

## **Tetraspanin CD151 and integrin $\alpha 3\beta 1$ contribute to the stabilization of integrin $\alpha 6\beta 4$ -containing cell-matrix adhesions**

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### **Keywords**

Hemidesmosome, tetraspanin, CD151, integrin, plectin, keratinocyte adhesion.

### **Summary Statement**

This study elucidates the role of tetraspanin CD151 in the formation and maintenance of cell-matrix adhesions in epidermal keratinocytes.

### **Abstract**

Tetraspanin CD151 has been suggested to regulate cell adhesion through its association with laminin-binding integrins  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$ ; 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  $\alpha 3\beta 1$ , 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- $\alpha 3\beta 1/\alpha 6\beta 4$  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  $\alpha 6\beta 4$ , plectin, BP180 and BP230, 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), multiprotein complexes that are associated with the keratin intermediate filament system within the cell.

The integrin  $\alpha 6\beta 4$ , a heterodimeric transmembrane protein, is a main component of HDs. In type I HDs, present in the epidermis,  $\alpha 6\beta 4$  binds extracellularly to laminin-332 and intracellularly via two associated proteins, plectin and BP230 (BPAG1e), to the keratin cytoskeleton. Besides  $\alpha 6\beta 4$ , type I HDs contain two other transmembrane proteins: the tetraspanin CD151 and BP180 (also known as BPAG1 or collagen XVII) (Litjens et al., 2006; Sterk et al., 2000; Walko et al., 2015). In more simple epithelia of the intestine or the mammary gland, a rudimentary form of HDs is present. These type II HDs contain  $\alpha 6\beta 4$  and plectin, but lack BP180 and BP230 (Fontao et al., 1997; Uematsu et al., 1994).

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) (Has and Fischer, 2018). Patients carrying null mutations in the genes for the integrin  $\alpha 6$  or  $\beta 4$  subunits, or the cytolinker protein plectin, suffer from a severe form of EB, which is indicated by the absence of HDs (Ashton et al., 2001; McLean et al., 1996; Nakamura et al., 2005; Niessen et al., 1996; Vidal et al., 1995). 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 (Groves et al., 2010; Jonkman et al., 1995; Liu et al., 2012; McMillan et al., 1998). 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 integrin  $\alpha 3$  gene (Crew et al., 2004; Has et al., 2012; Kagan et al., 1988; Vahidnezhad et al., 2017). Targeted deletion of genes encoding HDs and associated proteins in mice largely recapitulates the pathology observed in humans (Andrä et al., 1997; DiPersio et al., 1997; Dowling et al., 1996; Guo et al., 1995; Sachs et al., 2006; van der Neut et al., 1996; Wright et al., 2004).

Integrin  $\alpha 3\beta 1$ , another laminin-binding integrin expressed by keratinocytes, is a component of focal adhesions (FAs), but can also be found together with  $\alpha 6\beta 4$  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  $\alpha 6\beta 4$  and  $\alpha 3\beta 1$  in TEMs is facilitated through binding of their  $\alpha$  subunits to CD151 (Hemler, 2005). The association of CD151 with laminin-binding

integrins has been reported to affect integrin-dependent adhesion, migration and signaling (Berditchevski, 2001; Stipp, 2010; Termini and Gillette, 2017).

While it has been shown that CD151 is colocalized with  $\alpha 6\beta 4$  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 (Sterk et al., 2002). 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  $\alpha 3\beta 1$  and the HD proteins  $\alpha 6\beta 4$  and plectin, but which are not associated with the keratin cytoskeleton.

## **Results**

### *CD151 forms multi-protein complexes with both $\alpha 3\beta 1$ and $\alpha 6\beta 4$ in keratinocytes*

Previous studies have shown that CD151 forms stable complexes with the laminin-binding integrins  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  (Hemler, 2005; Sterk et al., 2002). We confirmed these findings by reciprocal co-immunoprecipitation (co-IP) assays using two different monoclonal antibodies against CD151 (5C11 and 11G5) and antibodies against the integrin  $\alpha 3$  and  $\alpha 6$  subunits (Fig. 1A). Furthermore, these assays showed that in PA-JEB/ $\beta 4$  keratinocytes the  $\alpha 6$  subunit does not form a heterodimer with  $\beta 1$ , which is consistent with the fact that  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$ , but not  $\alpha 6\beta 1$ , are the major laminin-binding integrins in skin (Sonnenberg et al., 1991) (Fig. 1A). Using immunofluorescence (IF) microscopy, we detected CD151 in a pattern of consecutive arches, in which it is partially co-localized with  $\alpha 3\beta 1$ . Both  $\alpha 3\beta 1$  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  $\alpha 3\beta 1$  and CD151 co-localized with the HD proteins  $\alpha 6\beta 4$  and plectin (Fig. 1C,D). In summary, these results confirm that in keratinocytes CD151 binds both  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$ , and is present in adhesion structures that contain characteristics of HDs.

### *CD151 regulates $\alpha 6\beta 4$ distribution.*

To determine to what extent CD151 impacts  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$ -containing adhesion complexes, we abrogated CD151 protein expression in PA-JEB/ $\beta 4$  and HaCaT keratinocytes using CRISPR-Cas9 (KO) and subsequently restored the expression of CD151 by retroviral-mediated gene transfer. Analysis by Western blot (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  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  at the plasma membrane (Fig. 2A,B). However, quantitative IF staining and confocal microscopy showed reduced clustering of  $\alpha 3\beta 1$  (and to a lesser extent of  $\alpha 6\beta 4$ ) at the basal cell surface

(Fig. 2C). Furthermore,  $\alpha 3\beta 1$  was no longer co-distributed with  $\alpha 6\beta 4$  but showed increased co-localization with phospho-paxillin in FAs (Fig. 2D,E). In the CD151-proficient cells,  $\alpha 6\beta 4$  was clustered in central and peripheral adhesions, but strikingly in the CD151-deficient cells it was almost exclusively present at the cell's periphery (Fig. 2F-H). These results show that, while not essential for  $\alpha 3\beta 1$  or  $\alpha 6\beta 4$  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.

#### CD151 is essential for the maintenance of central $\alpha 6\beta 4$ -containing adhesions

To gain real-time insight into how CD151 contributes to the formation of  $\alpha 6\beta 4$ -containing adhesions in the central region of the cells, we deleted CD151 in PA-JEB keratinocytes expressing GFP-tagged  $\beta 4$  (Geuijen and Sonnenberg, 2002). The absence of CD151 and presence of  $\beta 4$ -GFP was confirmed by FACS and WB analysis (Fig. 3A,B). With these cells, the clustering of  $\beta 4$ -GFP was followed over time by time-lapse imaging. During initial stages of cell spreading, both CD151-deficient and -proficient PA-JEB/ $\beta 4$ -GFP cells assembled  $\alpha 6\beta 4$ -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; Movie S1,2). Furthermore, in fluorescence recovery after photobleaching (FRAP) experiments, a spot in the central and peripheral  $\alpha 6\beta 4$ -containing adhesions was simultaneously bleached and the recovery of the  $\beta 4$ -GFP signal at these spots was followed over time (Fig. 3D,E). The results show that the mobile fraction of  $\alpha 6\beta 4$  in the central  $\alpha 6\beta 4$ -adhesions is larger than in the peripheral ones, indicating a higher dynamic nature of  $\alpha 6\beta 4$  in the central adhesions (Fig. 3E,F).

#### Peripheral but not central $\alpha 6\beta 4$ -adhesions contain BP230 and are associated with keratin.

We reasoned that the observed differences in the dynamics of  $\alpha 6\beta 4$  between central and peripheral adhesion clusters could be due to the association of  $\alpha 6\beta 4$  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  $\alpha 6\beta 4$ -containing adhesions, with laminin-332 being slightly more abundant under the central adhesion clusters and plectin at the peripheral adhesion clusters (Fig. 4A,B). Strikingly, keratin and BP230 (which, like plectin, can connect  $\alpha 6\beta 4$  to the keratin cytoskeleton) were associated solely with  $\alpha 6\beta 4$  in the peripheral adhesions (Fig. 4A,B). Interestingly, in the peripheral adhesions only approximately 30 and 50 percent of  $\alpha 6\beta 4$  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/ $\beta$ 4 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/ $\beta$ 4 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  $\alpha$ 6 $\beta$ 4-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  $\alpha$ 6 $\beta$ 4 in the peripheral adhesions of CD151-deficient PA-JEB/ $\beta$ 4 keratinocytes (Fig. S2). The central  $\alpha$ 6 $\beta$ 4-containing adhesion clusters, which share some of the characteristics of HDs, such as the clustering of  $\alpha$ 6 $\beta$ 4 and its association with laminin-332 and plectin, also have features in common with TEMs: they contain  $\alpha$ 3 $\beta$ 1 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.

