn is silenced, there was hardly any change in the level of β4 tyrosine phosphorylation. However, in agreement with the Giancotti lab, the PP2 inhibitor did reduce EGF-induced β4 tyrosine phosphorylation significantly. It is possible, that these results are explained by an earlier suggested functional redundancy between SFKs [461,462]. Because of functional redundancy, the contribution of each individual SFK to tyrosine phosphorylation of β4 can differ amongst cells and can be different in different circumstances. Alternatively, the PP2 inhibitor might inhibit kinases other than the SFKs, which could contribute to tyrosine phosphorylation of β4, e.g. CSK and PTK6 [463,464]. Furthermore, it is known that PP2 weakly inhibits EGFR, and thus possible that the EGFR itself phosphorylates β4. In this regard it is interesting to note that the tyrosine residues Y1343, Y1422 and Y1440 lie within an EGFR kinase consensus sequence (X[E/D]pYX) and that β4 and EGFR are often found in close proximity of each other. Alternatively, inhibition of EGFR by PP2 could reduce the activation of multiple kinases downstream of the EGFR important for β4 tyrosine phosphorylation.

In summary, we investigated whether tyrosine phosphorylation of β4 is contributing to EGFR induced intracellular tumorigenic signaling in epithelial cells. Combining our data with data from others, we conclude that, although β4 can contribute to PI3K or MAPK signaling under certain circumstances, EGF-induced tyrosine phosphorylation of β4 does not substantially contribute to pro-tumorigenic signaling downstream of EGFR and therefore the biological relevance of EGF-induced tyrosine phosphorylation of β4 remains unknown.

Materials and Methods

Reagents

The primary and conjugated antibodies used in this study are listed in Table 1. Secondary antibodies for western blot were goat anti-mouse IgG HRP (BioRad; 1:3000) and polyclonal goat anti-Rabbit IgG HRP (Dako; 1:5000). Secondary antibodies used for immunofluorescence were donkey anti-rabbit IgG Alexa 594 (Invitrogen A21207; 1:400), goat anti-mouse IgG Alexa 568 (Invitrogen A11004; 1:200) and goat anti-guinea pig Alexa 488 (Invitrogen A11073; 1:200). The PP2 and PP3 compounds used to treat cells for 1 h at 1, 5 and 10 μM were from Merck Chemical Ltd (Beeston, Nottingham, UK) were and EGF (Sigma-Aldrich), HGF (R&D systems) and PDGF-BB (Sigma-Aldrich) were used at 50 ng ml^{-1} concentrations to treat cells for the indicated times.

The polyclonal rabbit antibodies against the phosphorylated Y1440 and Y1422 sites on β4 were raised against synthetic peptides with the sequence SSTLTRD**pY**NSLTRSE and STTLPRD**pY**STLTSVS respectively. The two synthetic peptides were conjugated to maleimide-activated mcKLH and injected into rabbits. The rabbits received a booster immunization every 4 weeks and antisera were collected 1 week after the third booster. To prevent recognition of other epitopes by the antibody during immunoblotting, the antibodies were used in combination with 10 μM of both unphosphorylated synthetic peptides for β4 and 20 μM phosphorylated EGFR Y1068 peptides.

Cell culture

PA-JEB immortalized keratinocytes were isolated from a patient with Pyloric Atresia associated with Junctional Epidermolysis Bullosa [13,24]. PA-JEB/β4 keratinocytes stably expressing wild-type β4 were generated by retroviral transduction [3] and maintained in serum-free keratinocyte medium (KGM; Invitrogen), supplemented with 50 μg ml^{-1} bovine pituitary gland extract, 5 ng ml^{-1} EGF, and antibiotics (100 units ml^{-1} streptomycin and 100 units ml^{-1} penicillin; Sigma). HaCaT keratinocytes, A431 epidermal squamous carcinoma cells, HT29 colorectal adenocarcinoma cells and COS7 monkey kidney cells were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% heat-inactivated fetal calf serum (FCS; Serana Europe GmbH, Pessin, Germany), and antibiotics. DiFi rectal carcinoma and MDA-MB-231 breast adenocarcinoma were kindly provided by the laboratories of Rene Bernards and Jos Jonkers at the Netherlands Cancer Inst. (Amsterdam, the Netherlands) cell lines and were cultured in RPMI medium 1640 (Gibco #52400-025) containing 10% heat-inactivated fetal calf serum (FCS; Serana), and antibiotics. MCF10A mammary epithelial cells (obtained from ATCC) were cultured in HAM's medium; DMEM/F12 (Gibco #31331-028) supplemented with 5% horse serum (Sigma-Aldrich), EGF (20 ng ml^{-1}; Sigma-Aldrich), insulin (10 μg ml^{-1}; Sigma-Aldrich), hydrocortisone (0.5 mg ml^{-1}; Sigma-Aldrich), cholera toxin (100 ng ml^{-1}; Sigma-Aldrich) and antibiotics and MDA-MB-468 mammary adenocarcinoma

Target	Antibody	Application	Source
α-tubulin	Mouse mAb	WB (1:10000)	Sigma #t5168
EGFR (Ab-1)	528, Mouse mAb	IF (1:50)	Calbiochem
EGFR	528, mAb	IP (1 μg ml^{-1})	Santa Cruz #sc-120
EGFR	1005, Rabbit pAb	WB (1:250)	Santa Cruz #sc-03
Fyn	Rabbit pAb	WB (1:1000)	Cell Sign. #4023P
GAPDH	D16H11, Rabbit mAb	WB (1:1000)	Cell Sign. #5174S
Itg. β1	Mouse mAb, TS2/16	IP (1 μg ml^{-1})	ATCC
Itg. β4	450-11A, Mouse mAb	IF (1:500)	Kind gift from Steve Kennel
Itg. β4	450-11A, Mouse mAb	IP (1 μg ml^{-1})	BD Pharm. #555722
Itg. β4	Rabbit pAb	IF (1:2000)	Homemade
Itg. β4	Rabbit pAb	WB (1:2000)	Homemade
Itg. β4	PE-Rat anti-human-CD104	FACS (1:400)	BD Pharm. #555720
Itg. pβ4 (Y1440)	Rabbit pAb	WB	Homemade
Itg. pβ4 (Y1422)	Rabbit pAb	WB	Homemade
Lyn	H-6, Mouse mAb	WB (1: 500)	Santa Cruz #sc-7274
MAPK	Mouse mAb	WB (1:1000)	BD Trans. #610104
pAkt (S473)	Rabbit pAb	WB (1:500)	Cell Sign. #9271S
pEGFR (Y845)	Rabbit pAb	WB (1:1000)	Cell Sign. #2231
pEGFR (Y1045)	Rabbit pAb	WB (1:1000)	Cell Sign. #2237S
pEGFR (Y1068)	Mouse mAb	WB (1:1000)	Cell Sign. #2236S
Plcγ1	530, Rabbit pAb	WB (1:200)	Santa Cruz #sc-426
pMAPK (p44/p42) (T202/Y204)	Rabbit pAb	WB (1:1000)	Cell Sign. #9101
pPKCα,β II (T638/641)	Rabbit pAb	WB (1:1000)	Cell Sign. #9375
pPKC (pan) (βII S660)	Rabbit pAb	WB (1:1000)	Cell Sign. #9371S
pPKD (S744/748)	Rabbit pAb	WB (1:1000)	Cell Sign. #2054S
pPlcγ1 (Y783)	D6M9S, Rabbit mAb	WB (1:1000)	Cell Sign. #14008S
pTyrosine (pY)	4G10, Mouse mAb	WB (5 μg ml^{-1})	MilliporeSigma
Shc	Mouse mAb	WB (1:1000)	BD Trans. #S68020
Src	32G6, Rabbit mAb	WB (1:1000)	Cell Sign. #2123
Yes	Mouse mAb	WB (1:1000	BD Trans. #610375

Table 1 *Primary antibodies used in various techniques.*

cells (a kind gift from Jos Jonkers lab) in Leibovitz's L-15 medium (Gibco #11415-049) supplemented with 10% heat-inactivated fetal calf serum and antibiotics. All cells were cultured at 37°C in a humidified, 5% CO_2 atmosphere. When cells were serum starved for experimental reasons, the cells were washed two times with PBS and subsequently grown for 12-20h in medium without FCS or growth factors.

Preparation of cell lines

CRISPR-Cas9 mediated KO: The A431 and HaCaT β4 KO cells were prepared using CRISPR-Cas9 technology. Target sgRNAs against integrin β4 (5'-GACTCGCTCCCTCCGCGCCT-3') were cloned in the pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene plasmid #42230, deposited by Feng Zhang). The cells were transiently transfected with this plasmid using lipofectamine® 2000 (Invitrogen) in OptiMEM (Gibco), and bulk sorted, for the β4 negative population, using a Moflo Asterios (Beckman Coulter) or FACSAria Fusion (BD Biosciences) cell sorter.

siRNA KD of SFKs in A431 cells: The Yes (SI00302218) and Lyn (SI00660557O) siRNAs were purchased from QIAGEN. The human Src (L-003175-00-0005) and Fyn (L-003140-00-0005) SMARTpools and non-targeting control pool (D-001810-10-05) were from Dharmacon. A431 cells were transiently transfected with the siRNAs using lipofectamine® 2000 (Invitrogen). Lipofectamine and siRNA solutions in OptiMEM (Gibco) were mixed and incubated for 20-30 min at room temperature. Cells were incubated with the transfection solution for approximately 48h. The cells were serum starved for 20h, by two PBS washes and the replacement of the full medium for medium without supplements and serum, after which the cells were treated with EGF.

Stable (re)expression of proteins by retroviral transduction: For (re)expression of β4 wt and β4 mutants in β4 deficient A431 cells, we used stable cellular retroviral mediated transduction. Virus was produced by calcium phosphate transfection of Phoenix-ampho cells following the retroviral producer line protocol, and added to A431 β4KO cells. Cells which expressed the protein of interest were enriched by zeocin selection and bulk sorted by FACS. The constructs used were β4 WT, β4 4Y-F, β4 2Y14F and β4 2Y13F in LZRS-IRES-Zeo.

Transient overexpression of proteins in COS7 cells: COS7 cells were transfected using the DEAE-dextran method. The constructs used were β4 WT, β4 2Y14F and β4 4Y-F in pcDNA3.

cDNA constructs: The full-length β4 cDNA in pUC18 has been described previously [8]. Point mutants of β4 Y1343, Y1349, Y1422 or Y1440 were generated by site-directed mutagenesis with the PCR-based overlap extension method using Pwo DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN), and fragments containing the different mutations were exchanged with corresponding fragments in the β4 pUC18 vector. Retroviral vectors containing mutant β4 cDNAs were generated by subcloning the mutant β4 cDNAs into the *Eco*RI restriction site of the pcDNA3 vector or the LZRS-MS-IRES-ZEO vector as described before [3].

Flow Cytometry/FACS

Flow Cytometry/FACS was performed essentially as described previously [465]. Cells were collected after trypsinization, and incubated for 50-60 min with conjugated β4-PE antibody on ice in PBS containing 2% FCS. After incubation, cells were washed twice with 2% FCS in PBS. Finally, cells were passed through a nylon mesh filter and 50.000 positive cells were analyzed per sample using a FACSCalibur cell analyzer (BD Biosciences). β4 KO cells were used as a negative control. The graphs were made in FlowJo and adapted in Adobe Illustrator®.

Immunofluorescence imaging

Immunofluorescence imaging was performed essentially as described previously [465]. Cells were seeded on glass coverslips and grown for 40 h in the presence of DMEM+FCS. Cells were fixed wih 2% PFA for 10 min, permeabilized with 0,2% Triton X-100 and blocked wih 2% BSA (Serva) in PBS. Cells were incubated with primary and secondary antibodies for 50-60 min. In between and after, cells were washed three times with PBS. Nuclei were stained with DAPI (Sigma-Aldrich) and MOWIOL was used to mount the coverslips for confocal imaging. Imaging was performed using a Leica TCS SP5 confocal microscope with a 63× (NA 1.4) oil objective.

Immunoprecipitations and western blot

For analysis of proteins in whole cell lysates, subconfluent cells were washed in cold PBS, lysed in 1% Nonidet P-40, 100 mM NaCl, 4 mM EDTA, 20 mM Tris-HCl (pH 7.5), supplemented with 1.5 mM Na_3VO_4, 15 mM NaF as phosphatase inhibitors and a protease inhibitor cocktail (1:500; Sigma), whole cell lysates were cleared by centrifugation at 12.000 rpm for 60 min at 4°C and supplemented with SDS sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 12.5 mM EDTA, 0.02% Bromophenol Blue) with β-mercaptoethanol and heated at 95°C for 5 min [465].

Immunoprecipitations were performed with the cleared cell lysates after centrifugation. The lysates were incubated at 4°C for 1,5-2h with 1 µg antibody. Subsequently, the lysates were incubated 4-20h at 4°C with prewashed Protein G Sepharose 4 Fast Flow beads (GE Healthcare), beads were washed two times with lysis buffer and two times with PBS and bound proteins were dissolved in SDS sample buffer with β-mercaptoethanol and heated at 95°C for 5 min. Proteins were separated on 4-12% bolt gradient gels (Invitrogen) and transferred to Immobilon-P transfer membranes (Millipore). The membrane was blocked for at least 2 h in 2% BSA in TBST (10 mM Tris (pH 7.5), 150 mM NaCl, 0.05% Tween 20) before incubation with primary antibody overnight at 4°C and with secondary antibody for 1h at room temperature. After each incubation step, the membranes were washed twice with TBST and twice with TBS (TBST without Tween 20). Antibodies were detected using Clarity Western ECL Substrate (Bio-Rad). Quantification of western blot data was performed with ImageJ.

RT-PCR

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) analysis was performed as described previously [466]. Reactions were performed in triplo for determination of the levels of Yes, Lyn, Frk, Lck, Fgr, Hck, Blk and CyclofilinA (CF; control) in A431 cells. Cells were grown in complete medium and lysed in RNA-Bee (Tel-test Inc.) or Trizol® reagent (Ambion #15596018), following manufacturer's instructions. Total RNA was separated from DNA and proteins by the addition of chloroform (Sigma-Aldrich) and subsequent centrifugation at 12.000 x g for 15 min at 4°C. The RNA was precipitated with isopropanol and washed with 70% ethanol. Integrity of the isolated RNA was assessed by agarose gel electrophoresis.

First strand cDNA synthesis was performed with 3 µg of total RNA using the first strand cDNA synthesis kit K1612 (Thermo Fisher Scientific) following manufacturer's directions. The PCR reactions were run using SYBR® Advantage® qPCR premix (Clontech) on a 7500 Fast Real-Time PCR system (Applied Biosystems) and the primers (IDT) which were used, are shown in Table 2. Relative mRNA quantities were obtained using the $2^{-\Delta Ct}$ method. The ΔCt is the obtained Ct value of the SFK minus the obtained Ct value of CF (endogenous control).

Target	Forward primer sequence	Reverse primer sequence
Yes	5'-CCTCATTTTCAGTGGTGCCA-3'	5'-TGCTTCCCACCAATCTCCTT-3'
Lyn	5'-ACAGCTTGAGTGACGATGGA-3'	5'-ATTCTCCATGCTCCTCCAGG-3'
Lck	5'-GGGATCGTTTTCACTGTCGG-3'	5'-GCCCATCTGAAGCATTGGTG-3'
Fgr	5'-CATTCCCAGCAACTACGTGG-3'	5'-GACAGGGAGTAGGCACCTTT-3'
Hck	5'-TAGCCACAACAGCAACACAC-3'	5'-CACTCCCCGGATTCCTCTAG-3'
Blk	5'-AATGATCGGGACCTGCAGAT-3'	5'-GGCCTTGTTGATTGGAGCAA-3'
CF (control)	5'-CATCTGCACTGCCAAGACTGA-3'	5'-TTGCCAAACACCACATGCTT-3'

Table 2 *RT-PCR primers*

Peptide pulldowns and mass spectrometry analyses

Phosphorylated and unphosphorylated peptides based on sequences within the integrin β4 (Table 3) were synthesized by Fmoc chemistry in the laboratory of Huib Ovaa at the NKI and purified by HPLC. The peptides were synthesized with a cysteine followed by a caproic acid at the amino terminus. The cysteine was used for coupling to SulfoLink (Pierce, Rockford, IL), while the caproic acid was added to provide spacing between the peptide and the support material. The sequence of each peptide was verified by mass spectrometry.

For peptide pull downs, PA-JEB/β4 keratinocytes were grown to confluency on 15 cm tissue culture dishes, lysed in 3 ml 50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM sodium pyrophosphate,

500 µM sodium vanadate, 10 µg/ml aprotinin and 10 µg/ml leupeptin (PLC-lysis buffer) per 15 cm tissue culture dish. Lysates were precleared by centrifugation at 10,000 rpm in a microfuge at 4°C for 30 min. and incubated for 1 h with 100 µl of a 10% slurry of each of the different β4 peptides. Agarose beads were collected by centrifugation and washed four times with PLC buffer. Sample were boiled, resolved by SDS-PAGE and stained with Coomassie blue. Protein bands of interest were cut out, subjected to in-gel trypin digest and analyzed by mass spectrometry.

Tyrosine site	Unphosphorylated	Phosphorylated
1422	Cys-Caproic acid-SSTLTRDYNSLTRSE	Cys-Caproic acid-SSTLTRDpYNSLTRSE
1440	Cys-Caproic acid-STTLPRDYSTLTSVS	Cys-Caproic acid-STTLPRDpYSTLTSVS
1494	Cys-Caproic acid-CERPLQGYSVEYQLL	Cys-Caproic acid-CERPLQGpYSVEYQLL
1526	Cys-Caproic acid-DLLPNHSYVFRVRAQ	Cys-Caproic acid-DLLPNHSpYVFRVRAQ

Table 3 *β4 peptides used for peptide pulldowns*

Acknowledgements

We thank Monique Slijper of Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences for mass spectrometry, and NKI colleagues for sharing cells and reagents.



CHAPTER 5

Tetraspanin CD151 and integrin α3β1 contribute to the stabilization of integrin α6β4-containing cell-matrix adhesions

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Abstract

Tetraspanin CD151 has been suggested to regulate cell adhesion through its association with laminin-binding integrins α3β1 and α6β4; however, its precise function in keratinocyte adhesion remains elusive. In this study, we investigated the role of CD151 in the formation and maintenance of laminin-associated adhesions. We show that CD151, through binding to integrin α3β1, plays a critical role in the stabilization of an adhesion structure with a distinct molecular composition of hemidesmosomes with tetraspanin features. These hybrid cell-matrix adhesions, which are formed early during cell adhesion and spreading and at later stages of cell spreading, are present in the central region of the cells. They contain the CD151–α3β1/α6β4 integrin complexes and the cytoskeletal linker protein plectin, but are not anchored to the keratin filaments. In contrast, hemidesmosomes, keratin filament-associated adhesions that contain integrin α6β4, plectin, BP180 (encoded by COL17A1) and BP230 (encoded by DST), do not require CD151 for their formation or maintenance. These findings provide new insights into the dynamic and complex regulation of adhesion structures in keratinocytes and the pathogenic mechanisms underlying skin blistering diseases caused by mutations in the gene for CD151.

Introduction

The skin is our largest organ and its barrier function protects us from dehydration and pathogenic infections. To exert these functions, the skin needs to maintain its integrity, for which stable adhesion of basal keratinocytes to the underlying extracellular basal lamina is of paramount importance. This adhesion is largely mediated by hemidesmosomes (HDs), multi-protein complexes that are associated with the keratin intermediate filament system within the cell.

The integrin α6β4, a heterodimeric transmembrane protein, is a main component of HDs. In type I HDs, present in the epidermis, integrin α6β4 binds extracellularly to laminin-332 and intracellularly via two associated proteins, plectin and BP230 (BPAG1e, encoded by DST), to the keratin cytoskeleton. Besides integrin α6β4, type I HDs contain two other transmembrane proteins: the tetraspanin CD151 and BP180 (BPAG2 or collagen XVII, encoded by COL17A1) [1–3]. In more simple epithelia of the intestine or the mammary gland, a rudimentary form of HDs is present. These type II HDs contain integrin α6β4 and plectin, but lack BP180 and BP230 [317,467].

In humans, the importance of type I HDs for maintaining skin integrity is underscored by genetic evidence that demonstrates that mutations in any of the six genes encoding HD proteins cause the congenital inherited skin blistering disorder epidermolysis bullosa (EB) [21]. Patients carrying null mutations in the genes for the integrin α6 or β4 subunits, or the cytolinker protein plectin, suffer from a severe form of EB, which is indicated by the absence of HDs [23–25,468,469]. However, HDs with grossly normal HD structures, but reduced keratin filament association, are found in patients carrying mutations in the genes for BP180 or BP230, and accordingly, these patients only suffer from a mild form of EB [470–473]. Mutations in CD151 have not been associated with abnormalities in HDs but nevertheless cause a mild regional skin blistering phenotype, similar to that observed in patients with mutations in the gene encoding integrin α3 [52,474–476]. Targeted deletion of genes encoding HDs and associated proteins in mice largely recapitulates the pathology observed in humans [54,407,408,477–480].

Integrin α3β1, another laminin-binding integrin expressed by keratinocytes, is a component of focal adhesions (FAs), but can also be found together with integrin α6β4 in tetraspanin-enriched microdomains (TEMs) (also named tetraspanin webs). TEMs are formed by lateral associations of tetraspanins with one another and with other proteins. Incorporation of integrins α6β4 and α3β1 in TEMs is facilitated through binding of their α subunits to CD151 [206]. The association of CD151 with laminin-binding integrins has been reported to affect integrin-dependent adhesion, migration and signaling [83,481,482].

While it has been shown that CD151 is colocalized with integrin α6β4 in type I HDs, the exact function of CD151 in the formation of these adhesions or other cell-matrix adhesions in keratinocytes is not known [211]. In this study, we assessed the contribution of CD151 to the formation and maintenance of laminin-associated adhesion complexes in keratinocytes. In agreement with data obtained from CD151 knockout mice and patients with CD151 mutations, our findings confirm that CD151 is not critical for the formation of type I HDs. However, we show that CD151 is essential for the maintenance of adhesion complexes in which CD151 is present together with integrin α3β1 and the HD proteins integrin α6β4 and plectin, but which are not associated with the keratin cytoskeleton.