#### *$\alpha$ 3 $\beta$ 1 is localized in central HD-like adhesions but not in peripheral HDs*

Despite the fact that CD151 can form stable complex with both  $\alpha$ 3 $\beta$ 1 and  $\alpha$ 6 $\beta$ 4, type I HDs in the skin contain only complexes of CD151 with  $\alpha$ 6 $\beta$ 4 (Sterk et al., 2002; Underwood et al., 2009). To investigate the distribution of  $\alpha$ 3 $\beta$ 1 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  $\alpha$ 6 $\beta$ 4 in the central HD-like adhesions and the peripheral HDs almost completely overlapped each other (Fig. 4C). In contrast, the profiles of  $\alpha$ 3 $\beta$ 1 and  $\alpha$ 6 $\beta$ 4 only overlapped in the central HD-like adhesions (Fig. 4C). In the peripheral region the peak intensities of  $\alpha$ 3 $\beta$ 1 overlapped with that 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  $\alpha$ 3 $\beta$ 1,  $\alpha$ 6 $\beta$ 4 and CD151 cluster together, in the periphery they can be found in two distinct complexes:  $\alpha$ 3 $\beta$ 1 in FAs and  $\alpha$ 6 $\beta$ 4 and CD151 in type I HDs.

#### *CD151 stabilizes central HD-like adhesions through binding to $\alpha$ 3 $\beta$ 1 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  $\alpha$ 3 $\beta$ 1, we deleted and reconstituted integrin  $\alpha$ 3 in the HaCaT and PA-JEB/ $\beta$ 4 keratinocyte cell lines (Fig. 5A,B; Fig. S3A,B). In both these cell lines, the absence of  $\alpha$ 3 did not affect the total levels of  $\beta$ 4 at the cell surface and the formation of peripheral HDs was not obviously disturbed. However, as observed in the CD151-deficient cells, the absence of  $\alpha$ 3 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  $\alpha 3$ -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  $\alpha 3$ -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  $\alpha 3$ -deficient and -proficient cells (Fig. S3D,E).

Next, we investigated whether the role of  $\alpha 3\beta 1$  in maintaining the central HD-like adhesions requires binding of  $\alpha 3\beta 1$  to laminin-332. To this end, we introduced an  $\alpha 3$ -binding-deficient mutant (G163A) into the  $\alpha 3$ -deficient HaCaT cells (Zhang et al., 1999). Expression of the mutant  $\alpha 3\beta 1$  was verified by FACS analysis (Fig. 5B). Unlike the wild-type  $\alpha 3$  subunit, the  $\alpha 3$ -G163A mutant was unable to completely rescue the central HD-like adhesions, suggesting that  $\alpha 3\beta 1$  binding to laminin contributes to efficient maintenance of these adhesions (Fig. 5C,D). These findings were confirmed in PA-JEB/ $\beta 4$  cells using the  $\alpha 3$  blocking antibody, J143 (Fig. S3F,G).

Palmitoylation of CD151 is necessary for stabilization of the CD151 TEMs and hence for incorporation and clustering of  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  into these multi-molecular complexes of CD151 (Berditchevski et al., 2002; Yang et al., 2002). To study the role of this self-associating function of CD151 for the stabilization of the central HD-like adhesions, we introduced a CD151 palmitoylation mutant into the CD151-deficient cells (Fig. 5B) (Berditchevski et al., 2002). The expression of this mutant in 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). Together these results show that for efficient maintenance of the central HD-like adhesions, both laminin-332-associated  $\alpha 3\beta 1$  and self-associated and TEM-forming CD151 are needed.

#### *Laminin-332, but not plectin contributes to clustering of $\alpha 6\beta 4$ 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  $\alpha 6\beta 4$  in the different adhesions requires binding of  $\alpha 6\beta 4$  to laminin-332 or plectin. Therefore, we expressed the  $\beta 4$  mutants,  $\beta 4$ -AD and  $\beta 4$ -RW, in the  $\beta 4$ -deficient PA-JEB keratinocytes (Fig. 6A). The  $\beta 4$ -AD mutant is an adhesion-defective mutant that cannot bind to laminin-332, while the  $\beta 4$ -RW mutant, carrying a single missense mutation R1281W in the cytoplasmic domain of  $\beta 4$ , is unable to bind plectin (Geerts et al., 1999; Nievers et al., 2000; Russell et al., 2003). In the  $\beta 4$ -AD mutant expressing cells, central HD-like adhesions were less commonly present than in the  $\beta 4$ -WT expressing cells (Fig. 6B,C). Similar findings were obtained when PA-JEB/ $\beta 4$  and HaCaT cells were treated with the  $\beta 4$  blocking antibody ASC8 (Russell et al., 2003). 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  $\beta 4$ -RW mutant and in plectin-deficient PA-JEB/ $\beta 4$  keratinocytes, the  $\alpha 6\beta 4$  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  $\alpha 6\beta 4$  to laminin-332, like that of  $\alpha 3\beta 1$ , contributes to the stabilization of the central HD-like adhesions. On the contrary, binding of  $\alpha 6\beta 4$  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 $\alpha 6\beta 4$ -mediated cell adhesion*

Integrin clustering determines the strength by which cells adhere to the ECM (Puklin-Faucher and Sheetz, 2009). To investigate whether CD151-mediated clustering of  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  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  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$ , using a spinning disk device (Boettiger, 2007). CD151-deficient cells showed a reduced tolerance (albeit not statistically significant) to shear stress compared to the proficient cells (Fig. 7A), suggesting that CD151 plays only a minor role in adhesion strengthening when both  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  are present. Next, we investigated whether cell adhesion mediated by either  $\alpha 3\beta 1$  or  $\alpha 6\beta 4$  is strengthened by CD151. To resolve this, we individually deleted  $\alpha 3$  and  $\beta 4$  in the CD151-deficient HaCaT keratinocytes and confirmed the absence of these proteins by FACS and WB analysis (Fig. 7B,C). The deletion of  $\alpha 3$  in the CD151-deficient HaCaT keratinocytes had no effect on the adhesive strength of  $\alpha 6\beta 4$  to laminin-332 (Fig. 7D). However, in the absence of  $\alpha 6\beta 4$ , the additional loss of CD151 resulted in a significant reduction in  $\alpha 3\beta 1$ -mediated adhesion strengthening (Fig. 7E,F). We conclude that CD151 significantly strengthens  $\alpha 3\beta 1$ , but not  $\alpha 6\beta 4$ -mediated cell adhesion by facilitating the clustering of this integrin into central HD-like adhesions.

#### **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  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  (Sincoc et al., 1997). In skin, CD151 is complexed with  $\alpha 6\beta 4$  and is a component of HDs (Sterk et al., 2000). 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 in addition to the CD151- $\alpha 3\beta 1/\alpha 6\beta 4$  integrin complexes the cytolinker plectin, but are not associated with keratin filaments. We further show that CD151, through its binding to  $\alpha 3\beta 1$  is critical for the formation and stabilization of these central HD-like adhesions and strengthens  $\alpha 3\beta 1$ - but not  $\alpha 6\beta 4$ -

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 (Bouameur et al., 2013), 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 (Guo et al., 1995). Similarly, reduced amounts of keratins are associated with type II HDs, which contain plectin but lack BP230 (Fontao et al., 1997; Uematsu et al., 1994).

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  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  into central HD-like adhesions may strengthen keratinocyte adhesion to newly deposited laminin-332 before FAs and HDs are fully established but 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  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  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 (Michael et al., 2014; Seltmann et al., 2013), 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 HD-like adhesions, unlike type I HDs, contain  $\alpha 3\beta 1$  and are connected to  $\alpha 6\beta 4$  through CD151. Thus, they will likely contribute to its stability, as well.