Results

CD151 forms multi-protein complexes with both integrins α3β1 and α6β4 in keratinocytes

Previous studies have shown that CD151 forms stable complexes with the laminin-binding integrins α3β1 and α6β4 [206,211]. We confirmed these findings by reciprocal coimmunoprecipitation (co-IP) assays using two different monoclonal antibodies against CD151 (5C11 and 11G5) and antibodies against the integrin α3 and α6 subunits (Fig. 1A). Furthermore, these assays showed that in PA-JEB/β4 keratinocytes (immortalized keratinocytes isolated from a patient with pyloric atresia associated with junctional epidermolysis bullosa, and stably expressing wildtype integrin β4) the α6 subunit does not form a heterodimer with β1, which is consistent with the fact that integrins α3β1 and α6β4, but not α6β1, are the major laminin-binding integrins in skin [429] (Fig. 1A). Using immunofluorescence (IF) microscopy, we detected CD151 in a pattern of consecutive arches, in which it is partially co-localized with integrin α3β1. Both integrin α3β1 and CD151 are also found at the cell periphery where they do not colocalize and are present in distinctive adhesion structures (Fig. 1B). Furthermore, both integrin α3β1 and CD151 co-localized with the HD proteins integrin α6β4 and plectin (Fig. 1C,D). In summary, these results confirm that in keratinocytes CD151 binds both integrins α3β1 and α6β4, and is present in adhesion structures that contain characteristics of HDs.

CD151 regulates integrin α6β4 distribution

To determine to what extent CD151 impacts integrin α3β1- and α6β4-containing adhesion complexes, we abrogated CD151 protein expression in PA-JEB/β4 and HaCaT keratinocytes using CRISPR-Cas9 (KO) and subsequently restored the expression of CD151 by retroviral-mediated gene transfer. Analysis by western blotting (WB) and fluorescence-activated cell sorting (FACS) confirmed the absence and restoration of CD151 expression, and showed that the deletion of CD151 did not affect the expression of integrins α3β1 and α6β4 at the plasma membrane (Fig. 2A,B). However, quantitative IF staining and confocal microscopy showed reduced clustering of integrin α3β1 (and

to a lesser extent of integrin α6β4) at the basal cell surface (Fig. 2C). Furthermore, integrin α3β1 was no longer co-distributed with integrin α6β4 but showed increased co-localization with phospho-paxillin in FAs (Fig. 2D,E). In the CD151-proficient cells, integrin α6β4 was clustered in central and peripheral adhesions, but strikingly in the CD151-deficient cells it was almost exclusively present at the cell periphery (Fig. 2F–H). These results show that, while not essential for integrin α3β1 or α6β4 protein expression or their transport to the plasma membrane, CD151 is necessary for proper organization of these integrins in the central region of the cells.

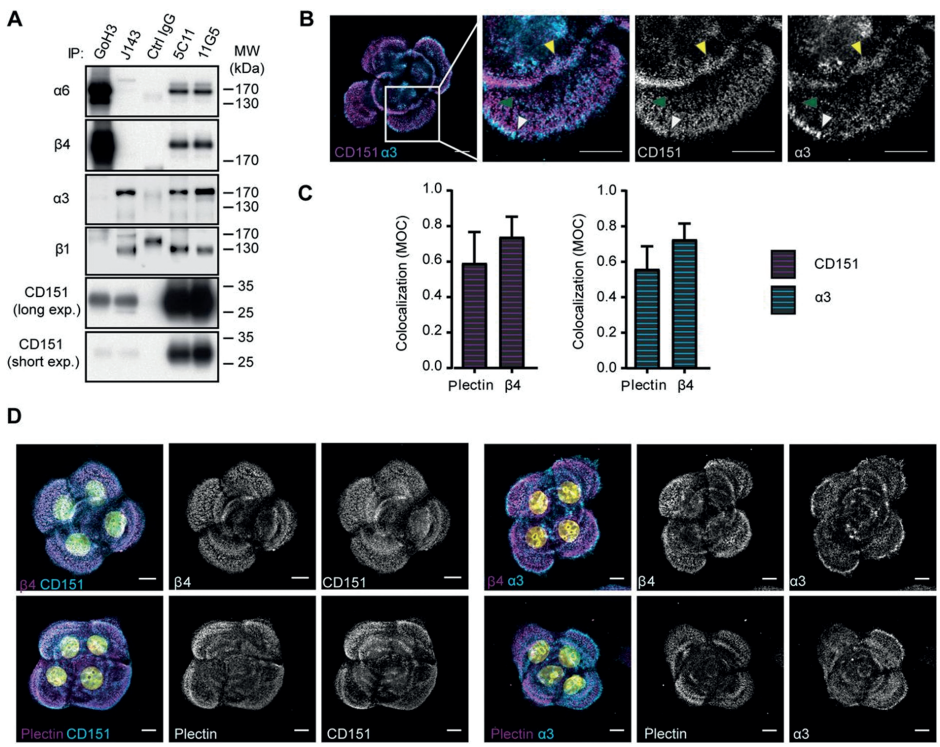


Figure 1 Association of CD151 with integrins α3β1 and α6β4 in adhesion complexes. *(A) Detection of integrin α3β1–CD151 and integrin α6β4–CD151 complexes in lysates of PA-JEB/β4 keratinocytes using immunoprecipitation and western blotting under non-reducing conditions. Antibodies raised against the following proteins were used for immunoprecipitation: GoH3 against integrin α6, J143 against integrin α3, and 5C11 and 11G5 against CD151. (B) Confocal overview and zoom-in image of PA-JEB/β4 cells stained for integrin α3 (cyan) and CD151 (magenta). Yellow arrowheads indicate adhesions containing integrin α3 and CD151 and white and green arrowheads indicate integrin α3 and CD151 located independently of each other in the periphery of the cells. Scale bars: 10 μm. (C) Quantification (mean±s.d.) of the colocalization (Manders' overlap coefficient, MOC) of integrin α3 [46 images from two independent experiments (N=2)] or CD151 (30 images; N=2) with plectin and integrin α3 (52 images; N=2) or CD151 (74 images; N=4) with integrin β4. (D) Confocal images of double-IF staining of CD151, integrin α3 (cyan) and HD components plectin and integrin β4 (magenta) in PA-JEB/β4 keratinocytes. Nuclei were counterstained with DAPI (yellow). Scale bars: 10 μm.*

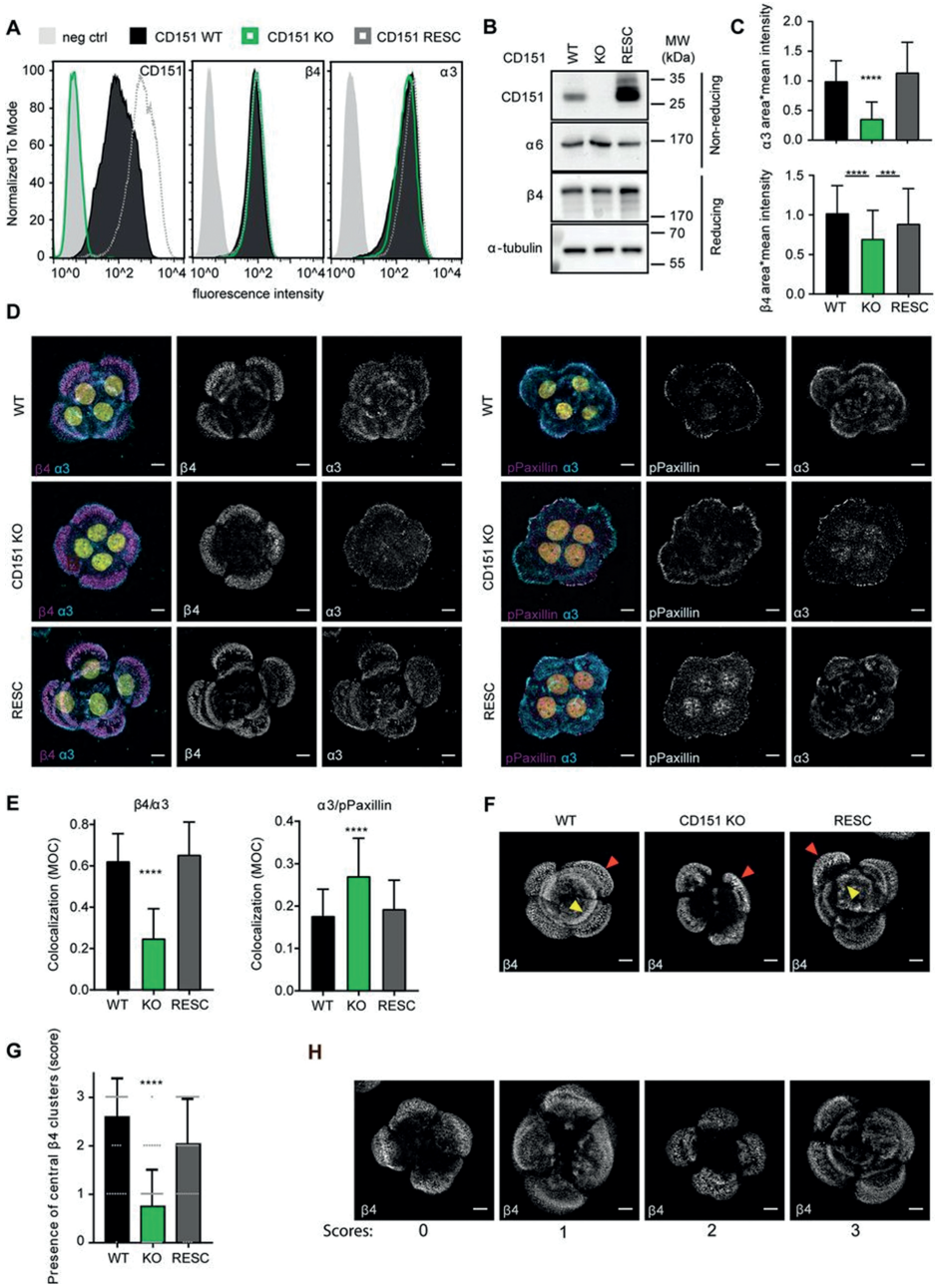


Figure 2 CD151 influences localization of adhesions containing integrins α3β1 and α6β4. *(A) FACS analysis of surface expression of CD151, integrin β4 and integrin α3 for wild-type (WT), CD151 knockout (CD151-KO) and rescue (CD151-RESC) PA-JEB/β4 cells. (B) Whole-cell lysate of WT, CD151-KO and CD151-RESC PA-JEB/β4 keratinocytes analyzed by western blotting for protein levels of CD151, integrin α6, integrin β4 and α-tubulin (loading control) under reducing or non-reducing conditions as indicated. (C) Quantification (mean±s.d.) of the amount of integrin α3 (WT, 55; KO, 57; RESC, 55 images; N=3) and integrin β4 (WT, 122;*

*KO, 122; RESC, 119 images; N=8) clustering at the basal cell surface (integrin α3 or integrin β4 area×intensity). ***P<0.001, ****P<0.0001 (Mann–Whitney U-test). (D) Confocal images of WT, CD151-KO and CD151-RESC PA-JEB/β4 keratinocytes stained for integrin α3 (cyan) and integrin β4 (magenta), or integrin α3 (cyan) and phosphorylated paxillin (magenta). Nuclei were counterstained with DAPI (yellow). Scale bars: 10 μm. (E) Quantification (mean±s.d.) of the colocalization (MOC) of integrin β4 and integrin α3 (WT, 56; KO, 55; RESC, 57 images; N=3) and of integrin α3 and phosphorylated paxillin (WT, 55; KO, 57; RESC, 55 images; N=3). ****P<0.0001 (Mann–Whitney U-test). (F) Confocal images of WTand CD151- KO and CD151-RESC PA-JEB/β4 keratinocytes stained for integrin β4. Red arrowheads show peripheral integrin α6β4-containing adhesions, while yellow arrowheads point to central integrin α6β4-containing adhesions. Scale bars: 10 μm. (G) Quantification (mean±s.d.) of the amount of central integrin α6β4- containing adhesions per cell island (dots represent individual cell islands) using images from WT (57 images; N=4), CD151-KO (56 images; N=4) and CD151-RESC (55 images; N=4) PA-JEB/β4 cells stained for integrin β4. ****P<0.0001 (Mann–Whitney U-test). (H) Scoring system for the amount of central integrin α6β4-containing adhesions present, with example images. 0=no central adhesion, 1=few central adhesions, 2=several central adhesions, 3=many central adhesions. Scale bars: 10 μm.*

CD151 is essential for the maintenance of central integrin α6β4-containing adhesions

To gain real-time insight into how CD151 contributes to the formation of integrin α6β4-containing adhesions in the central region of the cells, we deleted CD151 in PA-JEB keratinocytes expressing GFP-tagged integrin subunit β4 [315]. The absence of CD151 and presence of β4–GFP was confirmed by FACS and WB analysis (Fig. 3A,B). The clustering of β4–GFP was followed over time in these cells by time-lapse imaging. During initial stages of cell spreading, both CD151-deficient and -proficient PAJEB/β4–GFP cells assembled integrin α6β4-containing adhesions at the periphery of the keratinocyte islands. However, while these initial adhesion structures were maintained during later phases of cell spreading and island formation in the CD151-proficient cells, in the CD151-deficient cells, the central adhesion structures resolved and disappeared when the cells formed new peripheral adhesions after cell division and cell spreading (Fig. 3C; Movies 1 and 2). Furthermore, in fluorescence recovery after photobleaching (FRAP) experiments, a spot in the central and peripheral integrin α6β4-containing adhesions was simultaneously bleached and the recovery of the β4–GFP signal at these spots was followed over time (Fig. 3D,E). The results show that the mobile fraction of integrin α6β4 in the central integrin α6β4 adhesions is larger than in the peripheral ones, indicating a higher dynamic nature of integrin α6β4 in the central adhesions (Fig. 3E,F).

Peripheral but not central integrin α6β4-adhesions contain BP230 and are associated with keratin

We reasoned that the observed differences in the dynamics of integrin α6β4 between central and peripheral adhesion clusters could be due to the association of integrin α6β4 with different proteins. Analysis of the distribution of laminin-332 and plectin by total internal reflection fluorescence (TIRF) microscopy showed that both proteins are associated with central and peripheral integrin α6β4-containing adhesions, with laminin-332 being slightly more abundant under the central adhesion clusters

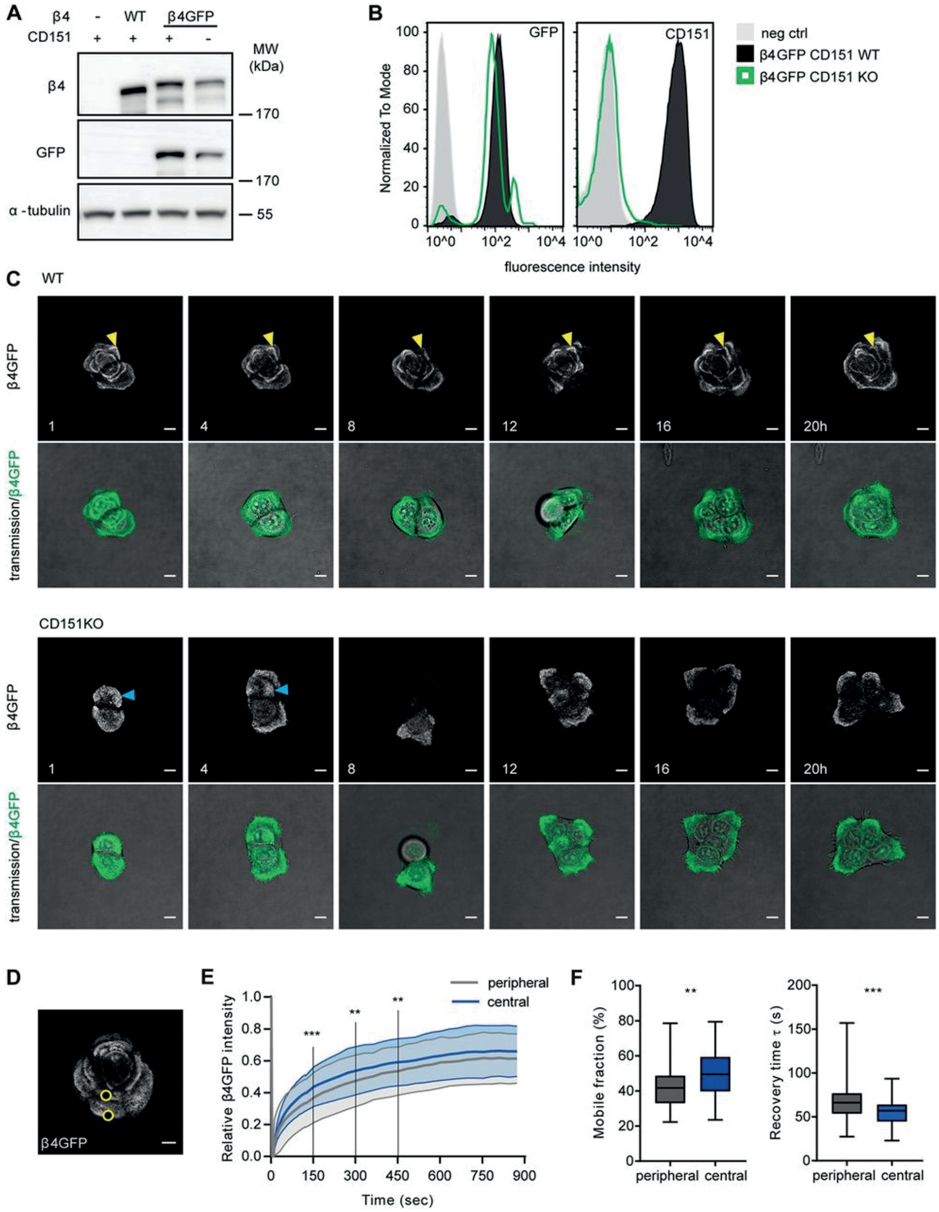


Figure 3 CD151 is essential for maintenance of early integrin α6β4-containing adhesions. *(A) Western blotting analyses of WT or CD151-KO PA-JEB keratinocytes, reconstituted with WT-β4 or integrin β4–GFP. Whole cell lysates were tested for integrin β4, GFP and α-tubulin protein levels. (B) FACS analyses of CD151 and integrin β4–GFP surface expression on WT and CD151-KO PA-JEB/β4–GFP cells. (C) Stills from a time-lapse experiment using WTand CD151-KO PA-JEB/β4–GFP cells. Top panels show the integrin β4–GFP signal and bottom panels the overlay of the integrin β4–GFP and DIC signal. Stills show images taken at 1, 4, 8, 12, 16 and 20 h after addition of DMEM+FCS (0 h time point, 20 h after seeding). The yellow arrowheads indicate a peripheral adhesion becoming a central adhesion over time in WT cells and the blue arrowheads indicate a peripheral adhesion disappearing over time in KO cells. Scale bars: 10 μm. (D) Example image of PA-JEB/β4– GFP*

*after the bleach step of a FRAP experiment; FRAP regions indicated in peripheral and central adhesions with yellow circles. Scale bar: 10 µm. (E) Integrin β4–GFP intensity (relative to the mean integrin β4–GFP intensity before bleaching) over time (in seconds) during FRAP experiments in both central (blue) and peripheral (grey) integrin α6β4-containing adhesions. Data was collected from 38 islands in three experiments, lineplot shows the mean+s.d. (F) Mobile fraction and recovery time of integrin β4–GFP in central and peripheral adhesions during the first 230 s of the same dataset used for E. Boxplots divide the data in quartiles and the median is indicated. The whiskers show minimal and maximal values. **P<0.01, ***P<0.001 (Wilcoxon matched-pairs signed rank test) in E,F.*

and plectin at the peripheral adhesion clusters (Fig. 4A,B). Strikingly, keratin and BP230 (which, like plectin, can connect integrin α6β4 to the keratin cytoskeleton) were associated solely with integrin α6β4 in the peripheral adhesions (Fig. 4A,B). Interestingly, in the peripheral adhesions only approximately 30 and 50 percent of integrin α6β4 was found to be associated with BP230 and keratin, respectively (Fig. 4B). The possibility that the peripheral adhesions lacking keratin and BP230 are associated with the IF protein vimentin can be largely excluded since few PA-JEB/β4 keratinocytes express vimentin and even fewer cells have it associated with peripheral adhesions. For similar reasons, we can exclude that the central adhesions are associated with vimentin (Fig. S1). F-actin, which in many PA-JEB/β4 keratinocytes is organized in a typical circumferential ring, was not obviously associated with either the central or peripheral adhesions (Fig. S1). These results characterize many of the peripheral but not the central integrin α6β4-containing adhesions as type I HDs. Consistent with the notion that type I HDs are present in CD151-deficient patients and mice, the HD proteins BP180, BP230 and plectin were all found to co-localize with integrin α6β4 in the peripheral adhesions of CD151-deficient PA-JEB/β4 keratinocytes (Fig. S2). The central integrin α6β4-containing adhesion clusters, which share some of the characteristics of HDs, such as the clustering of integrin α6β4 and its association with laminin-332 and plectin, also have features in common with TEMs: they contain integrin α3β1 and CD151 and therefore display a hybrid molecular composition. From this point forward, we will refer to these adhesions as central HD-like adhesions in this report.

Integrin α3β1 is localized in central HD-like adhesions but not in peripheral HDs

Despite the fact that CD151 can form a stable complex with both integrins α3β1 and α6β4, type I HDs in the skin contain only complexes of CD151 with integrin α6β4 [211,483]. To investigate the distribution of integrin α3β1 in relation to CD151 in the different cell-matrix adhesions in the cultured keratinocytes, we performed TIRF imaging and created intensity profiles along a line segment. As shown in Fig. 4, the intensity plots of CD151 and integrin α6β4 in the central HD-like adhesions and the peripheral HDs almost completely overlapped each other (Fig. 4C). In contrast, the profiles of integrins α3β1 and α6β4 only overlapped in the central HD-like adhesions (Fig. 4C). In the peripheral region the peak intensities of integrin α3β1 overlapped with those of tyrosine phosphorylated FA-associated protein paxillin, and not with HD markers BP230 and keratin (Fig. 4D). These results confirm that central and peripheral adhesions differ in protein composition. Furthermore, while in the central HD-like

adhesions integrin α3β1, integrin α6β4 and CD151 cluster together, in the periphery they can be found in two distinct complexes: integrin α3β1 in FAs and integrin α6β4 and CD151 in type I HDs.

CD151 stabilizes central HD-like adhesions through binding to integrin α3β1 and self-association

Our findings so far indicate that CD151 is essential for maintaining central HD-like adhesions. To determine whether CD151 stabilizes these adhesions through binding to integrin α3β1, we deleted and reconstituted integrin α3 in the HaCaT and PA-JEB/β4 keratinocyte cell lines (Fig. 5A,B; Fig. S3A,B). In both these cell lines, the absence of integrin α3 did not affect the total levels of integrin β4 at the cell surface and the formation of peripheral HDs was not obviously disturbed. However, as observed in the CD151-deficient PA-JEB/β4 keratinocytes, the absence of integrin α3 caused a loss of central HD-like adhesions, albeit less dramatically (Fig. 5B–D; Fig. S3C). To exclude the possibility that the absence of the central HD-like adhesions is caused by reduced spreading of the integrin α3-deficient cells, we also analyzed the cells that were plated on coverslips coated with a mixture of vitronectin and fibronectin. Under these conditions, cell spreading of the integrin α3-deficient cells was increased and found to be comparable to that of the wild- type cells on uncoated coverslips. Importantly, the difference in the amount of central HD-like adhesions persisted between the integrin α3-deficient and -proficient cells (Fig. S3D,E).