In central HD-like adhesions, CD151 and  $\alpha 3\beta 1$  colocalize, but in the periphery CD151 associates with type I HDs and  $\alpha 3\beta 1$  is localized into FAs. The localization of  $\alpha 3\beta 1$  into FAs coincides with the incorporation of BP230 in HDs. Since the localization of BP180 in type I HDs precedes that of BP230 (Koster et al., 2003), it is possible that BP180 displaces  $\alpha 3\beta 1$  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  $\alpha 3$ ,  $\alpha 6$  and  $\beta 4$  subunits (Yang et al., 2004), 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  $\alpha 3\beta 1$  in cultured keratinocytes, no obvious abnormalities in HDs have been observed in mice lacking integrin  $\alpha 3$  in basal keratinocytes (Conti et al., 2003; DiPersio et al., 1997; Margadant et al., 2009). Yet, genetic ablation of the  $\beta 1$  integrin subunit or its co-activator kindlin-1 in basal keratinocytes has been associated with reduced HD protein expression and formation of HDs (Brakebusch et al., 2000; Qu et al., 2012; Raghavan et al., 2000). This suggests that if  $\alpha 3\beta 1$  has a role in HD formation, this role could be fulfilled by other integrins, as well. Furthermore, our finding that CD151 contributes to  $\alpha 3\beta 1$ - but not  $\alpha 6\beta 4$ -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  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$ , 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  $\alpha 3\beta 1$  but not  $\alpha 6\beta 4$ , have been associated with progressive and irreversible kidney dysfunction (Crew et al., 2004; Kagan et al., 1988; Sachs et al., 2012; Vahidnezhad et al., 2017).

In conclusion, we show that CD151 stabilizes the central HD-like adhesions by strengthening the adhesion of  $\alpha 3\beta 1$  to laminin-332 and facilitating the incorporation of  $\alpha 6\beta 4$  into TEMs containing  $\alpha 3\beta 1$ . On the other hand, CD151 is not needed for the formation or stabilization of  $\alpha 6\beta 4$ -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 CD151- and  $\alpha 3\beta 1$ -mutant patients and mice 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 WB were goat anti-mouse IgG HRP (BioRad) and polyclonal goat anti-Rabbit IgG HRP (Dako). Donkey anti-mouse IgG PE (Jackson ImmunoResearch) was the secondary antibody used in FACS. For immunofluorescence (IF), rabbit anti-rat IgG (homemade), goat anti-guinea pig IgG Alexa Fluor (AF) 488, goat anti-human IgG AF488, goat anti-mouse AF568, goat anti-mouse AF647, goat anti-rabbit AF488, goat anti-rabbit AF647, goat anti-rat AF647 (Invitrogen), goat anti-mouse FITC, goat anti-rat FITC (Rockland) and the isotype specific goat anti-mouse IgG1 AF568 (ThermoFischer #A-21124) and goat anti-mouse IgG2a-FITC (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 (Niessen et al., 1996; Schaapveld et al., 1998). The derivation of this cell line was done for diagnostic purposes; thus, the research conducted using these cells was exempt of the requirement for ethical approval. PA-JEB/ $\beta$ 4 keratinocytes stably expressing wild-type  $\beta$ 4 were generated by retroviral transduction (Sterk et al., 2000) and maintained in serum-free keratinocyte medium (KGM; Invitrogen), supplemented with 50  $\mu$ g ml<sup>-1</sup> bovine pituitary gland extract, 5 ng ml<sup>-1</sup> EGF, and antibiotics (100 units ml<sup>-1</sup> streptomycin and 100 units ml<sup>-1</sup> penicillin; Sigma). 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), and antibiotics. All cells were cultured at 37°C in a humidified, 5% CO<sub>2</sub> atmosphere.

The  $\alpha$ 3-,  $\beta$ 4-, CD151- and plectin-deficient cells were prepared using CRISPR-Cas9 technology. Target sgRNAs against integrin  $\alpha$ 3 (5'-GCTACTCGGTCGCCCTCCAT-3'), integrin  $\beta$ 4 (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  $\alpha$ 3-,  $\beta$ 4- 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 cotransfected with a plasmid containing a blasticidin cassette flanked by TIA target sites (described by Blomen et al. 2015), blasticidin resistant cells were enriched by selection for four days in the presence of blasticidin (4  $\mu$ g ml<sup>-1</sup>; Sigma-Aldrich), clones were picked and analyzed for plectin expression by WB.

For (re)expression of proteins in protein deficient cells, we used stable cellular retroviral mediated transduction. Cells which expressed the protein were enriched by zeocin or blasticidin selection and/or by bulk sorting by FACS. The constructs used were: the  $\beta$ 4-AD (D230A, P232A and E233A) in LZRS-IRES-blasticidin, a kind gift of Peter Marinkovich (Russell et al., 2003), the CD151 wt 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 (Berditchevski et al., 2002)) and the  $\alpha$ 3 wt in LZRS-IRES-Zeo. The  $\alpha$ 3 cDNA has been described previously (Delwel et al., 1994) and the full-length cDNA encoding human  $\alpha$ 3 was released from pUC18 by digesting with *SphI*, made blunt by Klenow digestion and ligated into the *SwaI* site of the retroviral LZRS-IRES-zeo vector. Furthermore, the  $\alpha$ 3-G163A mutant in pBJ-1 expression vector (Zhang et al., 1999) was a gift from Yoshikazu Takada and transiently transfected in  $\alpha$ 3-deficient cells. Generation of PA-JEB keratinocytes stably expressing cells  $\beta$ 4-RW (R1281W) has been described previously (Geerts et al., 1999).

#### Immunofluorescence imaging of fixed samples

For image acquisition, cells were seeded, at approximately 10-20% confluency, onto glass coverslips, uncoated or coated with 10  $\mu$ g ml<sup>-1</sup> fibronectin from bovine plasma (F1141, Sigma) and 5  $\mu$ g ml<sup>-1</sup> vitronectin (SRP3186, Sigma) and grown for 40-44 hours of which at least the last 20 hours in the presence of DMEM+FCS. After the cells had been fixed with 2% PFA for 10 min., permeabilized with 0.2% triton and blocked with 2% BSA (resolved in PBS; Serva), they were incubated for 50-60 minutes with primary and secondary antibodies, with three PBS washes in between. Nuclei were counterstained with DAPI (Sigma) 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 63x (NA 1.4) oil objective. A Leica SR-GSD microscope (Leica Microsystems, Wetzlar, Germany) equipped with a 160x 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 equal conditions.

Image analyses were performed using ImageJ: Colocalization was assessed using Mander's 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  $\beta$ 4 staining in the peripheral adhesions was obtained by drawing a region of interest around the peripheral adhesions and measuring the mean intensity of the  $\beta$ 4 staining in this region. The percentage of integrin  $\beta$ 4 associated with plectin, BP230, keratin 14 or laminin-332 was obtained by calculating how much of the  $\beta$ 4 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  $\beta 4$  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 and accompanied standard deviation (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 63x (NA 1.4) oil objective equipped with a live cell chamber. Live imaging of CD151-proficient and -deficient PA-JEB/ $\beta 4$ GFP cells was started 20h after seeding and immediately after replacing the KGM medium by DMEM+FCS. Every hour the differential interference contrast (DIC) and GFP fluorescent signals were collected of multiple cell islands at different positions. Videos were made in ImageJ.

FRAP experiments were performed, using the FRAP wizard in LAS-AF, using PA-JEB/ $\beta 4$ GFP cells grown for 20h in KGM medium and subsequently grown for an additional 20h in DMEM+FCS. Simultaneously a region in one peripheral- and one central- $\alpha 6\beta 4$  containing adhesion was bleached and fluorescent recovery was followed over time. Two images as controls were taken before bleaching and then 1 image every 10 (first 2 minutes) or 30 seconds for 15 minutes after bleaching. For analyses, the background was subtracted from the measured intensities and the curves were normalized to the mean value before bleaching. Furthermore, the values of the first 230 seconds 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  $\tau$ . Graphs are made using GraphPad Prism; the line plot shows the mean with standard deviations 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 Wilcoxon matched-pairs signed rank test (at different time points); P values: \*\* < 0.01, \*\*\* < 0.001.

### Flow Cytometry/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 (Boettiger, 2007). Wild-type or KO HaCaT cells were grown to 60-100% confluence on 30 mm coverslips for two 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<sup>-1</sup> NaCl, 0.25 gL<sup>-1</sup> KCL, 0.95 gL<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>\*2H<sub>2</sub>, 0.25 gL<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>), 10% B (1.34 gL<sup>-1</sup> CaCl<sub>2</sub>.2H<sub>2</sub>O) and 10% C (2 gL<sup>-1</sup> MgCl<sub>2</sub>.6H<sub>2</sub>O)) containing 5% dextran from Leuconostoc Spp. Mr 450,000-600,000 (Sigma). After spinning, the cells were fixed in 2% PFA, permeabilized in 0.2% triton-X100, 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 confluency was measured per ring and normalized to the total area of the ring and to the confluency in the middle area (100% confluent). The average shear stress per ring was calculated using the formula  $\tau = 10 * (0,8 r \sqrt{\rho\mu\omega^3})$  with  $\tau$  is the shear stress in dynes/cm<sup>2</sup>,  $r$  is the radial distance from the center of rotation in m,  $\rho$  is the fluid density in kg m<sup>-3</sup>,  $\mu$  is the fluid viscosity in Pa\*s and  $\omega$  is the rotational velocity in radians per minute. The  $\tau_{50}$  was obtained by fitting the data points to a logistic function  $f = \frac{100}{1+\exp[(\tau-c)/b]}$  using MATLAB software with  $c$  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 WT line, the same dataset was used in the different graphs. The significance of the percentage of adherent cells in KO cells compared to the WT 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  $\tau_{50}$  was visualized in a bar graph showing the mean with standard deviation of 9-10 independent experiments. The significant different between the  $\tau_{50}$  in  $\beta_4$  KO and  $\beta_4$ +CD151 KO cells was calculated using the non-parametric Mann-Whitney U test; P value: \*\*\*\* < 0.0001.

### Immunoprecipitations and western blot

For analysis of proteins in whole cell lysates, 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 inhibitors (1.5 mM Na<sub>3</sub>VO<sub>4</sub>, 15 mM NaF and protease inhibitor cocktail (1:500)), whole cell lysates were cleared by centrifugation at 12000 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 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 12.000 rpm for 60 min at 4°C, the cleared lysates were incubated at 4°C for 1.5h with 1 μg ml<sup>-1</sup> antibody. Subsequently, the lysates were incubated overnight with Protein G Sepharose 4 Fast Flow beads (GE Healthcare), beads were washed 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 1h 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 weight markers (kDa) are indicated.

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### **Competing interests**

The authors declare no competing interests.

### **Author contributions**

Conceptualization: L.t.M., A.S.; Methodology: L.t.M., J.J., W.W., M.K.; Software: R.H.; Verification: L.t.M.; Analysis: L.t.M., R.H.; Investigation: L.t.M., J.J.; Writing-original draft: L.t.M., A.S.; Writing-review and editing: L.t.M., R.H., W.W., A.S.; Visualization: L.t.M.; Supervision: A.S.

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### **Data availability**

All relevant data are available from the authors on reasonable request.

### **List of abbreviations**

ECM, extracellular matrix; HD, hemidesmosome; EB, epidermolysis bullosa; FA, focal adhesion; TEM, tetraspanin enriched micro domain; WT, wild-type; KO, knock-out; MUT, mutant; RESC, rescue; WB, Western blot; IP, immunoprecipitation; FACS, fluorescence activated cell sorting; TIRF, total internal reflection fluorescence; IF, immune fluorescence; MOC, Mander's overlap coefficient; DAPI, 4',6-diamidino-2-phenylindole; FRAP, fluorescent recovery after photobleaching.

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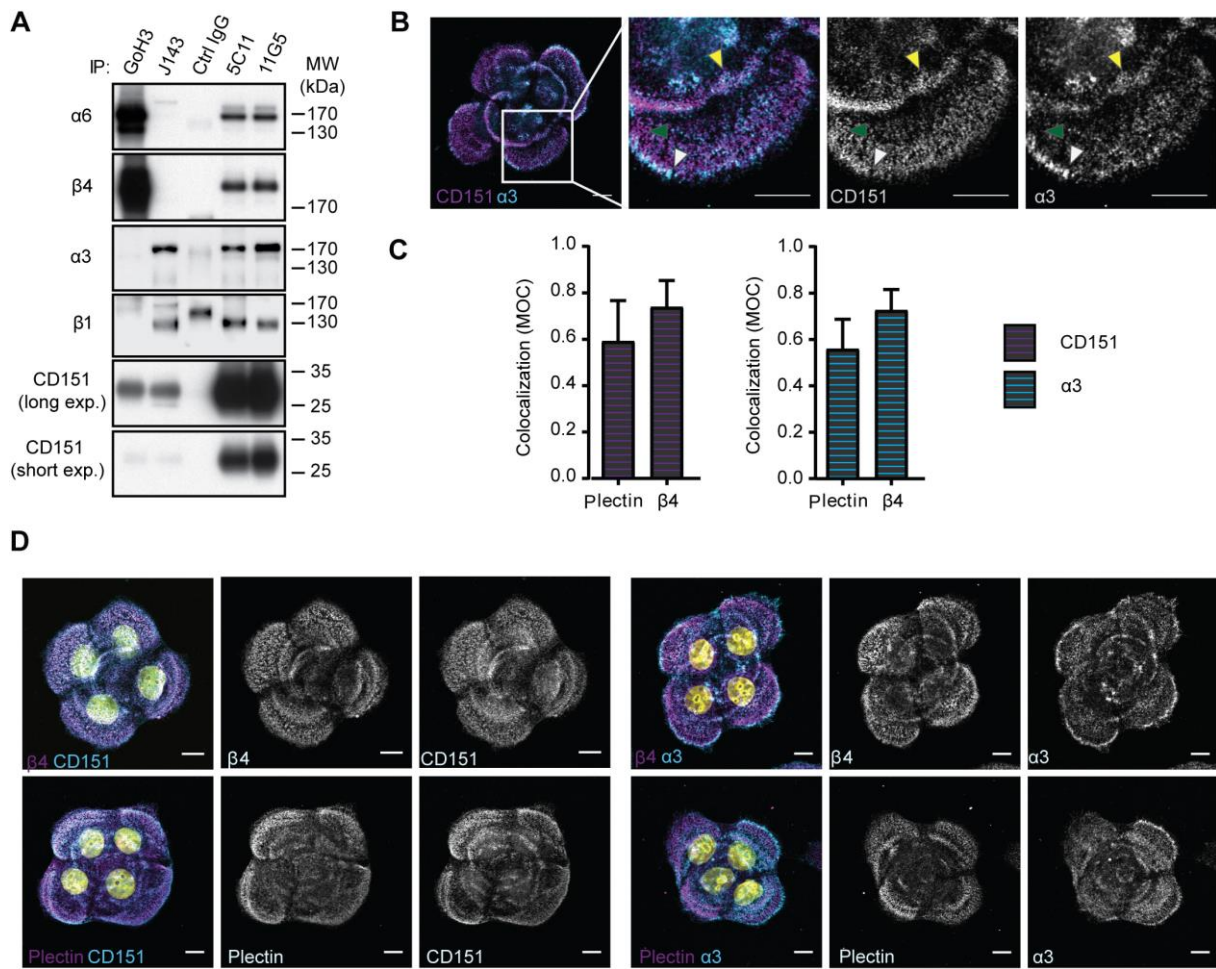
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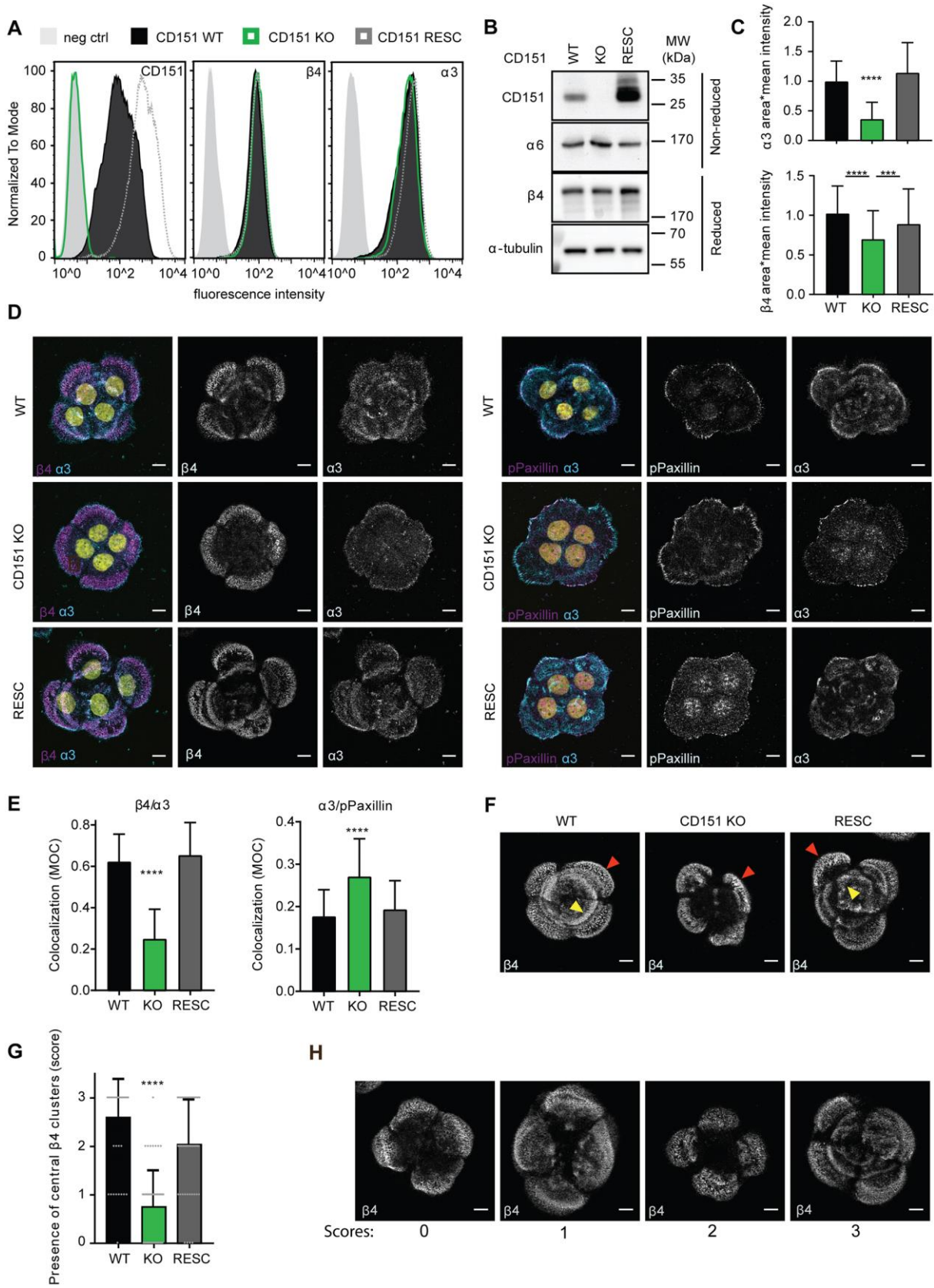
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## Figures