Next, we investigated whether the role of integrin α3β1 in maintaining the central HD-like adhesions requires binding of integrin α3β1 to laminin-332. To this end, we introduced a bindingdeficient integrin α3 mutant (G163A) into integrin α3-deficient HaCaT cells [484]. Expression of the mutant integrin α3β1 was verified by FACS analysis (Fig. 5B). Unlike the wild-type integrin α3 subunit, the integrin α3-G163A mutant was unable to completely rescue the central HD-like adhesions, suggesting that integrin α3β1 binding to laminin contributes to efficient maintenance of these adhesions (Fig. 5C,D). These findings were confirmed in PA-JEB/β4 cells using the integrin α3 blocking antibody, J143 (Fig. S3F,G).

Palmitoylation of CD151 is necessary for stabilization of the CD151 TEMs and hence for incorporation and clustering of integrins α3β1 and α6β4 into these multi-molecular complexes of CD151 [485,486]. To study the role of this self-associating function of CD151 for stabilization of the central HDlike adhesions,we introduced a CD151 palmitoylation mutant into the CD151-deficient PA-JEB/β4 keratinocytes (Fig. 5B) [486]. The expression of this mutantin CD151 KO cells did not rescue the central HD-like adhesions, suggesting that for the maintenance of these clusters CD151 needs to form multi-molecular complexes by oligomerization (Fig. 5E,F; Fig. S3H).

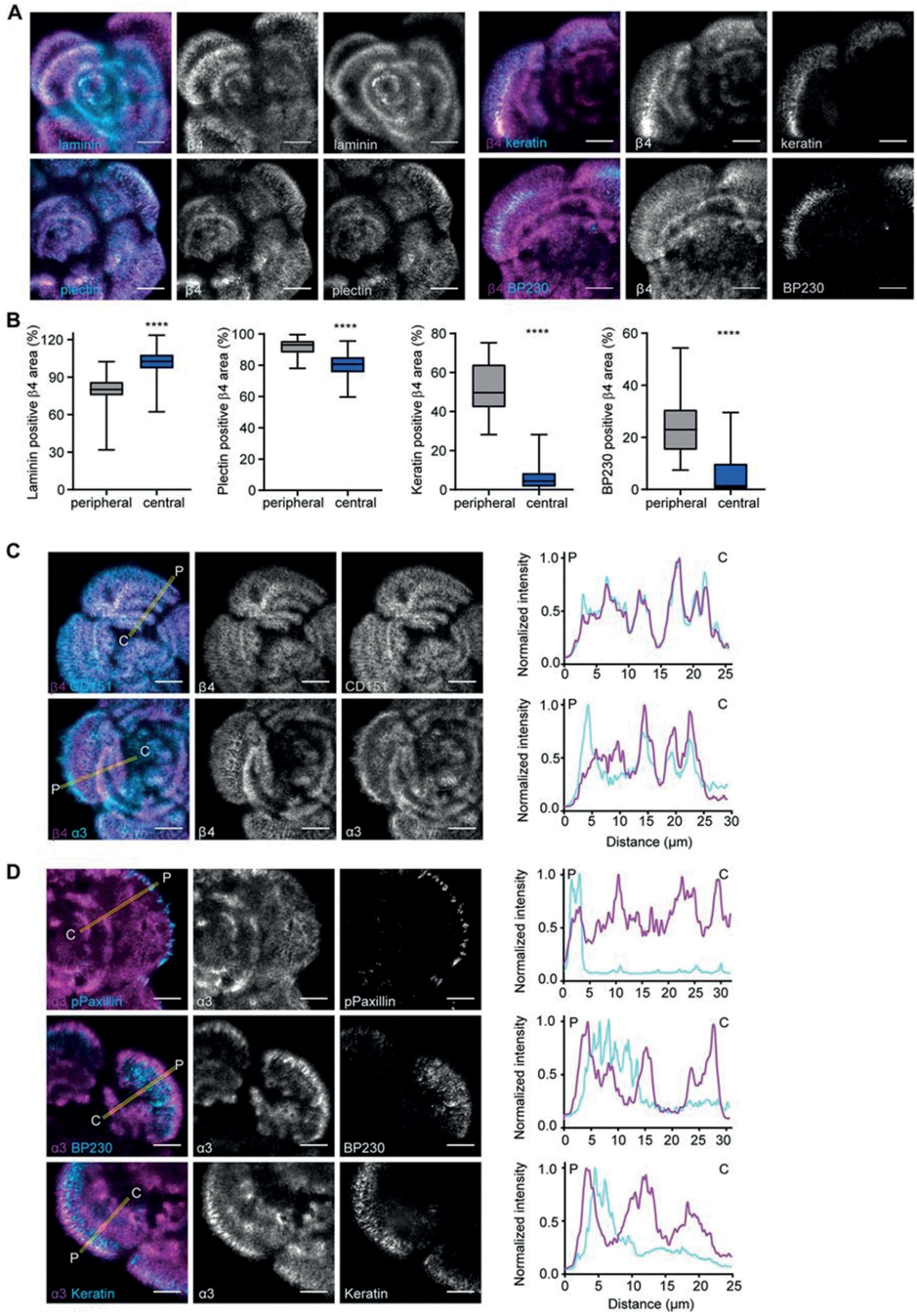


Figure 4 Central and peripheral adhesions differ in protein composition. *(A) TIRF images of PA-JEB/β4 keratinocytes stained for integrin β4 (magenta) and laminin-332, plectin, keratin-14 or BP230 (cyan). Scale bars: 10 μm. (B) Quantification of the integrin β4 area which is positive for laminin-332 (57 images; N=2), plectin (60 images; N=2), keratin-14 (59 images; N=2) or BP230 (61 images; N=2) in peripheral and central*

*integrin α6β4-containing adhesions. Boxplots divide the data in quartiles and the median is indicated. The whiskers show the minimal and maximal values. ****P<0.0001 (Mann–Whitney U-test). (C,D) TIRF images of PA-JEB/β4 cells stained for integrin β4 (magenta) and CD151 or integrin α3 (cyan) (C), or integrin α3 (magenta) and phosphorylated paxillin, BP230 or keratin (cyan) (D). Scale bars are 10 μm. Line plot shows the relative fluorescence intensities (normalized to the highest pixel intensity) of the different proteins along the line (thickness: 10 pixels) indicated in the overlay image. The line starts in the periphery (P) and is drawn towards the center (C) of the island.*

Taken together, these results show that for efficient maintenance of the central HD-like adhesions, both laminin-332-associated integrin α3β1 and self-associated and TEM-forming CD151 are needed.

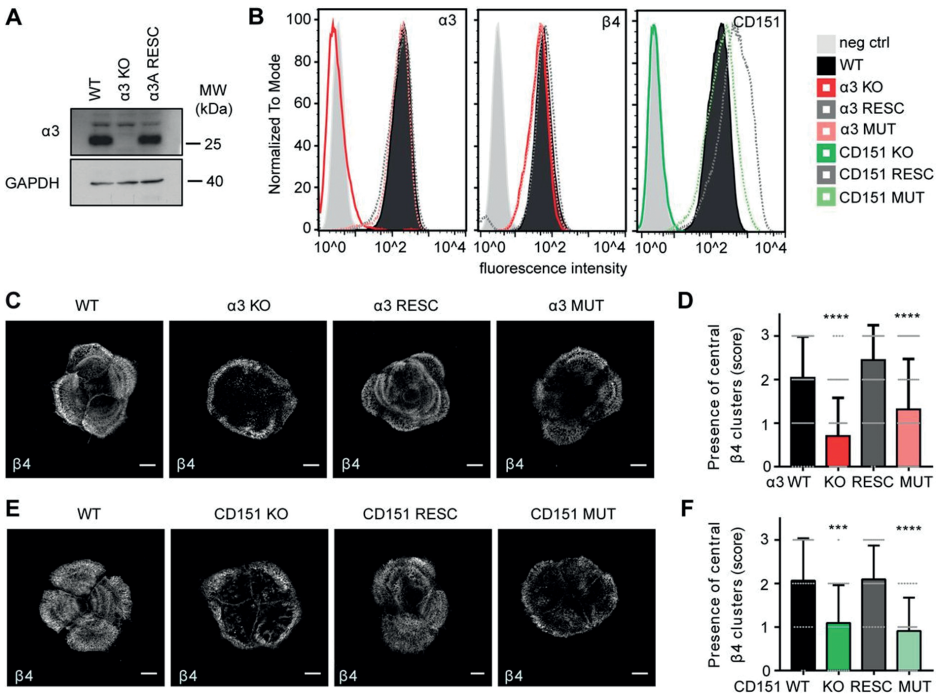


Figure 5 Integrin α3β1 and CD151-self association contribute to stabilization of central HD-like adhesions. *(A) Whole-cell lysate of WT, α3-KO and α3-RESC HaCaT keratinocytes analyzed for protein levels of integrin α3 and GAPDH (loading control) by western blotting. (B) FACS analyses of integrin α3, integrin β4 or CD151 surface expression on WT, α3-KO, α3-RESC, mutated integrin α3 (α3-MUT), CD151-KO, CD151-RESC and CD151-MUT HaCaT keratinocytes. (C) Confocal images of integrin β4 in WT and α3-KO, α3-RESC and α3-MUT HaCaT keratinocytes. Scale bars: 10 μm. (D) Quantification (mean ±s.d.) of the presence of central integrin β4 clusters (score 0–3, dots represent individual score values) in WT (101 islands; N=4) and α3-KO (98 islands; N=4), α3-RESC (103 islands; N=4) and α3-MUT (102 islands; N=4) HaCaT keratinocytes. ****P<0.0001 (Mann–Whitney U-test). (E) Confocal images of integrin β4 in WT and CD151-KO, CD151-RESC and CD151-MUT HaCaT cells. Scale bars: 10 μm. (F) Quantification (mean±s.d.) of the presence of central integrin β4 clusters (score 0–3) in WT (31 islands; N=2) and CD151-KO (33 islands; N=2), CD151-RESC (32 islands; N=2) and CD151-MUT (33 islands; N=2) cells. ***P<0.001, ****P<0.0001 (Mann–Whitney U-test).*

Laminin-332, but not plectin, contributes to clustering of integrin α6β4 in central HD-like adhesions

Since the peripheral HDs and central HD-like adhesions contain more plectin and laminin, respectively, we studied whether CD151- mediated clustering of integrin α6β4 in the different adhesions requires binding of integrin α6β4 to laminin-332 or plectin. Therefore, we expressed the integrin β4 mutants, β4-AD and β4- RW, in integrin β4-deficient PA-JEB keratinocytes (Fig. 6A). The β4-AD mutant is an adhesion-defective mutant that cannot bind to laminin-332, while the β4-RW mutant, carrying a single missense mutation R1281W in the cytoplasmic domain of integrin β4, is unable to bind plectin [9,327,487]. In the β4-AD mutant-expressing cells, central HD-like adhesions were less commonly present than in the wildtype integrin β4 (β4-WT)-expressing cells (Fig. 6B,C).

Similar findings were obtained when PA-JEB/β4 and HaCaT cells were treated with the integrin β4 blocking antibody ASC8 [327]. In the presence of this blocking antibody, the central HD-like adhesions are mostly absent, while there are abundant peripheral HDs (Fig. 6D,E). In the cells expressing the β4-RW mutant and in plectin-deficient PA-JEB/β4 keratinocytes, the integrin α6β4 staining was diffused and adhesions were less well-defined. Nevertheless, in many cell islands some central HD-like adhesions could be observed (Fig. 6B,C,F,H). In addition, the loss of plectin resulted in a reduction of peripheral HDs (Fig. 6G,H). These results indicate that the binding of integrin α6β4 to laminin- 332, as for integrin α3β1, contributes to the stabilization of the central HD-like adhesions. By contrast, binding of integrin α6β4 to plectin is not essential, but might contribute to the clustering of this integrin in the peripheral type I HDs.

CD151 does not affect the strength of integrin α6β4-mediated cell adhesion

Integrin clustering determines the strength with which cells adhere to the extracellular matrix (ECM) [488]. To investigate whether CD151-mediated clustering of integrins α3β1 and α6β4 strengthens the adhesion of keratinocytes to laminin-332 deposited by the cells to the substratum, we quantified the adhesion strength of CD151-proficient and -deficient HaCaT keratinocytes, which express both integrin α3β1 and α6β4, using a spinning disk device [489]. CD151-deficient cells showed a reduced tolerance (albeit not statistically significant) of shear stress compared to the CD151-proficient cells (Fig. 7A), suggesting that CD151 plays only a minor role in adhesion strengthening when both integrins α3β1 and α6β4 are present. Next, we investigated whether cell adhesion mediated by either integrin α3β1 or α6β4 is strengthened by CD151. To resolve this, we individually deleted integrin α3 and integrin β4 in the CD151-deficient HaCaT keratinocytes and confirmed the absence of these proteins by FACS and WBanalysis (Fig. 7B,C). The deletion of integrin α3 in the CD151-deficient HaCaT keratinocytes had no effect on the adhesive strength of integrin α6β4 to laminin-332 (Fig. 7D). However, in the absence of integrin α6β4, the additional loss of CD151 resulted in a significant reduction in integrin α3β1- mediated adhesion strengthening (Fig. 7E,F).

We conclude that CD151 significantly strengthens integrin α3β1-, but not integrin α6β4-mediated cell adhesion by facilitating the clustering of this integrin into central HD-like adhesions.

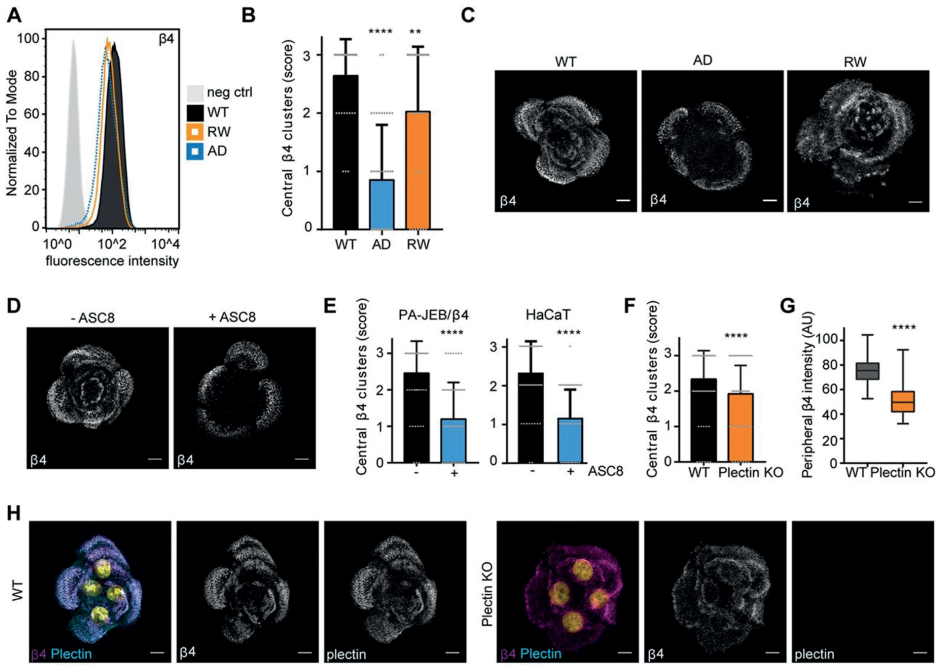


Figure 6 Laminin but not plectin binding to integrin α6β4 contributes to stabilization of central HD-like adhesions. *(A) FACS analyses of integrin β4 surface expression on WT, and β4-AD- and β4-RW-expressing PA-JEB keratinocytes. (B) Quantification (mean±s.d.) of the central integrin β4 clusters (score 0–3, dots represent individual score values) in WT (39 images; N=3), β4-AD (40 images; N=3) and β4-RW (42 images; N=3). **P<0.01, ****P<0.0001 (Mann–Whitney U-test). (C) Confocal images of integrin β4 inWT, β4-AD and β4-RWPA-JEB/β4 keratinocytes. Scale bars: 10 μm. (D) Confocal images of integrin β4 inWT PA-JEB/β4 keratinocytes grown on coverslips for 2 days in the absence (–) or presence (+) of integrin β4 blocking antibody ASC8. Scale bars: 10 μm. (E) Quantification (mean±s.d.) of the presence of central integrin β4 clusters (score 0–3) in WT PA-JEB/β4 treated without (59 images; N=3) or with (60 images; N=3) ASC8, and HaCaT keratinocytes treated without (60 images; N=3) or with (60 images; N=3) ASC8. ****P<0.0001 (Mann–Whitney U-test). (F) Quantification (mean±s.d.) of the presence of central integrin β4 clusters (score 0–3) in WT (110 images; N=6) and plectin-KO (102 images; N=6) PA-JEB/β4 keratinocytes. ****P<0.0001 (Mann– Whitney U-test). (G)Quantification of themean intensity of integrin β4 clustered in the peripheral adhesions inWT (73 images; N=4) and plectin-KO(71 images; N=4) PA-JEB/β4 cells. Boxplots divide the data in quartiles and the median is indicated. The whiskers show the minimal and maximal values. ****P<0.0001 (Mann–Whitney U-test). (H) Confocal images of integrin β4 (magenta) and plectin (cyan) in WT and plectin-KO PA-JEB/β4 keratinocytes. Scale bars: 10 μm.*

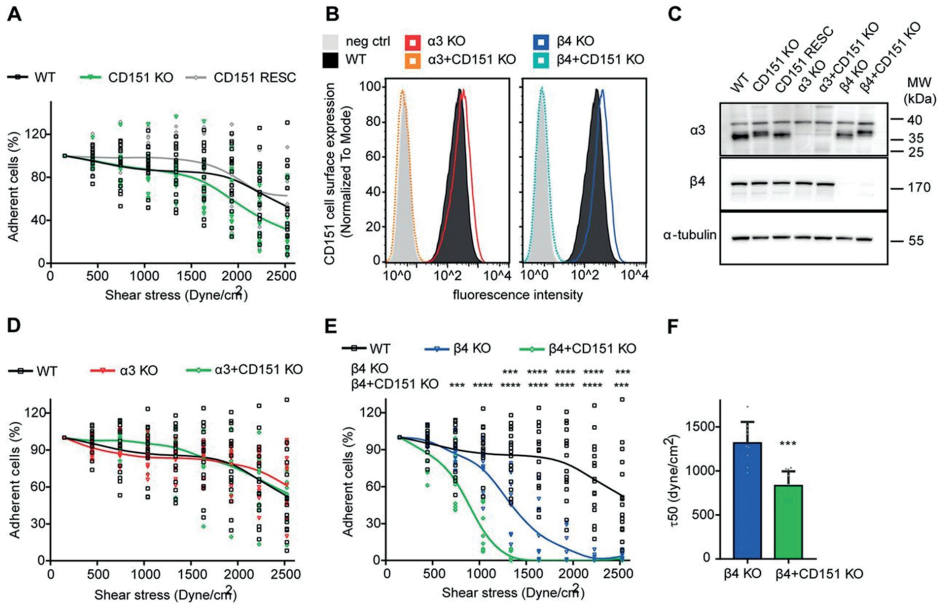


Figure 7 CD151 contributes to integrin α3β1-mediated adhesion strengthening. *(A) Percentage of WT (squares; n=16), CD151-KO (triangles; n=13) and CD151-RESC (diamonds; n=14) HaCaT keratinocytes that remained adherent under conditions of increasing shear stresses (shown in dynes/cm2). Line depicts the mean; no significant differences detected (unpaired t-tests corrected for multiple comparisons). (B) CD151 surface expression of WT, α3-KO, β4-KO and double α3+CD151 and β4+CD151 KO HaCaT keratinocytes analyzed by FACS. (C) Expression of integrin α3, integrin β4 and α-tubulin (loading control) in wholecell lysates from WT, CD151-KO and CD151-RESC, α3-KO, β4-KO and double α3+CD151 and β4+CD151 KO HaCaT keratinocytes analyzed by western blotting. (D,E) Percentage of WT (squares; n=16), α3-KO (triangles; n=11) and double α3+CD151 KO (diamonds; n=11) HaCaT keratinocytes (D) and of WT (squares; n=16), β4-KO (triangles; n=9) and double β4+CD151 KO (diamonds; n=10) HaCaT keratinocytes (E) that remained adherent after being submitted to increasing conditions of shear stresses (in dynes/cm2). Line depicts the mean; ***P<0.001, ****P<0.0001 (unpaired t-tests corrected for multiple comparisons). (F) Mean±s.d. τ50 calculated for β4-KO and double β4+CD151 KO HaCaT cells from the same dataset shown in E. ***P<0.001 (Mann–Whitney U-test).*

Discussion

CD151 has been shown to be broadly expressed by a variety of cell types and to form complexes with different laminin-binding integrins, including integrins α3β1 and α6β4 [490]. In skin, CD151 is complexed with integrin α6β4 and is a component of HDs [3]. In this study, we show that in cultured keratinocytes CD151 is not only present in HDs, but also in an adhesion structure with a distinct molecular composition of a HD with tetraspanin features. These hybrid adhesion structures, which are present in the central region of the cell, and referred to as central HD-like adhesions, contain the cytolinker plectin in addition to the CD151–α3β1/α6β4 integrin complexes, but are not associated with keratin filaments. We further show that CD151, through its binding to integrin α3β1, is critical for the formation and stabilization of these central HD-like adhesions and strengthens integrin α3β1- but not integrin α6β4-mediated

keratinocyte adhesion to laminin-332. CD151 is not needed for the formation of type I HDs that are formed in the periphery of the cells and are anchored to keratin filaments (Fig. 8).

The mechanistic reasons for the lack of association of keratin filaments with the central HD-like adhesions can be surmised based on previous studies. PKA-mediated phosphorylation of plectin at S4642 interferes with its interaction with intermediate filaments [71], and therefore, it is possible that plectin is phosphorylated at this residue in the central HD-like adhesions, which prevents its association with keratin filaments. Additionally, BP230, which is present in peripheral HDs but not central HD-like adhesions, may be required for efficient attachment of keratin filaments to the plasma membrane. In support of this supposition, BP230 knockout mice showed an impaired connection of keratin filaments with HDs [478]. Similarly, reduced amounts of keratins are associated with type II HDs, which contain plectin but lack BP230 [317,467].

Although an essential role of CD151 in the formation of type I HDs was not evident from our current experiments, a more subtle role of CD151 for the formation of HDs cannot be excluded. The ability of CD151 to facilitate the clustering of integrins α3β1 and α6β4 into central HD-like adhesions may strengthen keratinocyte adhesion to newly deposited laminin-332 before FAs and HDs are fully established; however, once integrins are connected to the cytoskeleton, the function of CD151 as a clustering molecule may no longer be required. This model would indicate an important role of CD151-mediated adhesion strengthening and clustering of integrins α3β1 and α6β4 into central HD-like adhesions in vivo. For example, during re-epithelization of wounded skin, when HDs are dissolved and stable adhesion of the keratinocytes to the basal lamina is no longer assured, the central HD-like adhesions may prevent detachment of migrating keratinocytes that are subject to external mechanical forces. At the same time, they may render the adhesion of keratinocytes sufficiently dynamic to allow integrin turnover and migration to occur. Consistent with a role of the central HD-like adhesions in migration, we found that these adhesions are more dynamic than the type I HDs.

Despite the fact that the association of BP230 with keratin filaments at type I HDs increases their stability [423,491], our FRAP analyses showed only a modest increase in the stability of the peripheral HD adhesions compared to the central HD-like adhesions. Since our FRAP analyses do not distinguish between peripheral adhesions that are or are not associated with keratin filaments, this apparent discrepancy may be explained by the notion that not all of the peripheral adhesions are associated with keratin filaments. Furthermore, it should be understood that the peripheral type I HDs lack integrin α3β1, whose presence in the HD-like adhesions contributes to their stability.

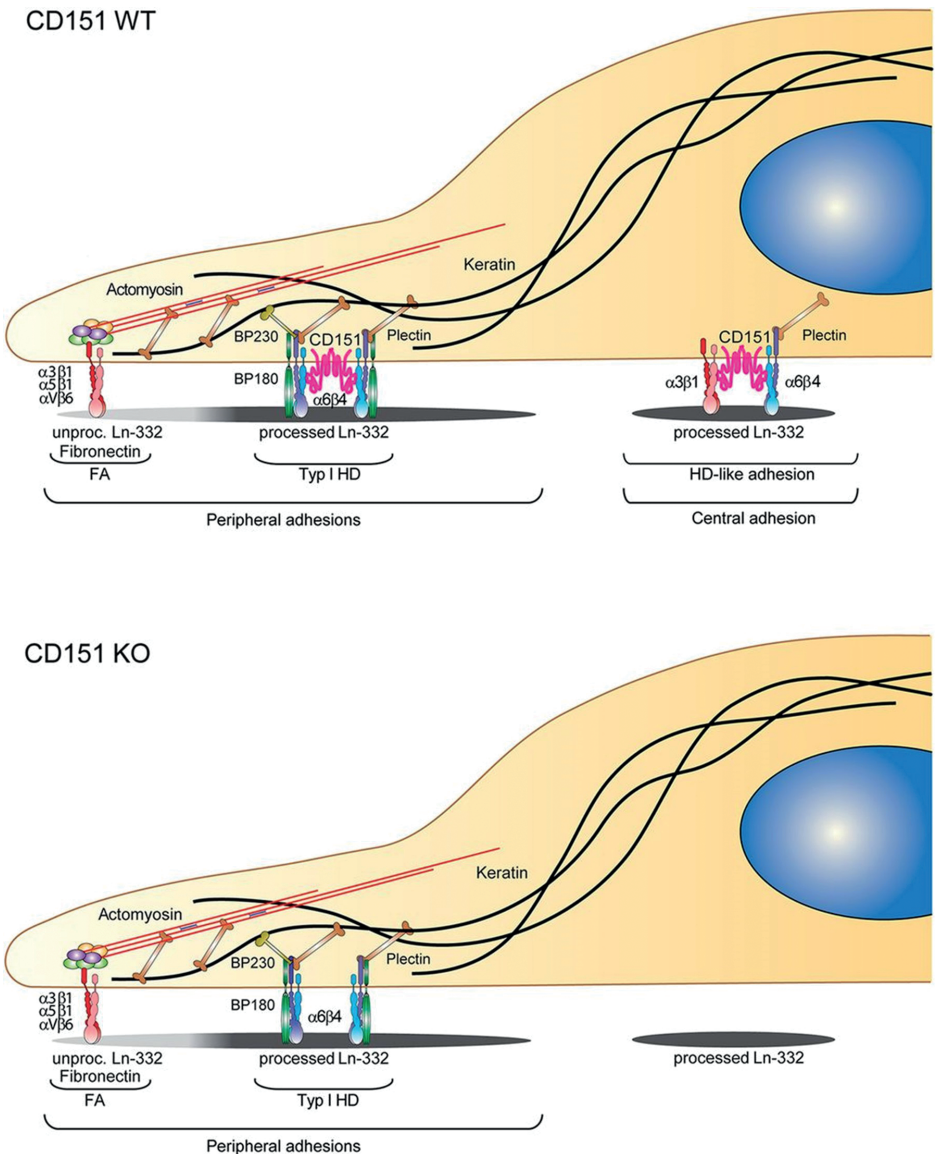


Figure 8 CD151 is essential for the maintenance of central HD-like adhesions but not the formation of peripheral FAs and type I HDs. *In the presence of CD151 (top panel), integrins α3β1 and α6β4 are present together with CD151 in central HD-like adhesions. Additionally, integrin α3β1 is present in FAs in the periphery of the cell island preceding the type I HDs in which integrin α6β4 is present also together with CD151. In the absence of CD151 (bottom panel), the peripheral adhesions are still formed but lack CD151, while the central HD-like adhesions are lost.*

In central HD-like adhesions, CD151 and integrin α3β1 colocalize, but in the periphery CD151 associates with type I HDs and integrin α3β1 is localized into FAs. The localization of integrin α3β1 into FAs coincides with the incorporation of BP230 in HDs. Since the localization of BP180 in type I HDs precedes that of BP230 [11], it is possible that BP180 displaces integrin α3β1 from CD151 and allows the integrin to become incorporated into FAs. In this respect it is interesting to mention that, like CD151 and the integrin α3, α6 and β4 subunits [492], BP180 contains several cysteine residues adjacent to its transmembrane domain that are potential sites of palmitoylation. The role of protein palmitoylation in the formation of HDs is not known, but it is reasonable to suggest that it might help to increase the local concentration of HD proteins, and facilitate the assembly and stability of HDs.

In agreement with our finding that HDs can be formed in the absence of integrin α3β1 in cultured keratinocytes, no obvious abnormalities in HDs have been observed in mice lacking integrin α3 in basal keratinocytes [54,55,493]. Yet, genetic ablation of the β1 integrin subunit or its co-activator kindlin-1 (encoded by FERMT1) in basal keratinocytes has been associated with reduced HD protein expression and formation of HDs [494–496]. This suggests that if integrin α3β1 has a role in HD formation, this role could be fulfilled by other integrins, as well. Furthermore, our finding that CD151 contributes to integrin α3β1- but not integrin α6β4-mediated strengthening of cell adhesions is consistent with the respective roles these proteins play in mediating cell-matrix adhesion in the skin and kidney. In the skin, where basal keratinocytes express both integrins α3β1 and α6β4, the effect of CD151 deletion is relatively mild, and patients display only a mild skin blistering disorder. In contrast, the loss of CD151 in glomerular podocytes, which express integrin α3β1 but not integrin α6β4, has been associated with progressive and irreversible kidney dysfunction [108,474–476].

In conclusion, we show that CD151 stabilizes the central HD-like adhesions by strengthening the adhesion of integrin α3β1 to laminin-332 and facilitating the incorporation of integrin α6β4 into TEMs containing integrin α3β1. By contrast, CD151 is not needed for the formation or stabilization of integrin α6β4-containing type I HDs in the periphery of the cells, which are linked to keratin filaments. These data provide new insights into the role of CD151 in the formation and maintenance of different populations of adhesions that are formed in cultured keratinocytes. Importantly, our study reflects the skin conditions observed in patients and mice with mutations in CD151 and integrin α3β1, and thus provides better understanding of the mechanisms underlying these conditions.

Material and Methods

Antibodies

The primary and conjugated antibodies used in this study are listed in Table S1. Secondary antibodies for western blotting were goat anti-mouse IgG HRP (1:3000; Bio-Rad, 170-6516) and polyclonal goat anti-Rabbit IgG HRP (1:5000; Dako, P044801-2). Donkey anti-mouse IgG PE (1:500; Jackson ImmunoResearch, 715-116-150) was the secondary antibody used in FACS. For immunofluorescence (IF), rabbit anti-rat IgG (1:1000; in-house), goat anti-guinea pig IgG Alexa Fluor 488, goat anti-human IgG Alexa Fluor 488, goat anti-mouse Alexa Fluor 568, goat anti-mouse Alexa Fluor 647, goat anti-rabbit Alexa Fluor 488, goat anti-rabbit Alexa Fluor 647, goat anti-rat Alexa Fluor 647 (1:200; Invitrogen, A-11073, A-11013, A-11004, A-21236, A-11034, A-21245, A-21247), goat anti-mouse FITC, goat anti-rat FITC (1:200; Rockland, 610-102-121 and 612-102-120) and the isotype-specific goat anti-mouse IgG1 Alexa Fluor 568 (1:500; Thermo Fisher Scientific, A-21124) and goat anti-mouse IgG2a FITC (1:250; Abcam, ab98697) were used secondary antibodies.

Cell lines

PA-JEB immortalized keratinocytes were isolated from a patient with pyloric atresia associated with junctional epidermolysis bullosa [13,24]. The derivation of this cell line was done for diagnostic purposes, thus, the research conducted using these cells was exempt from the requirement for ethical approval. PA-JEB/β4 keratinocytes stably expressing wild-type β4 were generated by retroviral transduction [3] and maintained in serum-free keratinocyte medium (KGM; Invitrogen), supplemented with 50 μg ml^{-1} bovine pituitary gland extract, 5 ng ml^{-1} EGF, and antibiotics (100 units ml^{-1} streptomycin and 100 units ml^{-1} penicillin; Sigma-Aldrich). Human HaCaT keratinocytes were obtained from Dr Fusenig of the German Cancer Center (DKFZ) in Heidelberg, Germany and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% heatinactivated fetal calf serum (FCS; Serana) and antibiotics. All cells were cultured at 37°C in a humidified, 5% CO_2 atmosphere.

The integrin α3-, integrin β4-, CD151- and plectin-deficient cells were prepared using CRISPR-Cas9 technology. Target sgRNAs against integrin α3 (5′-GCTACTCGGTCGCCCTCCAT-3′), integrin β4 (5′-GACTCGCTCCCTCCGCGCCT-3′), CD151 (5′-CAGGTTCCGACGCTCCTTGA-3′) and plectin (5′-GAGGTGCTTGTTGACCCACT-3′) were cloned into the pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene plasmid #42230, deposited by Feng Zhang). For the integrin α3-, integrin β4- and CD151- deficient cells, cells were transiently transfected with this plasmid using Lipofectamine® 2000 (Invitrogen) in OptiMEM (Gibco). The cells were bulk sorted for the population deficient of the protein of interest using a Moflo Asterios (Beckman Coulter) or FACSAria Fusion (BD Biosciences) cell sorter. For the preparation of the plectin-deficient cells, cells were co-transfected with a plasmid

containing a blasticidin cassette flanked by TIA target sites (described by Blomen et al. [497]), blasticidin-resistant cells were enriched by selection for four days in the presence of blasticidin (4 μg ml^{-1}; Sigma-Aldrich), clones were picked and analyzed for plectin expression by western blotting.

For (re)expression of proteins in protein-deficient cells, we used stable cellular retroviral-mediated transduction. Cells that expressed the protein were enriched by zeocin or blasticidin selection and/or by bulk sorting by FACS. The constructs used were: the β4-AD mutant (D230A, P232A and E233A) in LZRS-IRES-blasticidin, a kind gift from Peter Marinkovich [327], the CD151 wild-type and CD151 palmitoylation mutant in LZRS-IRES-Zeo, constructed by introducing the CD151 cDNA released from pZeo-CD151 or pZeo-CD151-PALM [gift from F. Berditchevski [486]] and the integrin α3 wild-type in LZRS-IRES-Zeo. The integrin α3 cDNA has been described previously [30] and the full-length cDNA encoding human integrin α3 was released from pUC18 by digesting with SphI, made blunt by Klenow digestion and ligated into the SwaI site of the retroviral LZRSIRES- zeo vector. Furthermore, the integrin α3-G163A mutant in pBJ-1 expression vector [484] was a gift from Yoshikazu Takada (University of California, Davis, USA) and transiently transfected in α3-deficient cells. Generation of PA-JEB keratinocytes stably expressing integrin β4 [β4RW (R1281W)] has been described previously [9].

Immunofluorescence imaging of fixed samples
For image acquisition, cells were seeded at ~10–20% confluence onto glass coverslips, uncoated or coated with 10 μg ml^{-1} fibronectin from bovine plasma (F1141, Sigma-Aldrich) and 5 μg ml^{-1} vitronectin (SRP3186, Sigma-Aldrich) and grown for 40–44 h of which at least the last 20 h in the presence of DMEM+FCS. After the cells had been fixed with 2% PFA for 10 min, permeabilized with 0.2% Triton X-100 and blocked with 2% BSA (resolved in PBS; Serva), they were incubated for 50–60 min with primary and secondary antibodies, with three PBS washes in between. Nuclei were counterstained with DAPI (Sigma-Aldrich) and coverslips were mounted with MOWIOL for confocal imaging. For TIRF imaging, coverslips were stored in PBS at 4°C until imaging.

Confocal imaging was performed using a Leica TCS SP5 confocal microscope with a 63× (NA 1.4) oil objective. A Leica SR-GSD microscope (Leica Microsystems, Wetzlar, Germany) equipped with a 160× oil immersion objective (NA 1.47) and an EMCCD Andor iXon camera (Andor Technology, Belfast, UK) was used to acquire TIRF images with an imaging depth of 200 nm. To be able to perform subsequent analyses, imaging was performed under consistent conditions.

Image analyses were performed using ImageJ: Co-localization was assessed using Manders' overlap coefficient (MOC) with the JACoP plugin. The amount of basal cell surface clustering was quantified by calculating the integrated density of the staining.

The intensity of the integrin β4 staining in the peripheral adhesions was obtained by drawing a region of interest around the peripheral adhesions and measuring the mean intensity of the integrin β4 staining in this region. The percentage of integrin β4 associated with plectin, BP230, keratin 14 or laminin-332 was obtained by calculating how much of the integrin β4-positive area was additionally positive for plectin, BP230, keratin or laminin-332. The quantification of the island sizes was performed by thresholding images based on their staining with Phalloidin-iFluor647 (AAT Bioquest) and measuring the surface area after selecting the outer region. Furthermore, the quantification of the amount of central integrin β4 clusters present per cell island was performed using an arbitrary scoring system (0=no central adhesions, 1=few central adhesions, 2=several central adhesions, 3=many central adhesions).

Graphs were made using GraphPad Prism and include bar graphs showing the mean±s.d. (including dots that represent independent measurement) or boxplots dividing the data in quartiles. The number of images used for analyses is specified in the figure legends and statistical significance was calculated using the non-parametric and non-paired Mann–Whitney U-test; P-values: *<0.05, **<0.01, ***<0.001, ****<0.0001.

Live imaging and FRAP

For live imaging and fluorescence recovery after photobleaching (FRAP), cells were seeded on 24 mm coverslips and imaging was performed using a Leica TCS SP5 confocal microscope with a 63× (NA 1.4) oil objective equipped with a live cell chamber. Live imaging of CD151-proficient and -deficient PA-JEB/β4–GFP cells was started 20 h after seeding and immediately after replacing the KGM medium with DMEM+FCS. Every hour the differential interference contrast (DIC) and GFP fluorescence signals were collected from multiple cell islands at different positions. Videos were made in ImageJ.

FRAP experiments were performed, using the FRAP wizard in LAS-AF (Leica), using PA-JEB/β4–GFP cells grown for 20 h in KGM medium and subsequently grown for an additional 20 h in DMEM+FCS. Simultaneously, a region in one peripheral and one central integrin α6β4- containing adhesionwas bleached and fluorescent recoverywas followed over time. Two images as controls were taken before bleaching and then one image every 10 s (first 2 min) or 30 s for 15 min after bleaching. For analyses, the background was subtracted from the measured intensities and the curves were normalized to themean value before bleaching. Furthermore, the values of the first 230 s were fitted with a single exponential decay using MATLAB software and the fitted curve was used to calculate the mobile fraction and the recovery time τ.Graphs were made using GraphPad Prism; the line plot shows the mean±s.d. and the boxplots divide the data in quartiles. The number of images used for analyses is specified in the figure legends and the significance difference between the central and peripheral adhesions

was calculated using the non-parametric Wilcoxonmatched-pairs signed rank test (at different time points); P-values: **<0.01, ***<0.001.

Flow cytometry and FACS

For FACS analyses, cells were collected after trypsinization, and incubated for 50–60 min with primary and secondary antibodies on ice in PBS containing 2% FCS. In between the antibody steps, cells were washed twice with 2% FCS in PBS. Finally, cells were passed through a nylon mesh filter and 50000+ cells were analyzed per sample using a FACSCalibur cell analyzer (BD Biosciences). Cells incubated with only secondary antibody were used as a negative control. Graphs include values of a representative experiment (out of three repetitions) normalized to the mode. The graphs were made in FlowJo and adapted in Adobe Illustrator.

Adhesion strengthening assay

To measure long-term adhesion strength of cells, we used the spinning disk assay described by Boettiger (2007). Wild-type or KO HaCaT cells were grown to 60–100% confluence on 30 mm coverslips for 2 days. Just before spinning, the cells were washed briefly with PBS and the coverslips were 'glued' to the stamp using grease. The cells were spun for 8 min at 4240 rpm in PBS-ABC buffer [80% A (10 gl^{-1} NaCl, 0.25 gl^{-1} KCl, 0.95 gl^{-1} $Na2HPO4*2H_2$, 0.25 gl^{-1} $KH2PO4$), 10% B (1.34 gl^{-1} $CaCl2.2H2O$) and 10% C (2 gl^{-1} $MgCl2.6H2O$)] containing 5% dextran from Leuconostoc spp. Mr 450,000–600,000 (Sigma-Aldrich). After spinning, the cells were fixed in 2% PFA, permeabilized in 0.2% Triton X-100, stained with DAPI and mounted on microscopic slides using MOWIOL. The complete coverslips were imaged using a Zeiss AxioObserver Z1. The coverslip area was divided in rings with a 1.5 mm diameter and the cell confluence was measured per ring and normalized to the total area of the ring and to the confluence in the middle area (100% confluent). The average shear stress per ring was calculated using the formula

$$\tau = 10\,x\,\left(0{,}8\,r\sqrt{\rho\mu\omega^3}\right)$$

with τ is the shear stress in dynes/cm^2, r is the radial distance from the center of rotation in m, ρ is the fluid density in kg m^{-3}, μ is the fluid viscosity in Pa s and ω is the rotational velocity in radians min^{-1}. The $\tau 50$ was obtained by fitting the data points to a logistic function

$$f = \frac{100}{1 + \exp\left[\frac{\tau - c}{b}\right]}$$

using MATLAB software, where c is the inflection point and equal to the point of 50% cell detachment ($\tau 50$) and b is the slope at the inflection point.

Graphs were generated and significance was calculated using GraphPad Prism. The line plots show the mean value of the 9–16 experiments per condition, which are individually shown as squares, diamonds or triangles. For the wild-type line, the same dataset was used in the different graphs. The significance of the percentage of adherent cells in KO cells compared to the wild-type cells was calculated at each data point using unpaired t-tests corrected for multiple comparisons using the Holm–Sidak method; P-values: ***<0.001, ****<0.0001. The τ50 was visualized in a bar graph showing the mean±s.d. of 9–10 independent experiments. The significant different between the τ50 in integrin β4 KO and integrin β4+CD151 KO cells was calculated using the non-parametric Mann–Whitney U-test; P-value: ****<0.0001.

Immunoprecipitations and western blotting
For analysis of proteins in whole cell lysates, sub-confluent cells were washed in cold PBS, lysed in RIPA (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 4 mM EDTA pH 7.5, 100 mM NaCl, 20 mM Tris-HCl pH 7.5) supplemented with inhibitors (1.5 mM Na_3VO_4, 15 mM NaF and 1:500 protease inhibitor cocktail), whole-cell lysates were cleared by centrifugation at 15,000 g for 60 min at 4°C and supplemented with SDS sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 12.5 mM EDTA, 0.02% Bromophenol Blue) with or without β-mercaptoethanol and heated at 95°C for 5 min. Immunoprecipitations were performed with cell lysates prepared in 1% NonidetP-40, 100 mM NaCl, 4 mM EDTA, 20 mM Tris-HCl (pH 7.5), supplemented with inhibitors. After centrifugation at 15,000 g for 60 min at 4°C, the cleared lysates were incubated at 4°C for 1.5 h with 1 µg ml^{-1} antibody. Subsequently, the lysates were incubated overnight with Protein G Sepharose 4 Fast Flow beads (GE Healthcare), beads werewashed three times with lysis buffer and two times with PBS and bound proteins were dissolved in SDS sample buffer without β- mercaptoethanol and heated at 95°C for 5 min.

Proteins were separated on 4–12% bolt Novex gradient gels (Invitrogen) and transferred to Immobilon-P transfer membranes (Millipore). The membrane was blocked for at least 2.5 h in 2% BSA in TBST (10 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween 20) before incubation with primary antibody overnight at 4°C and with secondary antibody for 1 h at room temperature. After each incubation, membranes were washed twice with TBST and twice with TBS (TBST without Tween 20). Antibodies were detected using Clarity Western ECL Substrate (Bio-Rad) and in the figures the molecular mass markers (kDa) are indicated.

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Supplementary information

Supplementary information available online at http://jcs.biologists.org/lookup/doi/10.1242/jcs.235366.supplemental



CHAPTER 6

Comparative interactomics analysis reveals potential regulators of α6β4 distribution in keratinocytes

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Abstract

The integrin α6β4 and cytoskeletal adaptor plectin are essential components of type I and type II hemidesmosomes (HDs). We recently identified an alternative type II HD adhesion complex that also contains CD151 and the integrin α3β1. Here, we have taken a BioID proximity labeling approach to define the proximity protein environment for α6β4 in keratinocytes. We identified 37 proteins that interacted with both α6 and β4, while 20 and 78 proteins specifically interacted with the α6 and β4 subunits, respectively. Many of the proximity interactors of α6β4 are components of focal adhesions (FAs) and the cortical microtubule stabilizing complex (CMSC). Though the close association of CMSCs with α6β4 in HDs was confirmed by immunofluorescence analysis, CMSCs have no role in the assembly of HDs. Analysis of the β4 interactome in the presence or absence of CD151 revealed that they are strikingly similar; only 11 different interactors were identified. One of these was the integrin α3β1, which interacted with α6β4 more strongly in the presence of CD151 than in its absence. These findings indicate that CD151 does not significantly contribute to the interactome of α6β4, but suggest a role of CD151 in linking α3β1 and α6β4 together in tetraspanin adhesion structures.

Introduction

The cytoskeleton of epithelial cells is an integrated network of actin microfilaments, keratin intermediate filaments and microtubules, which helps the cell to maintain its shape and internal organization. The actin and keratin networks are linked to the cytoskeletons of adjacent cells and the extracellular matrix by specialized junctional complexes and, thereby, contribute to tissue integrity and intracellular communication [498,499]. While the microtubule cytoskeleton is responsible for the intracellular transport of membrane-bound vesicles and organelles.