**Fig. 1. Association of CD151 with  $\alpha 3\beta 1$  and  $\alpha 6\beta 4$  in adhesion complexes.**

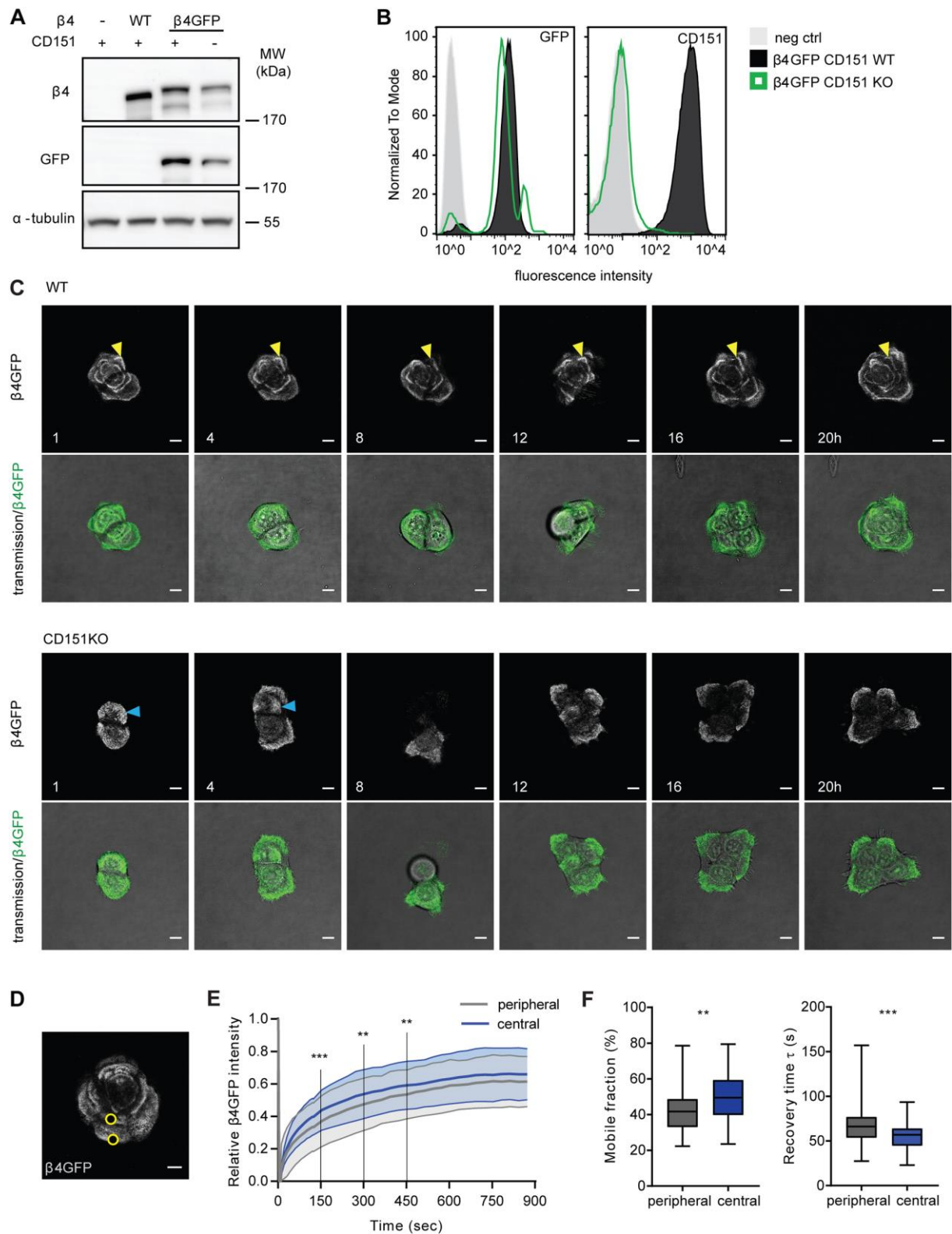
(A) Detection of  $\alpha 3\beta 1$ -CD151 and  $\alpha 6\beta 4$ -CD151 complexes in lysates of PA-JEB/ $\beta 4$  keratinocytes using immunoprecipitation and Western Blotting under nonreducing conditions. Antibodies used for immunoprecipitation were GoH3 against  $\alpha 6$ , J143 against  $\alpha 3$ , and 5C11 and 11G5 against CD151. (B) Confocal overview and zoom-in image of PA-JEB/ $\beta 4$  cells stained for  $\alpha 3$  (cyan) and CD151 (magenta). Yellow arrow heads indicate adhesions containing  $\alpha 3$  and CD151 and white and dark green arrow heads indicate  $\alpha 3$  and CD151 located independently of each other in the periphery of the cells. Scale bars are 10  $\mu\text{m}$ . (C) Quantification of the colocalization (MOC) of  $\alpha 3$  (46 images from 2 independent experiments (exp.)) or CD151 (30 images; 2 exp.) with plectin and  $\alpha 3$  (52 images; 2 exp.) or CD151 (74 images; 4 exp.) with  $\beta 4$ . (D) Confocal images of double-IF staining of CD151,  $\alpha 3$  (cyan) and HD components plectin and  $\beta 4$  (magenta) in PA-JEB/ $\beta 4$  keratinocytes. Nuclei were counterstained with DAPI (yellow) and scale bars are 10  $\mu\text{m}$ .