Hemidesmosomes (HDs) are integrin-based adhesion complexes that mediate stable anchorage of epithelial cells to the underlying basement membrane and serve as anchoring sites for the keratin cytoskeleton to the plasma membrane. Two types of HDs can be distinguished according to their protein composition. The integrin α6β4, a heterodimeric transmembrane protein, is the main component of both type I and type II HDs [1–3,317,467]. In addition to α6β4, these two structures share the tetraspanin CD151 and the cytoskeletal linker protein plectin, which through binding to β4 and keratin establishes a linkage between the extracellular matrix (ECM) and the keratin intermediate filament (IF) cytoskeleton. In type I HDs an additional linkage between the plasma membrane and keratin filaments through BP180 and BP230 enhances the stability of these adhesion structures. A third CD151-containing adhesion structure formed in the central region of cultured keratinocytes contains the integrin α3β1 in addition to the type II HD components α6β4 and plectin (te Molder *et al.*, 2019). Absence or defects of HD proteins compromises epithelial integrity, which results in a blistering disorder called Epidermolysis Bullosa [500]. Besides its role in mediating stable cell-matrix adhesion, the HD integrin α6β4 cooperates with growth factor receptors to promote pro-tumorigenic signaling [430].

Linkage of the actin cytoskeleton to the extracellular matrix occurs (amongst others) at integrin-based multiprotein complexes termed focal adhesions (FAs). The molecular composition of these adhesions may vary depending on external cues and cellular responses [501]. However, their core always consists of integrins and adaptor proteins such as talin and vinculin. In addition to providing adhesion of the cells, FAs are essential for cell migration.

In close proximity of FAs, cytoplasmic linker associated proteins (CLASPs) anchor the plus ends of microtubules to the plasma membrane through a complex of LL5α/β (also known as PHLDB1/2), liprin α1/β1 and ELKs (also known as ERC1) (Mimori-Kiyosue *et al.*, 2005; Lansbergen *et al.*, 2006; Akmanova and Steinmetz, 2008). KANK proteins have been identified as the adaptor proteins that can link this complex of proteins, known as the cortical microtubule stabilizing complexes (CMSCs), to FAs. They bind on the one hand to talin in FAs and on the other hand to liprin β1 in the CMSCs. By serving

as sites for the targeted delivery of microtubule bound vesicles containing MMPs at the cell membrane, the CMSCs are thought to play an important role in the turnover of FAs and actin reorganization [505–508].

Recently, we showed that FAs and HDs are mechanically coupled, and that plectin, which binds integrin β4 and F-actin in a mutual exclusive manner, plays a central role in this coupling [456]. In this study, we utilized the proximity dependent biotinylation (BioID) [509] to map the interactomes of the integrin α6 and β4 subunits in keratinocytes and assessed the contribution of CD151 to the β4 interactome. Furthermore, we investigated the role of CMSC components in HD dynamics.

Results

Identification of the β4 associated proteins by proximity-dependent biotinylation
To identify proteins that interact with integrin α6β4 in keratinocytes, we applied the BioID method [509] which has been successfully used to identify proteins that reside in close proximity of a specific protein of interest, including proteins that are only transiently associated [510–513]. BioID employs a modified biotin ligase BirA* fused to a protein of interest that biotinylates proteins in close proximity (20 nm) upon addition of biotin. We fused BirA* to the C-terminal tail of full-length integrin β4 (Fig. 1A) and stably expressed the fusion protein in β4-deficient PA-JEB keratinocytes by retroviral transduction. Expression of the β4-BirA* fusion protein (225 kDa) was confirmed by western blot (Fig. 1B). Like wild-type β4, the fusion protein localized in HDs as visualized by immunofluorescence (Fig. 1C).

Treatment of the β4-BirA* expressing cells with biotin resulted in a time-dependent increase in biotinylated proteins (Fig. 1D). Notably, a number of proteins were biotinylated in the absence of exogenously added biotin. In an effort to reduce the level of biotinylation of these background proteins, we depleted the culture medium of biotin by treatment with Streptavidin agarose beads, which reduced the amount of background proteins (Fig. 1E).

Biotinylated proteins were collected using streptavidin-Sepharose beads and analyzed by mass spectrometry. As expected, the integrin β4 subunit and its partner subunit α6, were identified among 130 other hits. Other interactors were components of HDs and multiple proteins of two other membrane complexes, CMSCs and FAs (Fig. 1F; Table S1).

Interactomics analysis of α6β4 associated proteins
To demonstrate the specificity of the β4 interactions with components of FAs and CMSCs, we performed an additional BioID experiment with an irrelevant transmembrane protein, the interleukin2 receptor (IL2R). Stable cell lines expressing

the IL2R fused to BirA* were generated by retroviral infection. The IL2R-BirA* fusion protein was expressed in PA-JEB/β4 keratinocytes. Pairwise comparison of the β4-BirA* and IL2R-BirA* datasets (obtained after treatment of the cells with 50 μM biotin) revealed that 115 proteins interacted specifically with integrin β4. Consistent with the results of the comparison of the β4 samples treated with or without biotin, we identified multiple HD proteins (5/6) and FA proteins (10/60), and all components of the CMSC (7/7) as proximity interactors of β4 (Fig. 2A; Table S2).

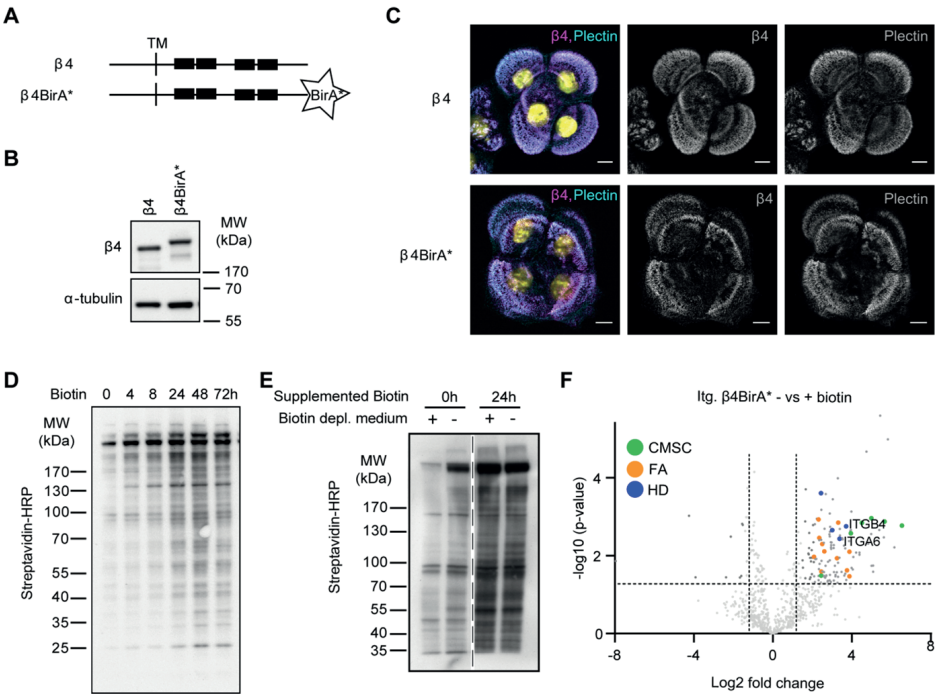


Figure 1 BioID method to identify β4 proximity interactors. *(A) Schematic representation of wild-type β4 and the β4-BirA* fusion proteins. Black boxes indicate the FnIII domains. TM is transmembrane domain. (B) Western blot analysis of whole cell lysates from PA-JEB/β4 and PA-JEB/β4-BirA* keratinocytes probed with anti-β4 and anti-α-tubulin (loading control) antibodies. (C) Representative confocal microscopy images of PA-JEB keratinocytes expressing wild-type β4 and β4-BirA* stained for β4 and plectin. Scale bars: 10 μm. (D) Whole cell lysates from PA-JEB/β4-BirA* keratinocytes treated for the indicated time points with 50 μM biotin and analyzed by western blot with streptavidin-HRP. (E) Western blot analysis of biotinylated proteins from PA-JEB/β4-BirA* cells, cultured in regular medium or biotin-depleted medium for 20h and subsequently treated with or without biotin for 24h. Biotinylated proteins were detected by probing the membrane with Streptavidin-HRP. (F) Volcano plot showing enrichment (log2) and corresponding significance (p-value, log10) of biotinylated proteins in biotin-treated and -untreated PA-JEB/β4-BirA* keratinocytes (n=3).*

Next, we determined the proximity interactors of the integrin α6 subunit. To this end, the α6 subunit fused to BirA* was expressed in PA-JEB/β4 keratinocytes in which

the α6 gene (*ITGA6*) had been disrupted by CRISPR/Cas9 technology. A total of 57 proximity interactors of α6 were identified that were absent from the negative IL2R-BirA* sample. 37 of these proteins were also identified in the β4 interactome. Again many of the shared proteins were components of HDs, CMSCs and FAs (Fig. 2C). The analysis also revealed a significant number of subunit-specific interactors, 20 for α6 and 78 for β4 (Fig. 2B). The fact that more proteins were identified with β4-BirA* than with α6-BirA* could be due to flexibility of the unusually long β4 cytoplasmic domain (1019 amino acids compared to 54 amino acids in α6), which allows the fused biotin ligase not only to biotinylate proteins that are spatially located near but also distant from the α6 subunit. It is important to note that keratinocytes do not express α6β1 and that, therefore, the interactors of α6 subunit can be considered to be specific for α6β4 (Table S3)[429,465].

In conclusion, the interactome of integrin α6β4 in keratinocytes contains 135 proteins and many of the previously mentioned HD, CMSC and FA proteins were amongst the significant interactors of both α6 and β4.

β4 proximity interactors in the presence and absence of CD151

Previously, we have shown that α6β4 resides in both central and peripheral adhesions in keratinocytes [465]. While the peripheral adhesions have all the hall marks of type I HDs, the central adhesions are more similar to type II HDs but are not associated with keratin filaments and additionally contain integrin α3β1. Because CD151 forms a complex with both α3β1 and α6β4, and plays an important role in promoting the formation of the central HD-like adhesions, we wondered whether some of the components of the β4 interactome depend on the presence of CD151. To this end, we generated CD151-deficient PA-JEB/β4-BirA* keratinocytes by CRISPR/Cas9-mediated gene disruption and subjected them to BioID. Deletion of CD151 was confirmed by FACS analysis (Fig. 3A). BioID results showed that the β4 interactomes in CD151-proficient and -deficient keratinocytes overlapped each other almost completely; only 11 different β4 interactors were identified (Fig. 3B Table S4). Integrin α3β1, a CD151 binding partner was significantly more associated with β4 in the presence of CD151 than in its absence. Additionally, several proteins associated with RhoA/ROCK-mediated contractility and trafficking were associated with β4 specifically in the presence or absence of CD151. Importantly, none of the identified proteins were components of HDs, CMSCs or FAs (Fig. 3B; Table S4). These results show that despite the fact that CD151 forms a complex with α6β4, this protein does not substantially contribute to the α6β4 interactome. Furthermore, these findings suggest that the CD151-containing adhesions in the central region of the cells hardly contribute to the interaction between HDs and CMSCs or FAs. Indeed, IF analysis in HaCaT keratinocytes revealed that CMSCs and FAs are assembled in close association with the peripheral HDs, but not the central tetraspanin adhesion structures (Fig. 3C).

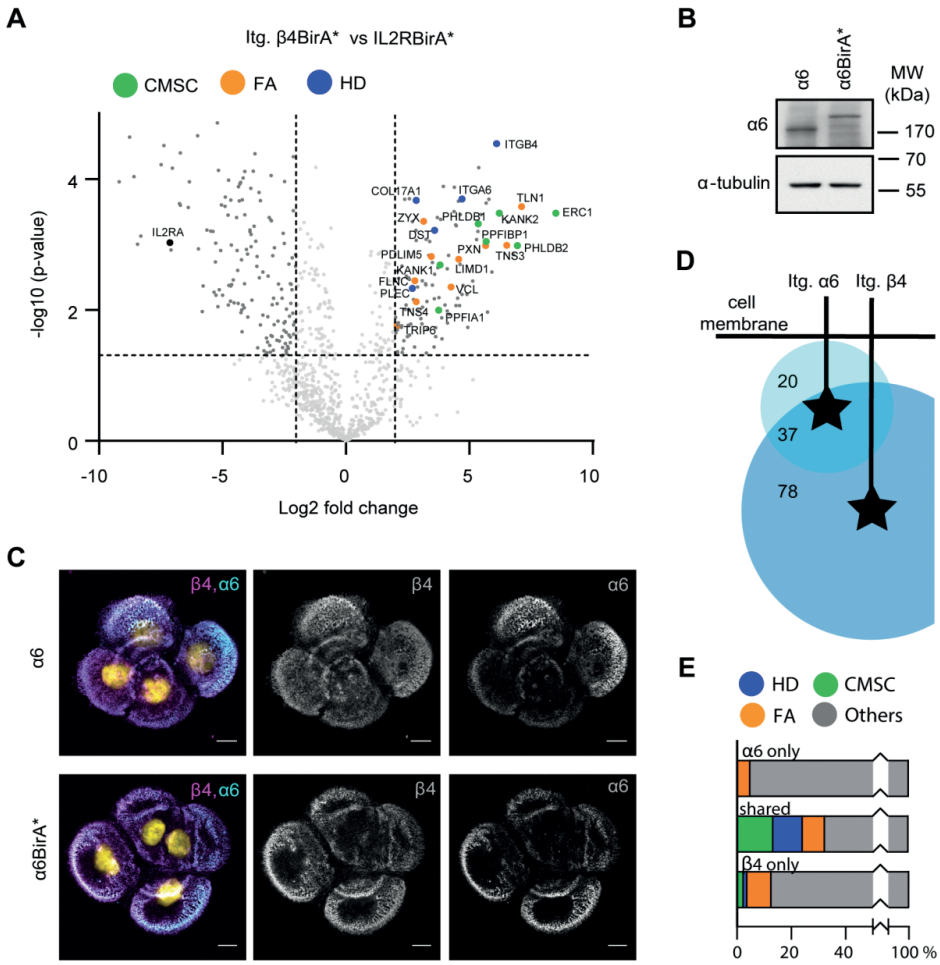


Figure 2 Interactomics analysis of α6β4 associated proteins. *(A) Volcano plot showing enrichment (log2) versus significance (p-value, log10) of proteins identified by β4-BirA* relative to control IL2R-BirA* (n=3). Components of the CSMCs, FAs and HDs are highlighted in green, orange and blue, respectively. (B) Western blot analysis of whole cell lysates from PA-JEB/β4 and PA-JEB/β4 α6-BirA* keratinocytes probed with anti-α6 and anti-α-tubulin (loading control) antibodies. (C) Representative confocal microscopy images of PA-JEB/β4 keratinocytes expressing endogenous α6 or α6-BirA* stained for α6 and β4. Scale bars: 10 μm. (D) Venn diagram depicting the number of overlapping hits between interacting proteins of the Itg. α6 and Itg. β4 subunit. Numbers of overlapping and specific proteins are presented. (E) Bar graphs showing the percentage of HD, CMSC and FA proteins in the α6 and β4 interactomes as well as their percentage among the interactors shared by the two interactomes.*

In simple epithelial tissues and cell lines, such as kidney or intestinal cells, integrin α6β4 typically resides in type II HDs [317,467]. The group of Aki Manninen used BioID to identify 91 significant cytoplasmic proximity interactors of α6β4 in Madin Darby Canine kidney epithelial cells [514]. We compared these interactors with the interactors identified for α6β4 in keratinocytes and found that only 30 proteins were

common between the two data sets (Fig. 3D; Table S5). Although more components of cell matrix complexes were found in keratinocytes than in kidney cells, many of the common hits were CMSC, FA and HD members. These results suggest that the interaction between these complexes is not restricted to type I HDs but also occurs at type II HDs (in kidney cells).

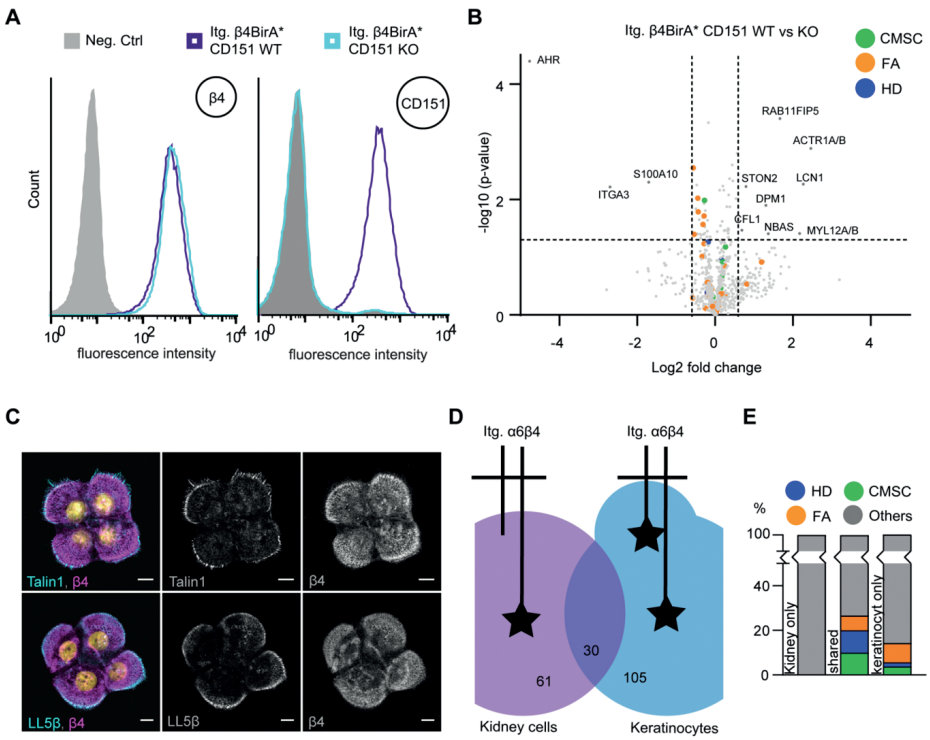


Figure 3: CMSC and FA proximity interaction with type I HDs formed at the cells' periphery. *(A) FACS analysis of β4 and CD151 in PA-JEB/β4-BirA* CD151 wild-type and CD151 KO cells. (B) Volcano plot showing β4 proximity interactors in the presence and absence of CD151 (n=3). (C) Immunofluorescence analysis of wild-type HaCaT keratinocytes showing colocalization of β4 with LL5β and talin1. Scale bars: 10 μm. (D) Venn diagram comparing the α6β4 interactome in keratinocytes with that of β4 in MDCK cells* **[514]**. *Numbers of overlapping and specific proteins are presented. (E) Bar graph showing the percentage of HD, CMSC and FA proteins among the unique interactors of α6β4 (105) and the β4 (61) in MDCK and PA-JEB/β4 keratinocytes, as well as their percentage among the interactors that are shared between the two interactomes.*

CMSCs are not required for the formation of HDs and vice versa.

Our finding that CMSC proteins are found in close proximity of α6β4 containing HDs, raises the question whether this complex plays a role in the formation of HDs by providing a platform at the plasma membrane for the delivery of exocytotic vesicles carrying specific HD components. To investigate the contribution of CMSCs in the formation of HDs, we generated stable liprin α1 and β1 knockdown PA-JEB/β4 keratinocytes by short hairpin RNA (shRNA)-mediated RNA interference. Efficient knockdown of these proteins was confirmed by western blot analysis (Fig. 4A,B).

Quantification of the protein levels showed that all shRNAs reduced the expression of their targeted proteins by at least 80% (Fig. 4B). Knockdown of liprin α1 or β1 almost completely prevented the formation of CMSCs as judged by immunofluorescence (Fig. 4C,D). However, HD formation, assessed by β4-plectin colocalization, was unaffected by the loss of the CMSCs (Fig. 4E).

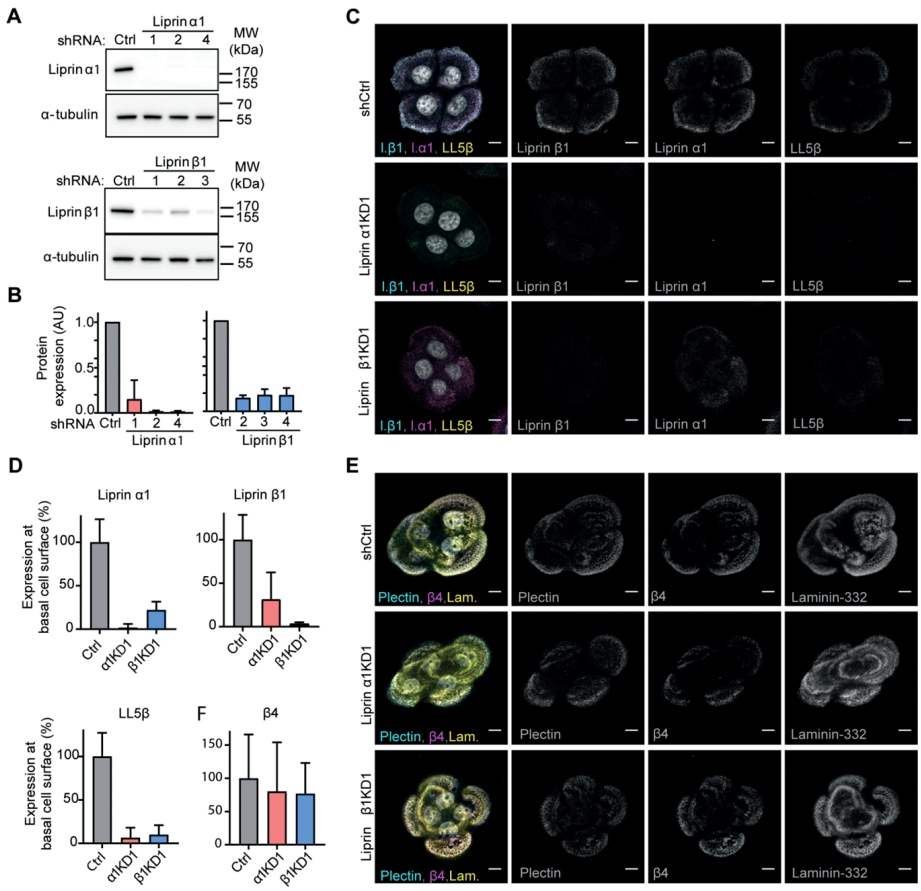


Figure 4: CMSCs are not required for the formation of HDs in keratinocytes. *(A) Western blot analysis of stable shRNA-expressing PA-JEB/β4 cell lines [control (Ctrl) and three knockdowns (KDs)] probed with antibodies against liprin α1, liprin β1 and α-tubulin. (B) Quantification of liprin protein expression normalized to α-tubulin protein expression levels in knockdown and control PA-JEB/β4 keratinocytes. Mean + SD, n=2. (C) Triple immunofluorescence detection of liprin α1, liprin β1 and LL5β in liprin α1 and β1 knockdown and control keratinocytes. Scale bars: 10 μm. (D) Quantification of immunofluorescence staining of liprin α1, liprin β1 and LL5β in knockdown and control PA-JEB/β4 keratinocytes (n=20). (E) Triple immunofluorescence detection of β4, plectin and laminin-332 in liprin α1 and β1 knockdown and control PA-JEB/β4 keratinocytes. Scale bars: 10 μm. (F) Quantification of immunofluorescence staining of β4 shows no significant difference (Mann-Whitney test used) between liprin α1 and β1 knockdown and control PA-JEB/β4 keratinocytes (n=22).*

To investigate if, conversely, HDs affect the formation of the CMSCs, we compared the presence of CMSCs in the presence or absence of HDs by using PA-JEB keratinocytes that express β4 upon doxycycline induction. The expression of β4 was time-dependent and reached a maximum at 24h after induction (Fig. 5A). Immunofluorescence analysis of β4-deficient and -proficient cells (assessed after 24h doxycycline induction) showed no obvious difference in the cellular distribution of CMSC protein localization. Moreover, no difference in the clustering intensities of CMSC proteins were observed (Fig. 5B,C).