**Fig. 2. CD151 influences localization of adhesions containing  $\alpha3\beta1$  and  $\alpha6\beta4$ .**

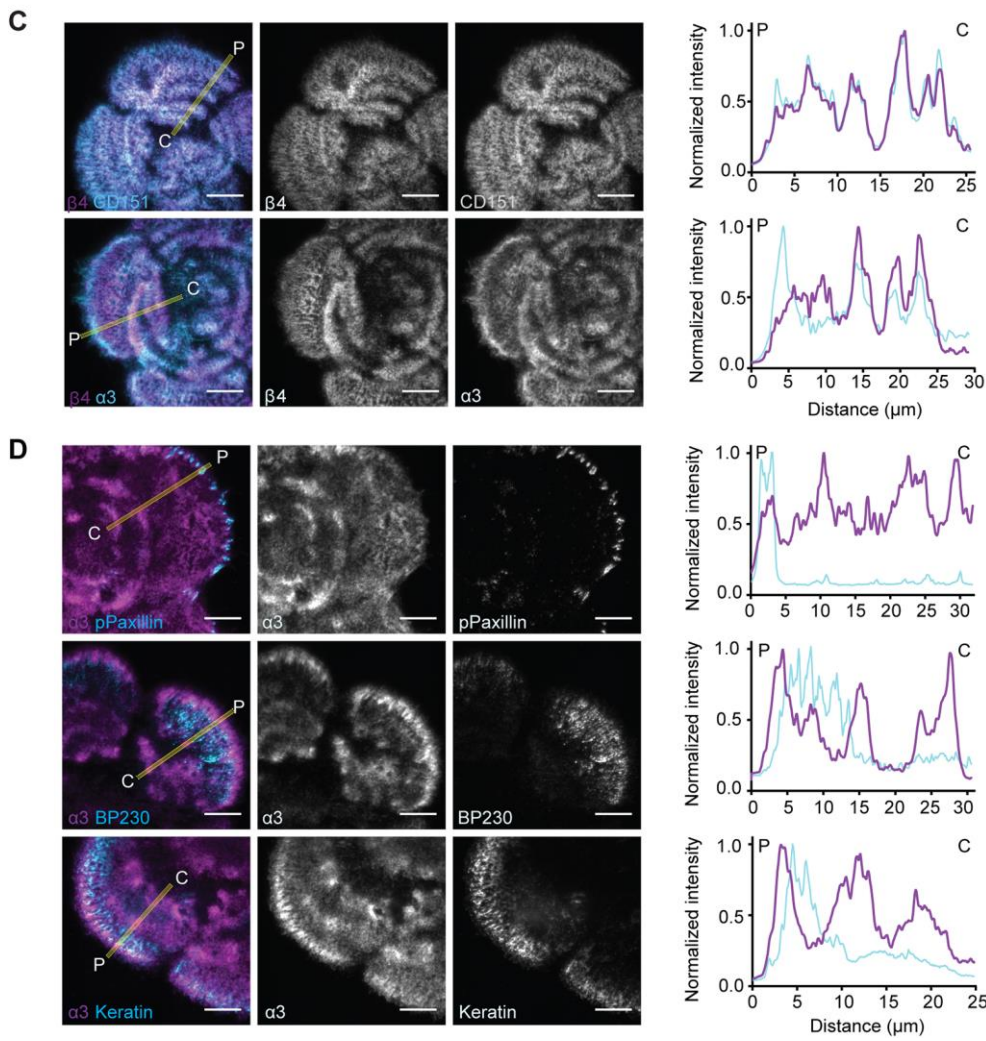
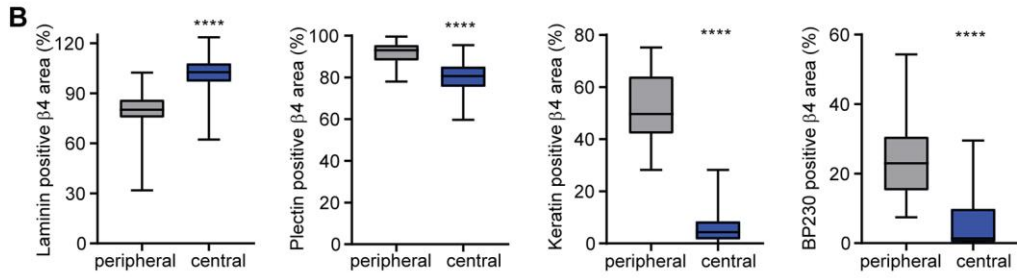
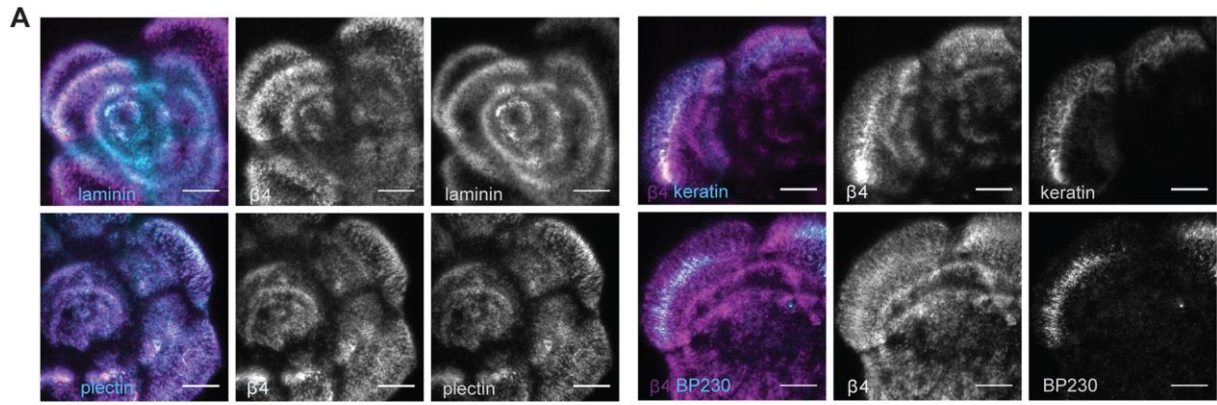
(A) FACS analysis of surface expression of CD151,  $\beta4$  and  $\alpha3$  for CD151-WT, -KO and -RESC PA-JEB/ $\beta4$  cells. (B) Whole cell lysate of WT, CD151-KO and CD151-RESC PA-JEB/ $\beta4$  keratinocytes analyzed by WB for protein levels of CD151,  $\alpha6$ ,  $\beta4$  and  $\alpha$ -tubulin (loading control) under reduced or non-reduced conditions as indicated. (C) Quantification of the amount of  $\alpha3$  (WT: 55, KO: 57 and RESC: 55 images; 3 exp. each) and  $\beta4$  (WT: 122, KO: 122 and RESC: 119 images; 8 exp. each) clustering at the basal cell surface ( $\alpha3$  or  $\beta4$  area\*intensity). Graph: Mean+s.d.; P values: \*\*\*< 0.001, \*\*\*\*< 0.0001 (Mann-Whitney U test). (D) Confocal images of WT, CD151-KO and CD151-RESC PA-JEB/ $\beta4$  keratinocytes stained for  $\alpha3$  (cyan) and  $\beta4$  (magenta), or  $\alpha3$  (cyan) and phosphorylated paxillin (magenta). Nuclei were counterstained with DAPI (yellow). Scale bars are 10  $\mu\text{m}$  (E) Quantification of the colocalization (MOC) of  $\beta4$  and  $\alpha3$  (WT: 56, KO: 55 and RESC: 57 images; 3 exp. each) and of  $\alpha3$  and phosphorylated paxillin (WT: 55, KO: 57 and RESC: 55 images; 3 exp. each). Graph: Mean+s.d.; P value: \*\*\*\*< 0.0001 (Mann-Whitney U test). (F) Confocal images of WT and CD151-KO and CD151-RESC PA-JEB/ $\beta4$  keratinocytes stained for  $\beta4$ . Red arrowheads show peripheral  $\alpha6\beta4$ -containing adhesions, while yellow arrowheads point to central  $\alpha6\beta4$ -containing adhesions. Scale bars are 10  $\mu\text{m}$ . (G) Quantification of the amount of central  $\alpha6\beta4$ -containing adhesions per cell island (dots represent individual cell islands) using images from WT (57 images; 4 exp.), CD151-KO (56 images; 4 exp.) and CD151-RESC (55 images; 4 exp.) PA-JEB/ $\beta4$  cells stained for  $\beta4$ . Graph: Mean+s.d.; P value: \*\*\*\*< 0.0001 (Mann-Whitney U test). (H) Scoring system for the amount of central  $\alpha6\beta4$ -containing adhesions present, with example images. 0 = no central adhesion, 1 = little central adhesions, 2 = several central adhesions, 3 = many central adhesions. Scale bars are 10  $\mu\text{m}$ .