In summary, this data show that under steady state conditions the assembly of HDs and CMSCs are not dependent on each other.

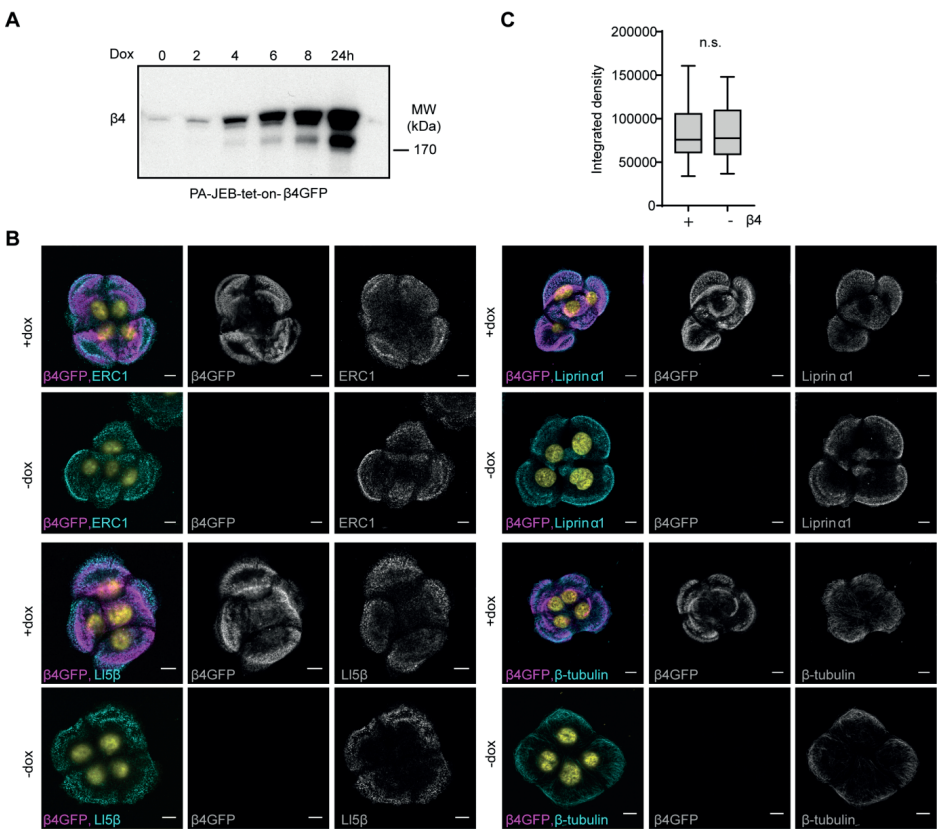


Figure 5: HDs are not required for the formation of CMSC. *(A) Western blot analysis of whole cell lysates from PA-JEB-tet-on-β4-GFP cells treated for different time points with doxycycline, and analyzed for β4. (B) Immunofluorescence analysis of CMSC members in PA-JEB keratinocytes treated with (+) or without (-) doxycycline. Cells were stained for ERC1, liprin α1, LL5β or β-tubulin. Scale bars: 10 μm. (C) Quantification of membrane associated liprin α1, integrated density (area*intensity), in PA-JEB-tet-on-β4-GFP treated with or without doxycycline for 24h (n=20). No significant difference; tested with Mann-Whitney test.*

Discussion

In this study we identified 135 interactors of the integrin α6β4, which include amongst others several FA proteins and all components of the CMSC complex. Recent evidence suggests that CMSCs contribute to FA turnover by facilitating integrin β1 recycling and enabling directional transport of MMPs via microtubules [515–517]. In a similar way CMSCs may regulate the turnover of HDs. Indeed, it has been shown that the recycling of α6β4 is dependent on Arf6, which cooperates with microtubules in adhesion-dependent trafficking [518,519]. Furthermore, CMSCs might play a role in the directional transport of HD-associated proteins to sites where new HDs are formed. In agreement with this hypothesis, basal microtubules have been shown to control the integrity of the basement membrane in avian development [520], and CMSCs are located at substratum adhesion sites composed of deposited laminin-5 and laminin-binding integrins [521]. It is perhaps interesting to mention here, that MTs have also been implicated in the assembly of desmosomes, intercellular junctions, which like HDs, are anchored to the intermediate filament system. The adhesion strength of desmosomes is dramatically weakened in the absence of the MT motor proteins kinesin-1 and kinesin-2, despite the fact that the desmosomal plaque proteins are not affected [522].

However, despite these findings and the fact that CMSCs are found in close proximity of type I HDs in the periphery of the cells, as determined by immunofluorescence and BioID, no defects in HD assembly were observed in keratinocytes depleted of liprin α1 or liprin β1, which causes the disruption of the CMSC. Likewise, the depletion of LL5 in MCF-10A cells did not affect the basal localization of α6β4 [521]. However, because our investigations involve the analysis of HDs under steady-state conditions, subtle differences in the dynamics and/or the rate of HD maturation may have been unnoticed. Furthermore, it is possible that the loss of CMSC function is compensated by other proteins that can anchor MTs at the plasma membrane or the actin cortex [523–528]. These proteins include APC, IQGAP and ACF7 (also known as MACF1), but none of them were identified in the interactome of the integrin α6β4.

The localization of CMSCs in the immediate vicinity of type I HDs raises the question whether these complexes are linked to each other. As far as we are aware, there are no CMSC-associated proteins identified that can link CMSCs directly to HDs. This in contrast to the interaction between CMSCs and FAs, which is mediated by the binding of KANK to talin in FAs [529]. Recently, we showed that in keratinocytes FAs and HDs are localized in close proximity to each other and that plectin plays a critical role in the mechanical coupling of these two adhesion structures [456]. Hence, we believe that the localization of CMSC near HDs is due to the interaction of CMSC with the HD-associated FAs.

Comparison of the β4 interactomes in keratinocytes and MDCK cells revealed an overlap of only 30 interactors. CMSC members were present in both interactomes. However, while in the keratinocytes all CMSC members were identified, in the kidney cells only LL5α/β and ERC1 were identified. This relatively small overlap in the two interactomes might be explained by the use of different cell lines and/or the subcellular localization of integrin α6β4 in type I versus type II HDs [514]. However, it could also have a technical reason, since it has been shown that proteomes identified by different hands and methods can differ considerably [530]. The approach employed by us to determine the β4 interactome differed from the one used by Myllymäki et al. [514] in that they used a myristoylated C-terminally BirA*-tagged GFP construct as a negative control, while we used an IL2R-BirA* construct. Furthermore, we expressed the β4-BirA* fusion protein in cells that are deficient in β4, which exclude possible competition between the fusion protein and endogenous β4 for expression at the cell surface because of the pairing of the α6 subunit with β4. Importantly, although the two β4 interactomes differ considerably, CMSC, FA and HD proteins were found as interactors of α6β4 in both datasets, suggesting that the association of CMSCs, FAs and HDs is not unique to keratinocytes. Besides the identification of the CMSC and FA proteins as proximity interactors of integrin α6β4, we observed several proteins that link α6β4 to cell-cell junctions, such as Discs Large Homolog 1 and 5, Afadin and ERBIN. These interactions might occur by a pool of α6β4 molecules that are not present in HD-like adhesions but are located at the lateral aspects of cells. ERBIN, which associate with p0071 at cell-cell junctions, has previously been shown to also bind the integrin β4 subunit [531,532].

Intriguingly, we also identified the palmitoyl acyltransferase ZDHHC5 as a proximity interactor of the α6 and β4 subunits. Previous studies have shown that in addition to CD151, both α6 and β4 subunits are also palmitoylated [492]. The enzyme responsible for the palmitoylation of the α6 and β4 subunits in MDA-MB-231 and PC3 cells has been identified as ZDHHC3 [533]. Unfortunately, ZDHHC5 has not been tested for its ability to palmitoylate the α6 and β4 subunits; thus, the possibility exists that this enzyme palmitoylates these subunits in a functional redundant manner. Since palmitoylation stabilizes tetraspanin-tetraspanin interactions as well as their interaction with partner molecules, the palmitoylation of the α6 and β4 subunits may contribute to their stable incorporation into CD151 TEMs that are formed in the central region of the cells.

Although only 11 statistically significant differences were detected between the interactomes of β4 in the presence and absence of CD151, some of them deserve particular attention because they provide further insights into the role of this tetraspanin in the formation and stabilization of the different α6β4-containing adhesions. For example, the identification of integrin α3β1 as a CD151-dependent interactor of integrin α6β4 supports the recently proposed role of CD151 in the clustering of α3β1, together with α6β4, in central HD-like adhesions [465]. Furthermore, the identification

of two downstream effectors of the RhoA-ROCK signaling pathway, the myosin regulator light chains 12A/B (MYL12A/B) and cofilin (CFL1), as proximity interactors of β4 in the absence of CD151 is in line with the proposed role of CD151 in regulating RhoA activation [534]. Indeed, it has been shown that deletion of CD151 results in elevated RhoA activity [534]. CD151 may regulate RhoA actvity by preventing α3β1 from becoming incorporated into FAs and thus to support high actomyosin contractility of cells cultured on laminin substrates. The finding that the myosin regulator light chains 12A/B and cofilin are CD151-dependent proximity interactors of integrin β4 may seem odd at first sight, but can be explained by the increased FA size and the close proximity of these FAs with HDs in the absence CD151. The ability of CD151 to suppress RhoA activity suggests that CD151 may also contribute to α6β4's ability to counteract cellular tension and traction force in keratinocytes [456]. Other β4 interactors in the absence of CD151 include several proteins involved in vesicle transport, which suggests that the α6β4 containing adhesions turn over more dynamically in the absence of CD151 than in its presence.

In conclusion, we show that the integrin α6β4 interactome comprises 135 proteins, and includes amongst others FA proteins and components of the CMSC complex, but also many novel proteins that offer opportunities for future research into their role in the assembly of the different α6β4 adhesions.

Material and Methods

Reagents

The primary antibodies used in this study are listed in Table 1. Conjugated antibody against biotinylated proteins was streptavidin-HRP (RPN1231 from GE Healthcare). Secondary antibodies for western blot were goat anti-mouse IgG HRP (Bio-Rad; 1:3000) and polyclonal goat anti-rabbit IgG HRP (Dako; 1:5000). Secondary antibody for FACS was donkey anti-mouse IgG PE (Jackson ImmunoResearch; 1:400). Secondary antibodies for IF (1:200) were goat anti-guinea pig IgG Alexa Fluor 488 (A-11073), goat anti-rabbit Alexa Fluor 647 (A-21245), goat anti-rabbit Alexa Fluor 594 (A-21207), goat anti-rat-TxR (T-6392) and goat anti-mouse Alexa Fluor 647 (A-21236) from Invitrogen, goat anti-mouse FITC (Rockland; 610-102-121) and Hamster-Cy5 (Jackson; 107-005-142).

Cell culture

PA-JEB immortalized keratinocytes were isolated from a patient with Pyloric Atresia associated with Junctional Epidermolysis Bullosa. PA-JEB/β4 keratinocytes stably expressing wild-type β4 were generated by retroviral transduction [3] and maintained in serum-free keratinocyte medium (KGM; Invitrogen), supplemented with 50 μg ml^{-1} bovine pituitary gland extract, 5 ng ml^{-1} EGF, and antibiotics (100 units ml^{-1} streptomycin and 100 units ml^{-1} penicillin; Sigma-Aldrich). HaCaT keratinocytes

(obtained from the American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% heat-inactivated fetal calf serum (FCS; Serana Europe GmbH, Pessin, Germany), and antibiotics. All cells were cultured at 37°C in a humidified, 5% CO_2 atmosphere.

Target	Antibody	Application	Source
α-tubulin	B-5-1-2; Mouse mAb	WB (1:10000)	Sigma-Aldrich T5168
β-tubulin	Mouse mAb	IF (1:200)	Amersham N357
CD151	5C11; Mouse mAb	FACS (1:1 Sup)	Kind gift from F. Berdichevsky
ERC1	Rabbit pAb	IF (1:500)	Gift from K. Hiroshi
Itg. α6	GoH3; Rat mAb	IF (1:1 Sup)	Homemade
Itg. α6	AA6NT; Rabbit pAb	WB (1:500)	Kind gift from A. Cress
Itg. β4	Rabbit pAb	IF (1:1000),WB (1:5000)	Homemade
Itg. β4	439-9B; Rat mAb	IF (1:200)	Kind gift from S. Kennel
Itg. β4	PE-conjugated Rat mAb	FACS (1:400)	BD Pharm. 555720
Laminin-332	R14; Rabbit pAb	IF (1:500)	Kind gift from M. Aumailley
Liprin α1	Rabbit pAb	IF (1:200), WB (1:1000)	Proteintech 14175-1AP
Liprin β1	Mouse mAb	IF (1:200), WB (1:200)	Santa Cruz Biotechnology 514575
LL5β (PHLDB2)	Hamster mAb	IF (1:200)	Kind gift from J. Sanes
Plectin	P1; Guinea Pig pAb	IF (1:500)	Kind gift from H. Herrmann
Talin1	8D4; Mouse mAb	IF (1:100)	Abcam 157808

Table 1 *List of primary antibodies, including application, dilution and source*

Cell culture and generation of stable cell lines

Cell lines stably expressing teton-β4-GFP, β4-BirA*, α6-BirA* or IL2R-BirA* we generated by retrovirus-mediated transduction. Virus was produced by transient transfection of Phoenix-ampho cells with retroviral vector constructs using calcium phosphate precipitation and added to the cells. Cells expressing the protein of interest were enriched by puromycin or zeocin selection and bulk sorted by FACS. The tetON-β4-GFP construct was cloned into the EcoRI site of pRetroX-Tight-Puro vector. The β4-BirA*, α6-BirA* and the IL2R-BirA* constructs used were cloned into the SnaBI site or Hind3/XbaI sites of the LZRS-IRES-Zeo vector.

PA-JEB/β4 α6KO or CD151KO cells were prepared using CRISPR-Cas9 technology. Target sgRNAs against integrin α6 (5'CGGTCGCGAGCTGCCCGCGA3') or CD151 (5'-CAGGTTCCGACGCTCCTTGA-3') were cloned in the pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene plasmid #42230, deposited by Feng Zhang). The cells were transiently transfected with this plasmid using lipofectamine® 2000 (Invitrogen) in OptiMEM (Gibco), and bulk sorted, for the negative population, using a Moflo Asterios (Beckman Coulter) or FACSAria Fusion (BD Biosciences) cell sorter.

Liprin α1 and liprin β1 were depleted by shRNA-mediated KD in PA-JEB/β4 cells. Recombinant lentiviruses were produced by transient co-transfection of HEK293T cells with plasmids encoding the vector and helper functions using lipofectamine 2000. Lentiviruses were added to the cells and infected cells were enriched by puromycin selection for 3 days. The shRNAs targeted: liprin α1: (1) 5′-GATGACAAGACAACCATAAAG-3′, (2) 5′-TGAGCCTTCCAAGGTACAAAC-3′, (3) 5′-GAGGAGATTGAAAGTCGAGTT-3′; Lirpin β1: (1) 5′-CATTGGCCTCCCTCAATATAA-3′, (2) 5′-CCAGAGTGTTTCCATTCATAT-3′, (3) 5′-GCCAAAGTGAAGCCAAAGAAA-3′ and the empty vector was used as negative control.

Western blot analyses

Subconfluent cells were washed in cold PBS, lysed in RIPA (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 4 mM EDTA pH 7.5, 100 mM NaCl, 20 mM Tris-HCl pH 7.5) supplemented with 1.5 mM Na_3VO_4, 15 mM NaF as phosphatase inhibitors and a protease inhibitor cocktail (1:500; Sigma-Aldrich). Cell lysates were cleared by centrifugation at 12000 rpm, 1 h at 4°C, supplemented with SDS sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 12.5 mM EDTA, 0.02% Bromophenol Blue) with β-mercaptoethanol and heated for 5 min at 95°C. Proteins were separated on 4-12% bolt gradient gels (Invitrogen) and transferred to Immobilon-P transfer membranes (Millipore). The membrane was blocked for 3 h in 2% BSA in TBST (10 mM Tris (pH 7.5), 150 mM NaCl, 0.05% Tween 20) before incubation with primary antibody overnight at 4°C and with secondary antibody for 1h at room temperature or with conjugated Ab for 4h. After each incubation step, the membranes were washed twice with TBST and twice with TBS (TBST without Tween 20). Antibodies were detected using Clarity Western ECL Substrate (Bio-Rad). Quantification of protein expression was performed on two independent experiments using ImageJ.

Flow cytometry analyses

Subconfluent cells were trypsinized and collected in PBS supplemented with 2% FCS. Cells were incubated for 50-60 min with primary antibody on ice, washed twice with ice-cold PBS/2% FCS and subsequently incubated with secondary antibody for 50 min on ice. After washing with 2% FCS in PBS, the cells were passed through a nylon mesh filter and 50.000 positive cells were analyzed per sample using a FACSCalibur cell analyzer (BD Biosciences). Unstained cells or cells treated only with secondary antibody were used as negative controls.

Immunofluorescence

Cells were grown on uncoated coverslips for 20h before changing the medium to DMEM supplemented with 10% FCS to facilitate HD formation. 20h after changing the medium the cells were washed with PBS, fixed with 2% PFA for 10 min, permeabilized with 0,2% triton for 5 min and blocked with 2% BSA (Serva) in PBS for 1h. Cells were

incubated with primary and secondary antibodies for 50-60 min each with PBS washing steps in between. Nuclei were stained with DAPI for 20 seconds and coverslips were mounted on slides using MOWIOL. After drying the slides overnight, cell islands of four cells were imaged using a Leica TCS SP5 confocal microscope with a 63x objective. Quantification of the intensity, integrated density/expression at the basal cell surface in 20-22 islands was performed using ImageJ.

BioID sample preparation

Cells were seeded for 24h in biotin depleted keratinocyte medium before being treated with 50 µM biotin (Sigma-Aldrich #B4501) for 20h in DMEM/10% FCS. After washing with cold PBS, cells were lysed at 4°C in NP40 lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 4 mM EDTA) supplemented with Na_3VO_4 (1.5 mM), NaF (15 mM), and protease inhibitor cocktail (1:1,000; Sigma-Aldrich). Collected whole cell lysates were cleared by centrifugation at 12.000 rpm, 60 min at 4°C and cleared cell lysates were incubated with Streptavidin Sepharose High Performance beads (GE Healthcare) overnight at 4°C. After incubation beads were washed with NP40 lysis buffer and cold PBS and beads were stored at -80°C.

Mass spec analyses

Peptide mixtures were prepared from three replicate experiments, measured and analyzed as previously described [456], with the following exceptions. The MS/MS data were searched against the human SwissProt database (20.367 entries, release 2020_01) complemented with a list of common contaminants and concatenated with the reversed version of all sequences. Differentially expressed proteins were determined using a Student's *t*-test (minimum threshold $P \leq 0.05$ and $[x/y] \geq 0.6 | [x/y] \leq -0.6$.

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Supplemental information

Supplementary information available online at https://bio.biologists.org/lookup/doi/10.1242/bio.054155.supplemental. Supplemental Tables: Table S1 β4BirA* minus versus plus biotin (n=3). Table S2 β4BirA* plus biotin versus IL2R-BirA* plus biotin (n=3). Table S3 α6BirA* plus biotin versus IL2R-BirA* plus biotin (n=3). Table S4 β4BirA* plus biotin CD151 proficient versus deficient (n=3). Table S5 Identified α6β4 interactome in keratinocytes versus hβ4-BirA interactors in MDCK cells



CHAPTER 7

Summary and general discussion

Regulators of the dual function of integrin α6β4: adhesion and/or signaling

The integrin α6β4 is a major component of hemidesmosomes (HDs), which provide stable adhesion of epithelial cells to the underlying basement membrane. However, when localized outside the confines of HDs, the integrin was found to contribute to growth factor signaling which regulates diverse cellular processes, including proliferation and survival. Regulation of α6β4 function is complex and not yet fully understood. A wide range of mechanisms, including posttranslational modifications, clustering via its binding partners, gene expression, calcium levels, and protein trafficking have been proposed to regulate α6β4 function. In this thesis, we have investigated the role of clustering and phosphorylation in the regulation of α6β4 function.

HD disassembly due to phosphorylation of HD components by kinases downstream of RTKs

Phosphorylation of the integrin β4 cytoplasmic domain as a regulatory mechanism for HD disassembly has been extensively studied in keratinocytes. In these cells, it was shown that HD assembly is initiated by the binding of the cytoplasmic domain of the β4 subunit to plectin and that these adhesions mediate stable anchorage of keratinocytes to the underlying basement membrane [9,13,405,406]. To enable migration and cell division, HDs have to be disassembled and disruption of β4 binding to plectin is a crucial step in this process.

The β4 cytoplasmic domain harbors several residues that are phosphorylated in keratinocytes in response to various stimuli such as PMA, HGF, MSP and EGF [68,69,72,273,279,397]. Most of these stimuli activate receptor tyrosine kinases (RTKs) and, subsequently, members of the PKC and MAPK signaling pathways which have been shown to directly phosphorylate β4 at S1356, S1364 and T1736 [68,69,72,397,535]. Phosphorylation of S1356 and S1364, located in the connecting segment (CS), is mediated by ERK1/2 and RSK1/2 and prevents interaction of β4 with the actin-binding domain (ABD) of plectin [68]. T1736 is located in the C-terminal end of the β4 cytoplasmic domain and its phosphorylation causes dissociation of β4 from the plakin domain of plectin, located adjacent to the ABD [69]. We defined MAPK-RSK1 and PKCδ-PKD2 as the main axes involved in phosphorylation of T1736 [466] (Ch.3).

In addition to the phosphorylation-dependent regulation of the binding of β4 to plectin, other phosphorylation events downstream of RTK activation contribute to the disassembly of HDs [6,70,71]. Integrin β4 is phosphorylated at S1424 which interferes with the binding of β4 to BP180 and BP230 [70]. Furthermore, plectin is phosphorylated by the ERK-MNK2 pathway at S4642, which reduces its binding to keratin filaments [71]. BP180 is phosphorylated by PKC leading to its translocation

from HDs [536]. Additionally, α6 is phosphorylated by PKC, which might contribute to HD disassembly, as well [74].