**Fig. 3. CD151 is essential for maintenance of early  $\alpha 6\beta 4$ -containing adhesions**

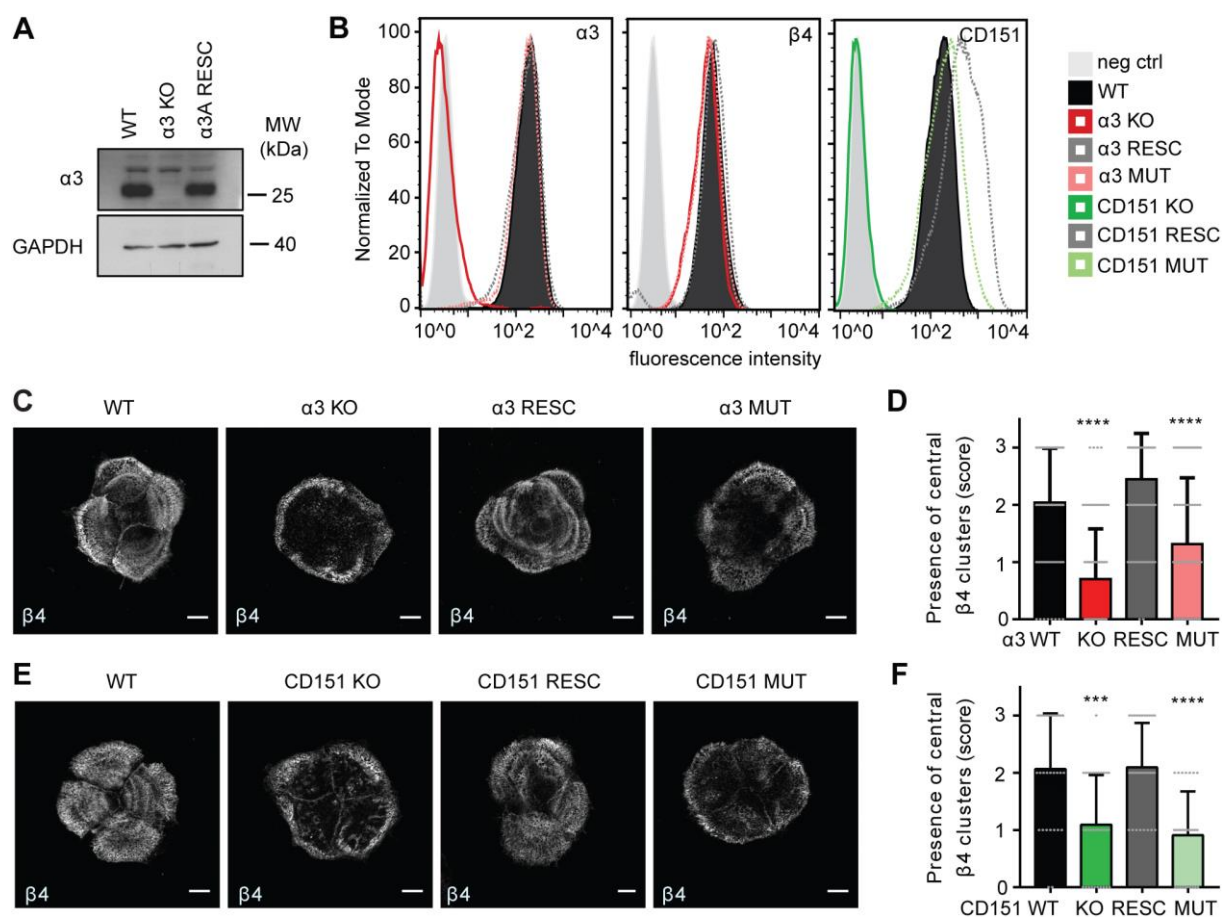
(A) WB analyses of WT or CD151-KO PA-JEB keratinocytes, reconstituted with WT- $\beta 4$  or  $\beta 4$ -GFP. Whole cell lysates were tested for  $\beta 4$ , GFP and  $\alpha$ -tubulin protein levels. (B) FACS analyses of CD151 and  $\beta 4$ -GFP surface expression on CD151-KO and WT PA-JEB/ $\beta 4$ -GFP cells. (C) Stills from a time-lapse

experiment using WT and CD151-KO PA-JEB/ $\beta$ 4-GFP cells. Top panels show the  $\beta$ 4-GFP signal and bottom panels the overlay of the  $\beta$ 4-GFP and DIC signal. Stills show images taken at 1, 4, 8, 12, 16 and 20 hours after addition of DMEM+FCS (0h time point, 20h 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 are 10  $\mu$ m. (D) Example image of PA-JEB/ $\beta$ 4-GFP after the bleach step of a FRAP experiment; FRAP regions indicated in peripheral and central adhesions with yellow circles. Scale bar is 10  $\mu$ m. (E) The relative  $\beta$ 4-GFP intensity over time (in seconds) during FRAP experiments in both central (blue) and peripheral (grey)  $\alpha$ 6 $\beta$ 4-containing adhesions. Data was collected from 38 islands in 3 experiments., lineplot shows the mean+s.d and P values are \*\*< 0.01, \*\*\*< 0.001 (Wilcoxon matched-pairs signed rank test). (F) Mobile fraction and recovery time of  $\beta$ 4-GFP in central and peripheral adhesions during the first 230 seconds of the same dataset used for E. Graph: boxplots dividing the data in quartiles and significance was calculated the same as for E.