In our studies, we have focused on the effects of the EGFR stimulation on β4 phosphorylation and HD disassembly. However, in an *in vivo* situation, multiple signals, such as EGF, MSP and TGFα/β, likely will act in concert to disassemble HDs and enable cell migration. In agreement with this, we found that EGF stimulation alone does not induce the complete disruption of HDs in keratinocytes [6,466] (Ch.3).

Tyrosine phosphorylation and its role in signaling

It has been suggested that Y1422 and Y1440 in the connecting segment (CS), Y1257 in the FnIII-2 domain and Y1494, Y1526 and Y1642 in the FnIII-3,4 domains of the β4 cytoplasmic domain serve as phosphorylation-dependent docking sites for the adaptor proteins Shc and IRS1 to activate the MAPK and PI3K signaling pathways [89–91,277,403,432,436]. Tyrosine phosphorylation occurs downstream of RTK activation but also by β4 ligation to laminin-332 [90,287,357]. We showed that the level of tyrosine phosphorylation of β4 upon RTK activation is strongly correlated with the level at which RTKs are expressed by cancer cells and therefore occurs predominantly in those cells that overexpress the relevant RTK and minimally in cells expressing the RTK at reduced, more physiological levels (Ch.4). Different from the PKC- and MAPK-dependent phosphorylation on threonine and serine residues, it has been shown that the tyrosine residues in the β4 cytoplasmic tail are phosphorylated by Src family kinases (SFKs) [277,278]. We confirmed that the SFK-inhibitor PP2 inhibits tyrosine phosphorylation of β4, but could not pinpoint the involvement of one or more specific SFKs (Src, Fyn, Yes, Lyn), which suggests that other kinases, sensitive to inhibition by PP2 (e.g. CSK and PTK6), might be involved as well [463,464] (Ch.4).

Although multiple tyrosine phosphorylation sites have been identified at the cytoplasmic domain of β4, our data shows that four sites in the CS, Y1422, Y1440, Y1343 and Y1349, are primarily responsible for the total tyrosine phosphorylation levels of β4 upon EGF stimulation (Ch.4). In agreement with this, structural data indicates that the tyrosine-based protein binding sites, located in the FNIII-3,4 domains, are not accessible for protein kinases due to steric hindrance [404] and, therefore, likely are only phosphorylated *in vivo* under non-physiological conditions, i.e. stress conditions. For the same reason, binding of the SH2 and PTB domain of adaptor proteins to the phosphorylated Y1494, Y1526 and Y1642 residues is unlikely to occur under physiological conditions. In contrast, there are no structural constraints for kinases and adapter proteins to bind the phosphorylation sites in the CS. Y1422 and Y1440 have been previously shown to act as a docking platform for Shc [89,279,436]. Using peptide pull down assays we identified PLCγ1, but not Shc, as a candidate binding partner for these tyrosine residues (Ch.4). However, the binding of PLCγ1 to β4 could not be confirmed in A431 epidermoid cancer cells, bearing high levels of the EGFR

(Ch.4). Furthermore, there is data suggesting that tyrosine phosphorylation of β4 contributes to the activation of MAPK and PI3K signaling [283,435]. However, we found no evidence for a role of tyrosine phosphorylation of β4 in augmenting EGFR-mediated activation of MAPK or PI3K signalling in A431 cells (Ch.4).

Additionally, β4 may contribute to pro-tumorigenic signaling via FAK [278,339,340,537–539]. Activation of FAK downstream of β4 has been observed upon ligation of α6β4 by laminin and β4 tyrosine phosphorylation at Y1494 [537,539], by the calcium-activated chloride channel regulator 1 (mCLCA1) [339], or by the C-terminal tensin-like protein (TNS4) [538], but also by direct binding of FAK to a region close to the transmembrane domain of β4 [278]. These findings suggest that both direct binding and signaling via tyrosine phosphorylation of β4 might contribute to FAK activation. However, mutation of the four dominant tyrosine phosphorylation sites on the β4 cytoplasmic tail in A431 cells did not affect FAK signaling downstream of EGFR activation (unpublished data).

Although our findings (and findings of others [327,451]) argue against an important role of β4 tyrosine phosphorylation in RTK-mediated signaling, they do not exclude the possibility that β4 contributes to signaling pathways via other mechanisms or downstream of other RTKs. Therefore the biological relevance of β4 tyrosine phosphorylation is still uncertain.

Tyrosine phosphorylation and its potential role in HD disassembly

Alternatively, β4 tyrosine phosphorylation might be relevant for HD dynamics. Although a possible role of tyrosine phosphorylation of β4 in HD dynamics has gained little attention, two groups initially suggested that tyrosine phosphorylation at Y1422 and Y1440 is critical for HD assembly [444,540]. In contrast, our group showed that the formation of HDs is not disrupted in cells expressing a phosphorylation-defective β4 mutant (Y1422F; Y1440F) [13]. Moreover, no tyrosine mutations on β4 have been identified in patients who suffer from a blistering disorder named Epidermolysis Bullosa (EB); this in contrast to the identification of non-tyrosine mutations that interfere with the binding of β4 to plectin.

Several studies suggested a role for β4 tyrosine phosphorylation in the disassembly of HDs [89,277]. In line with this, it was shown that in cancer cells, which exhibit abnormal high RTK activation and, therefore, likely also have high levels of β4 tyrosine phosphorylation (Ch.4), HDs are reduced in number and appear rudimentary [84,88,456]. Interestingly, the Y1343 and Y1349 phosphorylation sites, which we identified in Chapter 4, and have been identified in multiple mass spec analyses before [443], are located in the region (1328-1355) that follows the second FnIII domain and was found to be important for β4-plectin binding [17]. Therefore, the phosphorylation of these sites might contribute to β4-plectin dissociation [541].

Palmitoylation to increase the local concentration of HD and signaling proteins
The integrin β4 subunit is palmitoylated at several cysteines in the membrane-proximal regions [284,492]. (Z)DHHC3 was identified as the responsible palmitoyl acyltransferase for palmitoylation of the integrin β4 subunit in breast and prostate carcinoma cells [382]. We identified not ZDHHC3 but ZDHHC5 as a proximity interactor of both the α6 and β4 subunits in keratinocytes [542] (Ch.6), suggesting that this acyltransferase might contribute to α6β4 palmitoylation as well. In accordance, ZDHHC3 ablation resulted in only a partial reduction of β4 palmitoylation [382]. The function of β4 palmitoylation still has to be defined. However, it is reasonable to suggest that it contributes to both HD assembly and signaling since palmitoylation promotes secondary association between palmitoylated proteins in cholesterol and sphingolipid-rich microdomains [206,450].

Palmitoylation could play a role in α6β4-mediated signaling by promoting the association of palmitoylated integrin α6β4 with other palmitoylated proteins including the EGFR and the SFK members Yes, Lyn and Fyn [284,447,492,543]. In support of this, mutation of the palmitoylation sites on β4 affects β4 signaling downstream of Src family kinases (SFKs) [284]. However, mutation of β4 palmitoylation sites did not alter EGFR activation in EGF-treated A431 cells [492]. Additionally, palmitoylation likely contributes to HD formation and stability by increasing the local concentration of HD proteins, since in addition to the integrin β4 subunit multiple HD-associated proteins are palmitoylated (CD151 and the integrin α3 and α6 subunits) or contain potential palmitoylation sites (BP180) [465,492,544] (Ch.5). Furthermore, palmitoylation of the α6 and β4 subunits prevents degradation of α6β4 and therefore affects its expression levels [382]. However, mutation of β4 palmitoylation sites did not interfere with the formation of HD-like structures in A431 cells [492].

Palmitoylation of tetraspanins contributes to the formation of TEMs by promoting hydrophobic interactions between tetraspanins [450,545,546]. Next to the tetraspanins, also tetraspanin associated proteins such as the laminin-binding integrins α3β1, α6β1 and α6β4 become incorporated into TEMs and this clustering was shown to affect their ability to mediate adhesion and signaling [486,481,214–216,83,482]. In case of CD151, palmitoylation leads to clustering of both α6β4 and α3β1 in an alternative HD-like adhesion structure that is formed in the center of keratinocytes [465] (Ch.5). It is likely that palmitoylation of both α6β4 and α3β1 contributes to their stable incorporation into these alternative HD like adhesions as well.

Integrin α6β4 clustering by integrin α3β1-CD151
Previous work from our group suggested a role for α3β1-CD151 complexes in the formation of pre-HD clusters in which, upon maturation of these pre-HD clusters into HDs, α3β1 is replaced by α6β4 [1,3]. Our work indicates that the α3β1 and α6β4 integrins are not mutually exclusively associated with CD151-rich microdomains and

that neither α3β1 nor CD151 are needed for the formation of mature HDs. However, our data does show that for the maintenance of the alternative HD-like structures, α3β1, CD151 and laminin binding are essential [465] (Ch.5).

Integrin clustering determines the strength by which cells adhere to the extracellular matrix (ECM) [488]. The contribution of CD151 to the clustering of integrin α3β1 for adhesion strengthening is clear from studies in kidney cells and confirmed by us in α6β4-deficient keratinocytes [465,479,547] (Ch5). CD151-mediated clustering of α6β4 does not significantly contribute to the strength of α6β4-mediated adhesion under steady state conditions when mature (type I) HDs are present [465] (Ch.5). However, we suggest that CD151-mediated clustering of α6β4 in the alternative HD-like adhesions is essential for establishing and maintaining cell-matrix interactions in tissue reorganization in response to injury e.g. during wound healing when cells have to migrate and divide and mature HDs are dismantled [465] (Ch.5). Furthermore, CD151 might contribute to subtle regulation of integrin α6β4 function by stimulating integrin recycling, association of α6β4 with signaling molecules, RTK-mediated β4 phosphorylation and/or by stimulating ligand binding activity [3,212,214,217,223,394–396].

Clustering and signaling via laminin and plectin

In addition to CD151-mediated clustering of α6β4, integrin clustering is induced by binding of α6β4 to laminin-332, deposited by keratinocytes into the ECM. Laminin-332 is an important regulator of dermal-epidermal adhesion and plays a critical role in the formation and turnover of HDs [1,315,548]. Laminin-332 is deposited by keratinocytes in an unprocessed form which then is proteolytic processed into mature laminin-332. The unprocessed laminin-332 is the preferred ligand of integrin α3β1, which mediates keratinocyte migration on this ligand, while the processed laminin-332 is the preferred ligand for integrin α6β4 [548–552]. This does not mean that α6β4 cannot bind unprocessed laminin-332 or associate with HD-associated proteins on unprocessed laminin-332. However, since mature HDs are not formed on unprocessed laminin-332, HDs remain dynamic [553].

Besides providing a substrate for cell adhesion, laminin-332 also contributes to signaling. Upon binding ligand, the β1 and β3 integrins that associate with actin, trigger FAK autophosphorylation and subsequent activation of the FAK/Src complex [82], which leads to activation of downstream effectors such as MAPKs and Rho-GTPases. Although α6β4 contributes to MAPK signaling and might bind FAK, conventional integrin signaling as observed for β1/3 integrins does not apply to integrin α6β4. Signaling by β1/3 integrins requires the assembly of a multiprotein complex (FAs) at the integrin cytoplasmic tail, which includes the binding of talin to the membrane proximal NPXY motif of the β-integrin tail. Integrin α6β4 does not contain a binding site for talin, and there is no evidence that for signaling by integrin α6β4 a multiprotein

complex (HD) has to be formed. However, integrin α6β4 binding to laminin-332 does contribute to anchorage-dependent cell proliferation, and the prevention of cell death by loss of adhesion (anoikis) could very well be stimulated by the binding of integrin α6β4 and β1 integrins to their cognate ligands [274,300,430] (Ch.2).

In the absence of binding to laminin-332, α6β4 clustering is primarily mediated by plectin [315,327,487,554,555]. Indeed, a β4 mutant, which is unable to bind to laminin-332 was found to be clustered at the basal membrane, albeit less abundantly [465] (Ch.5). Compared to the inhibition of β4 binding to either laminin or plectin, clustering of β4 is significantly reduced when binding of β4 to both laminin-332 and plectin was prevented (unpublished data).

Protein trafficking in regulating HD dynamics

HD dynamics might be regulated by several molecular mechanisms, including microtubule-based trafficking of HD proteins. Microtubules have been shown to affect the integrity of the basement membrane including laminin organization, which contributes to HD formation [520]. These data might be explained by microtubule-mediated transport of matrix metallopeptidases (MMPs) to the basal membrane, which contributes to the degradation of ECM and hence to the disassembly of cell-matrix adhesions [515–517]. Additionally it was shown that in Zebrafish, the transport of Lgl2, a protein associated with exocytic vesicles, to the basolateral membrane is critical for correct HD formation [556–558].

Microtubules might also be involved in the regulation of HD dynamics by facilitating intracellular transport of HD proteins. Indeed, recycling of α6β4 depends on Arf6, which cooperates with microtubules in adhesion-dependent trafficking [518,519]. Additionally, it has been shown that microtubule-dependent rab5- and rab11-mediated trafficking of α6β4 affects the expression of this integrin at the cell surface [63,64]. The identification of several proteins involved in vesicular transport and the complete CMSC as close proximity interactors of integrin α6β4 at the basal membrane supports this hypothesis. However, we showed that interference with CMSC formation does not reduce the amount of HDs formed under steady state conditions [542] (Ch.6).

Calcium concentration regulates integrin α6β4 phosphorylation and HD formation

Calcium plays an important role in epidermal homeostasis. In the epidermis, a steep calcium gradient exists in the uppermost layers of the epidermis [559,560], which plays a crucial role in keratinocyte differentiation and formation of the epidermal barrier [561,562]. Calcium has also been shown to specifically affect HDs. During calcium-induced terminal differentiation of cultured keratinocytes, integrin α6β4 was found to be rapidly downregulated which resulted in the dissolution of HD-like structures [2,563]. However, in more short term experiments in cell culture, calcium promotes the

formation and maturation of HDs [466,564] (Ch.3). It is possible that calcium regulates the assembly or disassembly of HDs by controling the binding of specific proteins to the membrane-proximal Calx-β motif in the β4 cytoplasmic domain [565]. However, structural and biophysical analyses indicated that the Calx-β domain of β4 does not bind calcium [566]. Moreover, no specific β4 binding partners have been identified for this domain.

Interestingly, HD formation is affected by several proteins, whose activities are regulated by calcium. Calcium affects the activity of Rho GTPase [567,568], and therefore might influence β4's contribution to cell migration (discussed later in this chapter). Calcium also induces the activation of caspases which cleaves β4 and causes the disassembly of HDs [2,66]. Furthermore, calcium affects β4-plectin binding. On the one hand, calcium may activate calmodulin, which has been shown to interfere with β4-binding to plectin [563,569], while on the other hand, due to activation of calcium-dependent phosphatases [410], it may inhibit phosphorylation of β4 and thus prevent HD disassembly. We and others identified calcineurin as a calcium-dependent phosphatase which partially prevents HD disassembly by promoting the dephosphorylation of β4 at T1736 [69,466] (Ch.3). Inhibition of calcineurin by Calyculin A also inhibits β4 phosphoryation at different phosphorylation sites [410].

Other regulators:
Several other molecular mechanisms may affect α6β4 function and therefore its ability to mediate cell adhesion, signaling and migration. Increased protein expression can regulate α6β4 function by increasing the local concentration of β4 in HDs and, therefore, the strength by which cells adhere to laminin-332 or by increasing the amount of β4 outside the confines of HDs and therefore stimulate its signaling properties. Expression levels of β4 are affected by multiple mechanisms, such as epigenetic and miRNA regulation of β4 expression [383–385,570]. Furthermore, in tumors the expression and activation of an array of molecules have been identified that can affect β4 protein expression including Rac1 [374], TR3/Nur77 [375], PTHrP [369,376,377], IL24 [378] and H-Ras [379], ARRDC3 [380] and ZEB1 [381].

Additionally, α6 and β4 can exist as different isoforms due to alternative mRNA splicing [38,41]. The expression of different isoforms of β4 has been shown to differently affect the proliferative status of colon cancer cells [388]. Furthermore, both α6 and β4 subunits are glycosylated on several asparagine residues in their extracellular domains [571], and α6β4 clustering mediated by galectin-3 binding to the glycosylated α6 and β4 subunits promotes intracellular signaling and augments cell-matrix adhesion [571,572]. Finally, multiple unknown mechanisms could be in place and any of the newly identified proximity interactors of integrin α6β4 might be involved in the regulation of signaling by integrin α6β4 or the assembly or disassembly of HDs [542] (Ch.6).

Integrin α6β4 and forces

Association of β4 with the actin cytoskeleton and its role in migration
Besides its contribution to firm adhesion, α6β4 has been implicated in cell migration [84,88,319]. Migration is mediated by remodeling of the actin cytoskeleton and traction-forces generated by the actin-myosin cytoskeleton. Remodeling of the actin cytoskeleton is regulated by Rho family GTPases including RhoA, Rac1 and Cdc42. Various Rho GTPases are simultaneously present in the same cell and cellular actions are dependent on time- and concentration-dependent activation of the various Rho GTPases [573]. Integrin α6β4 has been implicated in actin cytoskeletal dynamics during migration through (EGF-mediated) regulation of Rac1 and RhoA [287,303,319,327,329,341]. RhoA contributes to the deposition of laminin-332, which can be used to drive migration of cells [322], while Rac1 contributes to migration on laminin-332 by promoting the formation of lamellipodia and by affecting α3β1 localization to lamellipodia [322,549,574].

RhoA activation stimulates myosin activity and actin-myosin contractility and therefore the formation of actin stress fibers and FAs [575,576]. Although, β4 has been suggested to contribute to RhoA activation, the opposite has also been shown. Recent work from our group showed that integrin β4 together with plectin decreases cell contractility by inhibition of RhoA–ROCK–Myosin Light Chain (MLC) phosphorylation and FAK-PI3K signaling, two mechanosignaling pathways downstream of FAs [456]. Furthermore, it has been shown that CD151 can regulate the expression and activity of Rho GTPases [83,183,218,219,226,227], and that integrin α3β1 affects the actin cytoskeleton differently in the presence of CD151 [83,206]. In support of a role of CD151 in modulating α6β4 function, we identified by BioID, two downstream effectors of the RhoA-ROCK signaling pathway, MYL12A/B and cofilin, as proximity interactors of α6β4 in keratinocytes lacking CD151 (Ch.6).

Interestingly, integrin α6β4 clustering in mature HDs also affects actomyosin contractility, since the number of intact and mature HDs is closely linked with the degree of actomyosin contractility [456]. The proposed mechanism by which integrin α6β4 in HDs affects intracellular forces and migration is through its ability to bind the cytoskeletal linker protein plectin. Beause plectin not only binds to the IF cytoskeleton at HDs but also links the IF cytoskeleton to the actomyosin cytoskeleton, it provides a mechanical link between integrin β4-containing HDs and the actin-associated FAs. In support of a mechanical coupling between these two adhesion systems, it was found that HDs and FAs are in close proximity of each other and that interference of β4 binding to the ECM or plectin increases cellular tension exerted by the actomyosin cytoskeleton through FAs [456]. Moreover, it has been shown that aberrant intermediate filament organization or plectin expression resulted in changes in actin filament dynamics and increased cellular tension [577–580].

Although these findings indicate that α6β4 affects migration and the actin cytoskeleton indirectly via plectin while present in HDs, α6β4 has also been shown to regulate migration independently of HDs at actin-rich protrusions [74,273,319,581]. These data suggest that various mechanisms might be in place to regulate the association of β4 with the actin cytoskeleton and its role in migration.

Formation of HDs is affected by tension
Evidence shows that cellular tension modulates the formation of different integrin-containing structures at the basal membrane such as FAs and clathrin lattices [456,582–585]. We hypothesize that similarly to how HDs affect cellular tension, vice versa, tension may modulate HD formation. Indeed, the expression of α6β4 in vascular tissues has been correlated to the amount of shear stress in the vessels [44,430] (Ch.2). Furthermore, in cell culture: BP230, which provides an additional link to the keratin cytoskeleton in type I HDs [478], is primarily located in the periphery of the cells where traction forces are highest (unpublished data) [456].

How does β4 contribute to signaling?

β4 signaling downstream of RTKs
Although the integrin α6β4 lacks enzymatic activity, it can promote the activity (and inhibition) of several signaling molecules, such as PI3k/AKT, MAPK, FAK, PLCγ, and Rho GTPases (reviewed in [430] (Ch.2). How β4 contributes to the activation of intracellular signaling pathways is still unclear. Cooperation of α6β4 with RTKs could be a mechanism by which β4 mediates signaling [272,277]. Synergy between β4 and RTKs has gained much attention because β4 is strongly tyrosine phosphorylated in RTK overexpresssing cancer cells stimulated with their cognate ligands. Several studies have proposed an association between RTKs, β4 tyrosine phosphorylation and pro-tumorigenic signaling (Ch.4).

I already discussed the role of tyrosine phosphorylation of β4 in signaling downstream of RTK activation and concluded that tyrosine phosphorylation of β4 did not augment EGFR intracellular signaling in cells overexpressing this receptor. Furthermore, we found no effect of β4 on the signaling by the EGFR in two non-cancerous epithelial cells (HaCaT and PA-JEB/β4) (Ch.4). Similar findings were reported for β4 and another RTK, the Met receptor [451].

One possible explanation for the absence of an effect is that in cell culture assays, cells are usually stimulated with an excess of GF. Such an excess of GF would cause robust RTK activation and more direct RTK induced signaling compared to the situation *in vivo*. The strong signaling therefore might override the possibility of a more subtle contribution of β4 to intracellular signaling. Additionally, the relevance of using an excess of GF in assays is questionable, since this does not mimic a pro-tumorigenic

situation in which RTKs are the drivers of tumor potential. Several reports showed that in cells that overexpresss the EGFR, EGF does not stimulate proliferation, but instead induces growth arrest [453–455,460] (Ch.4). This suggests that the low level of activation of the overexpressed RTKs in the absence of their cognate ligands is sufficient to support tumor cell growth. In conclusion, the tyrosine phosphorylation of β4 might not be functionally relevant but be the result of abnormal high levels of RTK activation and downstream signaling due to an excess of their cognate ligands (Ch.4).

This explanation disagrees with the hypothesis that β4 signaling is relevant only in carcinoma cells where RTKs are expressed at high levels and β4 tyrosine phosphorylation is strongest [88]. However, it agrees with the data that suggested that β4 signaling is only relevant when growth factors and hormones are limited by low availability [289,328]. Important to mention is that under such circumstances β4 is hardly phosphorylated (Ch.4).

Signaling upon ligand binding

There is data suggesting that in the absence of RTK activation, ligation of α6β4 by antibodies or ligand (laminin-332) may also activate downstream signaling pathways. Indeed binding of α6β4 to laminin causes β4 tyrosine phosphorylation, which results in Shc-dependent Ras/MAPK signaling [89,436,438] and IRS-induced PI3K signaling [90]. Furthermore, several adapter proteins (PI3K, FAK) have been shown to bind β4 upon RTK activation. However, the region on β4, to which these adaptor proteins bind, does not harbor any tyrosine residue that can be phosphorylated [278,357]. Whether independent of tyrosine phosphorylation, the engagement of α6β4 with ligand can induce binding of signaling proteins to β4 is not clear. However, a conformational change of the cytoplasmic tail of β4 cannot be excluded. Such conformational changes have been shown for several other integrins upon ligand binding.

Signaling in or outside of HDs

Current understanding is that α6β4 directly activates signaling proteins and needs to be released from HDs to fulfill its role in signaling [84]. However, in contrast, we recently showed that in PA-JEB/β4 keratinocytes, β4 impacts PI3K/Akt signaling through its ability to modulate cellular tension, via RhoA-ROCK-MLC and FAK-PI3K signaling pathways rather than through direct association with signaling intermediates [456]. In agreement with this, the effect of β4 on cellular tension is specifically dependent on β4 being incorporated in HDs and forming a link between the ECM and the IF cytoskeleton [456,577–580].

Different α6β4-containing complexes

Distribution of HD-like complexes

Adhesion of basal epithelial cells to the ECM is essential to maintain tissue integrity. The importance of α6β4 for stable cell-matrix adhesion is clear from studies with patients suffering from Junctional Epidermolysis Bullosa (JEB) [21,23–25,468,469]. JEB patients exhibit not only skin detachment, but also defects in the gastro-intestinal tracks, the airways and the eyes. In agreement with the disease phenotypes, HDs are not only found in skin but also in other tissues, including lung [586], intestine [4] and mammary gland [587].

The type of HDs differs between different tissues and cell types. In the epidermis, integrin α6β4 resides in type I HDs, while in simple epithelial tissues, such as the intestine, integrin α6β4 typically resides in type II HDs [317,467]. Type I HDs contain α6β4, CD151, plectin, BP180 and BP230 and form a link between laminin-332 in the ECM and the keratin IFs. Type II HDs are similar to Type I but lack BP180 and BP230 and therefore their link to the keratin IFs is less robust [473]. In cultured keratinocytes, we recently identified an alternative HD-like structure (type III HDs), which contains CD151, α3β1, α6β4 and plectin and is associated with laminin-332 in the ECM. These structures are not obviously linked to the keratin IF system [465] (Ch.5).

HD-like complexes in adhesion and migration

The different α6β4-containing protein complexes contribute differently to adhesion and migration of cells, and exhibit different dynamic properties. While HDs provide stable adhesion of the basal cells to the ECM, individual components of HDs, such as α6β4 and BP180, continuously renew themselves [315,588]. Turnover of HDs might enable cells to switch easily from a stationary to a migratory phenotype for example during wound healing [410,581]. While Type I HDs hold cells in place to prevent migration [315,316], this may not necessarily be true for type II HDs [317]. The turnover rate of type II HDs is sufficiently fast not to limit the migration of cells [70,72,318]. The dynamic nature of type III HD-like adhesions is also higher than that of (type I) HDs and these structures do not significantly contribute to α6β4-mediated adhesion [465] (Ch.5).

Although, the function of the type III HD-like adhesions is not clear, we proposed that their dynamic nature in combination with their α3β1-mediated adhesion and persistence over time during cell-island formation, could indicate a role for these complexes in strengthening keratinocyte adhesion to newly deposited laminin-332 before FAs and HDs are fully established for example during wound healing *in vivo*.

Concluding remarks and perspectives

The work presented in this thesis provides novel insights into the mechanisms involved in the regulation of integrin α6β4 function and are therefore relevant for understanding the cellular events that control skin integrity and tumor progression. We have focused our investigations on the role of RTKs and direct binding partners of α6β4 in the dynamics of HDs and discussed the controversial role of β4 in signaling. Additionally, we identified multiple new interactors of α6β4 which lays the foundation for better understanding of integrin α6β4 and its role in important biological processes.

However, to really understand these biological processes, understanding of integrin α6β4 by itself is not enough. It is likely that crosstalk between multiple integrins and multiple RTKs occur simultaneously in the same cell and that several integrin-containing complexes exist simultaneously and affect or supplement each other. Since little is known about how these different complexes interact, a better understanding of how the balance between these complexes is established and how they influence oncogenic signaling pathways in cells will be necessary to fully understand the role of laminin-binding integrins in adhesion and in the development and progression of tumors. Furthermore, most knowledge, about the laminin-binding integrin-containing adhesion complexes, comes from cell culture assays, and still very little is known about their dynamics *in vivo*.



ADDENDUM

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Nederlandse samenvatting voor leken
Curriculum Vitae
List of Publications
Dankwoord

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Nederlandse samenvatting

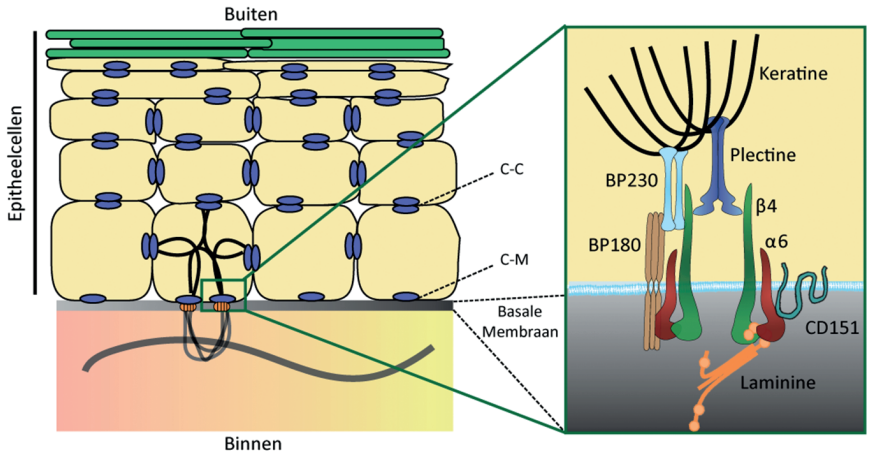
Hemidesmosomen zijn essentieel voor het verankeren van je huidcellen aan het onderliggende basale membraan. Integrine α6β4 vormt de kern van deze hemidesmosomen en is daardoor essentieel voor de integriteit van de huid. Daarnaast kan integrine α6β4, los van hemidesmosomen, bijdragen aan versterking van signaalroutes. Maar te sterke signaalroutes kunnen leiden tot kanker. Dit proefschrift beschrijft onze zoektocht naar mechanismen die de functie van integrine α6β4 reguleren zowel bij het vormen of ontmantelen van hemidesmosomen als in het versterken van signaalroutes. Kennis over deze eiwitten en interacties draagt bij aan ons begrip van (huid)kanker en Epidermolysis Bullosa (vlinderziekte).

Achtergrond
Het menselijk lichaam bestaat uit meer dan 30 miljard cellen van verschillende types en met verschillende functies, waaronder epitheelcellen. Epitheelcellen zijn de cellen die de holtes en weefsels bedekken die in contact staan met de buitenwereld, zoals het maag-darmkanaal, de urinewegen en de huid. De epitheelcellen vormen daar een laag van op elkaar aansluitende cellen, die fungeert als beschermende barrière. Deze laag voorkomt vochtverlies uit de onderliggende weefsels, maakt gecoördineerde uitwisseling van stoffen mogelijk en beschermt het lichaam tegen het ongewenst binnenkomen van schadelijke invloeden (zoals virussen, chemische stoffen en UV-straling).

De precieze vorm, functie en schikking van de epitheelcellen verschilt per weefsel. In de darmen bestaat het epitheel bijvoorbeeld uit één laag langwerpige epitheelcellen die, met een extra groot oppervlak, zeer geschikt zijn voor het opnemen van voedingstoffen. Het epitheel van de huid bestaat echter uit meerdere lagen cellen waarbij iedere laag zijn eigen vorm en specialisatie heeft (Fig. 1 links). Neem bijvoorbeeld de buitenste laag cellen. Die bestaat uit dode, verhoornde cellen die de daaronder gelegen levende cellen beschermen tegen onder andere zonlicht.

Om een continue sluitende laag te vormen, verankeren de epitheelcellen zich stevig aan elkaar (met cel-cel adhesies). Om goed vast te zitten aan de rest van het lichaam verankeren de epitheelcellen zich aan het onderliggende basale membraan. Deze verankering aan het basale membraan gebeurt door cel-matrix adhesies (Fig. 1 links). Het gespecialiseerde type cel-matrix adhesie waar dit proefschrift zich op richt zijn de hemidesmosomen (HD's).

Een HD bestaat uit verschillende eiwitten: het integrine α6β4, plectine, BP180, BP230 en tetraspanine CD151. Gezamenlijk vormen deze eiwitten een fysieke link tussen het keratine cytoskelet van de epitheelcel en laminine in het basale membraan (Fig. 1 rechts). Het integrine α6β4 vormt de kern van een HD en is essentieel voor de vorming van deze structuren.

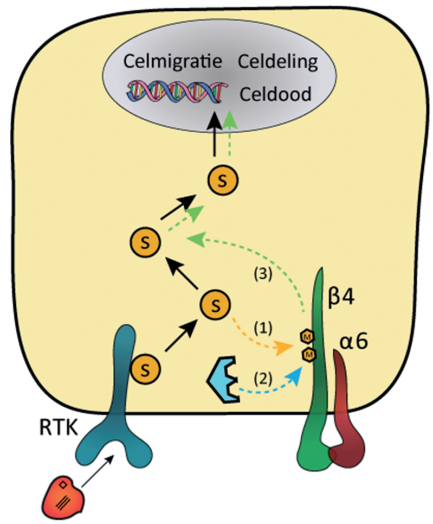


Figuur 1 Hemidesmosomen in de huid verzorgen adhesie van epitheelcellen aan het basale membraan. *Links: illustratie van de huid bestaande uit verschillende lagen epitheelcellen, voorzien van cel-cel (C-C) en cel-matrix (C-M) adhesies. Rechts: een uitvergroting van één type cel-matrix adhesie: een hemidesmosoom bestaande uit BP180, BP230, Plectine, het integrine α6β4 en CD151. Gezamenlijk vormen deze eiwitten een fysieke link tussen het keratine cytoskelet van de epitheelcel en laminine in het basale membraan.*

Integrines zijn eiwitten die door het celmembraan heen steken, waardoor een deel van het eiwit zich binnen de cel (intracellulair) en een deel zich buiten de cel (extracellulair) bevindt. Ze bestaan altijd uit twee subeenheden: één α-subeenheid en één β-subeenheid. De β_4-subeenheid, die een paar vormt met de α6-subeenheid, is uniek onder de β-subeenheden door zijn extreem lange intracellulaire staart. Deze staart bevat bindingsplaatsen voor andere eiwitten, waaronder het eiwit plectine. Door binding van plectine aan de intracellulaire staart van de β_4-subeenheid vormt zich een link tussen het integrine $\alpha 6\beta_4$ en het keratine cytoskelet in de epitheelcel. Extracellulair, vormen de α6- en β_4-subeenheid gezamenlijk een bindingsplaats voor laminine in het basale membraan.

Zoals eerder genoemd, zijn HD's essentieel voor de stabiele verankering van cellen aan het basale membraan. Afwijkingen in de eiwitten van een HD, kunnen leiden tot de ziekte Epidermolysis Bullosa (EB; vlinderziekte). Bij deze ziekte laten verschillende huidlagen los van elkaar, wat resulteert in blaarvorming. Kinderen die aan deze ziekte lijden, worden vanwege hun fragiele huid ook wel vlinderkinderen genoemd. EB gaat gepaard met symptomen als jeuk, blaarvorming, darm-, oog- en luchtwegproblemen, extreme littekenvorming en huidkanker. De symptomen en de ernst van de ziekte variëren van mild tot dodelijk. De uiting van de ziekte is afhankelijk van welk eiwit afwijkt en hoe het eiwit afwijkt. Verlies van het integrine $\alpha 6\beta_4$ (de kern van een HD) resulteert bijvoorbeeld in een ernstige vorm van EB, waarbij patiënten al kort na de geboorte overlijden. De oorzaak hiervan is dat, zonder het integrine $\alpha 6\beta_4$, HD's niet gevormd worden, waardoor de epitheelcellen loslaten van het basale membraan bij de minste aanraking.

Wanneer het integrine α6β4 niet aanwezig is in HDs, kan het een bijdrage leveren aan signaalroutes. Een signaalroute wordt geactiveerd door een 'bericht' van buiten de cel die op het oppervlak van de cel een tyrosine kinase receptor (RTK) activeert. Deze RTK start vervolgens een kettingreactie van signaaleiwitten die elkaar activeren en het signaal overbrengen naar de kern van de cel waar celfuncties gereguleerd worden. Verschillende signaalroutes beslissen gezamenlijk over het lot van een cel door bijvoorbeeld aan te zetten tot celdeling, celmigratie of celdood (Fig. 2). Overactivatie van specifieke signaalroutes kan echter leiden tot ongecontroleerde celdeling, oftewel kanker.



Figuur 2 Het integrine α6β4 kan signaalroutes versterken. *Tyrosine kinase receptoren (RTKs) worden geactiveerd door extracellulaire binding van een 'bericht'. Vervolgens activeert de RTK een signaalroute aan signaaleiwitten (S). De signaaleiwitten brengen het signaal naar de kern waar het functioneren van de cel wordt bepaald. Daarnaast kunnen de signaaleiwitten ook de integrine β4-subeenheid markeren (1)(M) waardoor hier andere eiwitten (2) aan kunnen binden die de signaalroutes kunnen versterken (3).*

Een RTK die vaak aanwezig is op het oppervlak van epitheelcellen heet de epidermale groeifactor receptor (EGFR). Te veel of overactief EGFR is een belangrijke stap in het ontstaan van verschillende epitheliale tumoren, zoals huidkanker. Signaaleiwitten die geactiveerd worden door de EGFR kunnen, behalve het signaal doorgeven naar de kern, ook het integrine α6β4 markeren. Deze markeringen bepalen welke eiwitten aan integrin α6β4 kunnen binden en welke juist niet. Door markering van integrin α6β4 kunnen HD-eiwitten niet meer binden, terwijl signaaleiwitten mogelijk juist wel kunnen binden.

Door binding van signaaleiwitten aan het integrine α6β4 kan het integrine α6β4 weer andere signaaleiwitten activeren en de al aanwezige signaalroutes in de cel versterken (Fig. 2). Het integrine α6β4 kan dus tumorformatie en kwaadaardigheid van een tumor stimuleren, wanneer het integrine α6β4 bijdraagt aan signaalroutes die aanzetten tot bijvoorbeeld celdeling.

Zoektocht om mechanismen te begrijpen die de functie van integrine α6β4 reguleren

Kortom, het integrine α6β4 is belangrijk om de integriteit van de huid te waarborgen, maar is ook geïdentificeerd als een eiwit dat bijdraagt aan tumorgroei. Hoe bepaald wordt wanneer integrine α6β4 een goedaardige of kwaadaardige rol uitoefent, is nog

onduidelijk. In dit proefschrift wordt onze zoektocht beschreven naar het identificeren en begrijpen van mechanismen die de rol van integrine α6β4 in cel-matrix adhesie en in signaalroutes reguleren.

Dit proefschrift begint, na een algemene introductie in hoofdstuk 1, in hoofdstuk 2 met een literatuuronderzoek naar de rol van integrine α6β4 (en andere integrines) in de verschillende stappen van tumorformatie en metastasering. Hierbij komen verschillende factoren aan bod die de functie van integrines beïnvloeden, waardoor integrines soms als een tumoronderdrukker en soms als tumorversterker kunnen optreden. We concluderen dat voor de bijdrage van het integrine α6β4 aan tumorgenese twee aspecten van belang zijn: ten eerste het uiteenvallen van HD's waardoor integrine α6β4 'de handen vrij heeft' om bij te dragen aan signaalroutes, en ten tweede de aanwezigheid van al geactiveerde signaalroutes.

Het uiteenvallen van HD's gebeurt door te voorkomen dat het integrine α6β4 aan andere HD-eiwitten zoals plectine bindt. Binding van plectine aan de β4-subeenheid kan worden voorkomen door β4 te markeren op specifieke plaatsen (serines/treonines). In hoofdstuk 3 hebben we onderzocht welke signaaleiwitten verantwoordelijk zijn voor het markeren van de integrine β4 subeenheid op een specifieke plek (T1736). We vonden onder andere dat het signaaleiwit RSK1 na activatie van de EGFR verantwoordelijk is voor markering van de β4-subeenheid op T1736. Deze data geeft inzicht in hoe HD's en specifiek de binding van plectine aan het integrine α6β4 wordt gereguleerd.

Wanneer de HDs uiteengevallen zijn, heeft het integrine α6β4 'de handen vrij' om bij te dragen aan signaalroutes. Andere gemarkeerde plekken (tyrosines) op de integrine β4-subeenheid zouden vervolgens kunnen dienen als bindingsplaats voor signaaleiwitten die op hun beurt tumorstimulerende signaalroutes kunnen versterken (Fig. 2). Echter of deze markeringen van β4 daadwerkelijke bijdragen aan het versterken van signaalroutes is al jaren onderdeel van het wetenschappelijke debat. Op basis van onze data, beschreven in hoofdstuk 4, concluderen we dat deze markeringen van β4 geen effect hebben op de sterkte van de door de EGFR verzonden signalen. Dit heeft tot gevolg dat, ook na dit onderzoek, niet bekend is wat de biologische relevantie is van deze markeringen van β4.

Behalve eiwitten die het integrine α6β4 markeren, kunnen ook andere eiwitten, zoals de tetraspanine CD151, de functie van integrines beïnvloeden. In hoofdstuk 5 hebben we onderzocht hoe de tetraspanine CD151 de adhesie van huidcellen door het integrine α6β4 aan het onderliggende basale membraan beïnvloedt. CD151 blijkt niet essentieel te zijn voor de vorming van HD's en niet significant bij te dragen aan de kracht waarmee het integrine α6β4 bindt aan het basale membraan. Echter, samen met het integrine α3β1, speelt CD151 een belangrijke rol bij het stabiliseren van een nog niet eerder

beschreven HD-type. In dit hoofdstuk hebben we de samenstelling van dit HD-type gekarakteriseerd, inzichten verkregen in de dynamiek van de verschillende types HD's en opperen we een mogelijke functie van dit nieuwe type HD.

Het is mogelijk dan er nog onbekende eiwitten zijn die de functie van het integrine α6β4 reguleren en daardoor effect hebben op de integriteit van de huid of formatie van (huid)kanker. In hoofdstuk 6, hebben we alle eiwitten die in de buurt komen van het integrine α6β4 in kaart gebracht met behulp van proteomics. We hebben 135 eiwitten geïdentificeerd als het "interactoom" van het integrine α6β4. De identificatie van het interactoom van het integrine α6β4 biedt een basis voor een hele nieuwe reeks onderzoeken naar mogelijke regulatoren van het integrine α6β4.

Afsluitend zijn de resultaten van dit onderzoek samengevat en bediscussieerd in Hoofdstuk 7. Concluderend, kunnen we stellen dat interessante nieuwe inzichten zijn verschaft in de mechanismen die het integrine α6β4 reguleren, en daardoor de integriteit van de huid of ontwikkeling van (huid)kanker beïnvloeden. Echter, om volledig te begrijpen hoe de integriteit van de huid wordt gewaarborgd, of hoe (huid)kanker zich ontwikkeld, is begrip van alleen het integrine α6β4 niet voldoende. Hiervoor zijn o.a. inzichten nodig in de communicatie tussen alle verschillende RTK's, verschillende integrines, en verschillende cel-matrix adhesies die gelijktijdig actief zijn in dezelfde cel.

Curriculum Vitae

Lisa te Molder werd geboren op 8 februari 1990 te Grave. In 2008 behaalde zij haar gymnasiumdiploma aan het MaasWaal College te Wijchen. In datzelfde jaar startte zij met de opleiding (Medische) Biologie aan de Radboud Universiteit te Nijmegen. Gedurende haar bachelor liep zij een 3 maanden durende stage op de afdeling Endocrinologie van het Radboudumc waar zij onder leiding van dr. Jyotsna Rao en Prof. dr. Henri Timmers onderzoek deed naar de immunohistochemische karakterisatie van feochromocytomen (zeldzame bijniertumor). Aanvullend op het curriculum voor de bachelor volgde zij in 2011 gedurende 6 maanden extracurriculaire vakken aan de Cardiff University in Wales. Na het behalen van haar bachelordiploma startte zij in 2012 haar masteropleiding Medische Biologie aan de Radboud Universiteit te Nijmegen. Gedurende deze periode doorliep zij 2 stages. De eerste stage van 7 maanden liep zij in het lab van dr. Colin Logie, op de afdeling Moleculaire Biologie in het Nijmegen Centre for Molecular Life Sciences (NCMLS, nu RIMLS). Daar deed zij, met behulp van gist (*S. Cerevisiae*), onderzoek naar de rol van het RSC-complex en de ubiquitine ligase UBR1 in mitose en meiose. De tweede stage, ook van 7 maanden, liep zij in het lab van Prof. dr. Arnoud Sonnenberg op de afdeling Celbiologie bij het Nederlands Kanker Instituut (NKI) te Amsterdam. Deze stage vormde de voorloper voor haar promotieonderzoek, waar zij in 2014 aan begon onder leiding van Prof. dr. Arnoud Sonnenberg.

List of Publications

Te Molder L, Kreft M, Heemskerk N, Schuring J, de Pereda J, Wilhelmsen K, Sonnenberg A. *EGFR-dependent tyrosine phosphorylation of integrin β4 is not required for downstream signaling events in cancer cell lines.* Manuscript in revision

Te Molder L, Hoekman L, Kreft M, Bleijerveld O, Sonnenberg A. *Comparative interactomics analysis reveals potential regulators of α6β4 distribution in keratinocytes.* Biol Open. 2020 Aug. 13;9(8).

Wang W, Zuidema A, **Te Molder L**, Nahidiazar L, Hoekman L, Schmidt T, Coppola S, Sonnenberg A. *Hemidesmosomes modulate force generation via focal adhesions.* J Cell Biol. 2020 Feb 3;219(2).

Te Molder L, Juksar J, Harkes R, Wang W, Kreft M, Sonnenberg A. *Tetraspanin CD151 and integrin α3β1 contribute to the stabilization of integrin α6β4-containing cell-matrix adhesions.* J Cell Sci. 2019 Oct 10;132(19).

Zuidema A, Wang W, Kreft M, **Te Molder L**, Hoekman L, Bleijerveld OB, Nahidiazar L, Janssen H, Sonnenberg A. *Mechanisms of integrin αVβ5 clustering in flat clathrin lattices.* J Cell Sci. 2018 Nov 5;131(21).

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[&] These authors contributed equally

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Lisa