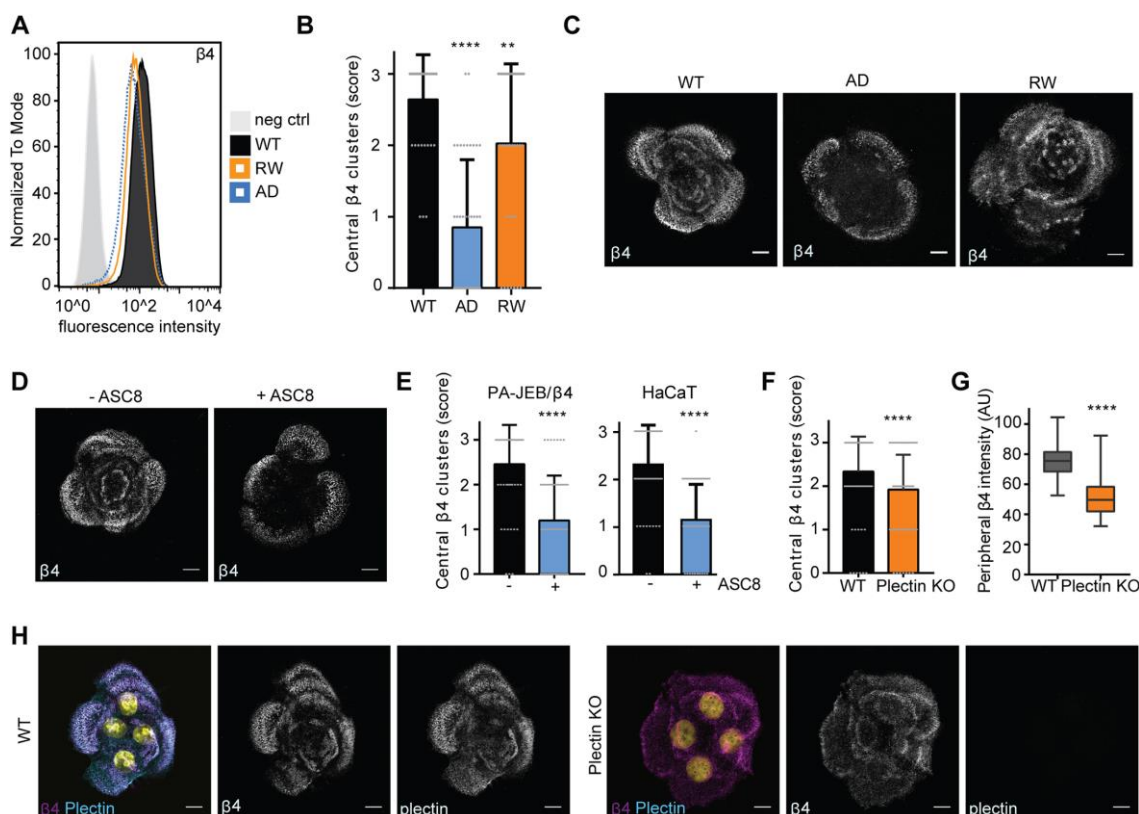
**Fig. 4. Central and peripheral adhesions differ in protein composition**

(A) TIRF images of PA-JEB/ $\beta$ 4 keratinocytes stained for  $\beta$ 4 (magenta) and laminin-332, plectin, keratin-14 or BP230 (cyan). Scale bars are 10  $\mu$ m. (B) Quantification of the  $\beta$ 4 area which is positive for laminin-332 (57 images; 2 exp.), plectin (60 images; 2 exp.), keratin-14 (59 images; 2 exp.) or BP230 (61 images; 2 exp.) in peripheral and central  $\alpha$ 6 $\beta$ 4-containing adhesions. Graph: boxplots dividing the data in quartiles; P value: \*\*\*\* $<$  0.0001 (Mann-Whitney U test). (C) TIRF images of PA-JEB/ $\beta$ 4 cells stained for  $\beta$ 4 (magenta) and CD151 or  $\alpha$ 3 (cyan). Scale bars are 10  $\mu$ m. (C+D) Line plot shows the normalized intensities of the different proteins along the line (thickness: 10 pixels) indicated in the overlay image. The line starts in the periphery and is drawn towards the center of the island. (D) TIRF images of PA-JEB/ $\beta$ 4 cells stained for  $\alpha$ 3 (magenta) and phosphorylated paxillin, BP230 or keratin (cyan). Scale bars are 10  $\mu$ m.



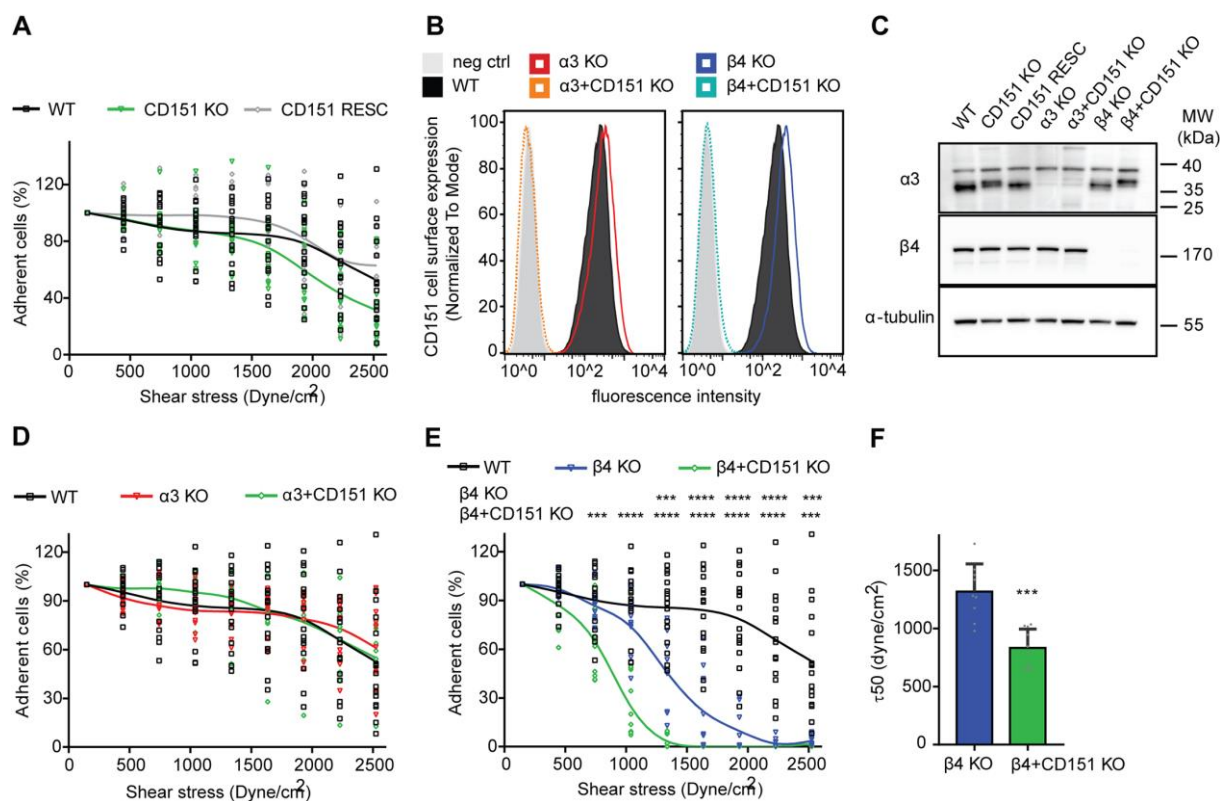
**Fig. 5.  $\alpha 3\beta 1$  and CD151-self association contribute to stabilization of central HD-like adhesions.**

(A) Whole cell lysate of WT,  $\alpha 3$ -KO and  $\alpha 3$ -RESC HaCaT keratinocytes analyzed for protein levels of  $\alpha 3$  and GAPDH (loading control) by WB. (B) FACS analyses of  $\alpha 3$ ,  $\beta 4$  or CD151 surface expression on WT,  $\alpha 3$ -KO/-RESC/-MUT and CD151-KO/-RESC/-MUT HaCaT keratinocytes. (C) Confocal images of  $\beta 4$  in WT and  $\alpha 3$ -KO/-RESC/-MUT HaCaT keratinocytes. Scale bars are 10  $\mu\text{m}$ . (D) Quantification of the presence of central  $\beta 4$  clusters (score 0-3) in WT (101 islands; 4 exp.) and  $\alpha 3$ -KO (98 islands; 4 exp.) /-RESC (103 islands; 4 exp.) /-MUT (102 islands; 4 exp.) HaCaT keratinocytes. Graph: Mean+s.d.; P value: \*\*\*\*< 0.0001 (Mann-Whitney U test) (E) Confocal images of  $\beta 4$  in WT and CD151-KO/-RESC/-MUT HaCaT cells. Scale bars are 10  $\mu\text{m}$ . (F) Quantification of the presence of central  $\beta 4$  clusters (score 0-3) in WT (31 islands; 2 exp.) and CD151-KO (33 islands; 2 exp.)/-RESC (32 islands; 2 exp.)/-MUT (33 islands; 2 exp.) cells. Graph: Mean+s.d.; P values: \*\*\*< 0.001, \*\*\*\*< 0.0001 (Mann-Whitney U test).



**Fig. 6. Laminin- but not plectin binding to  $\alpha 6\beta 4$  contributes to stabilization of central HD-like adhesions**

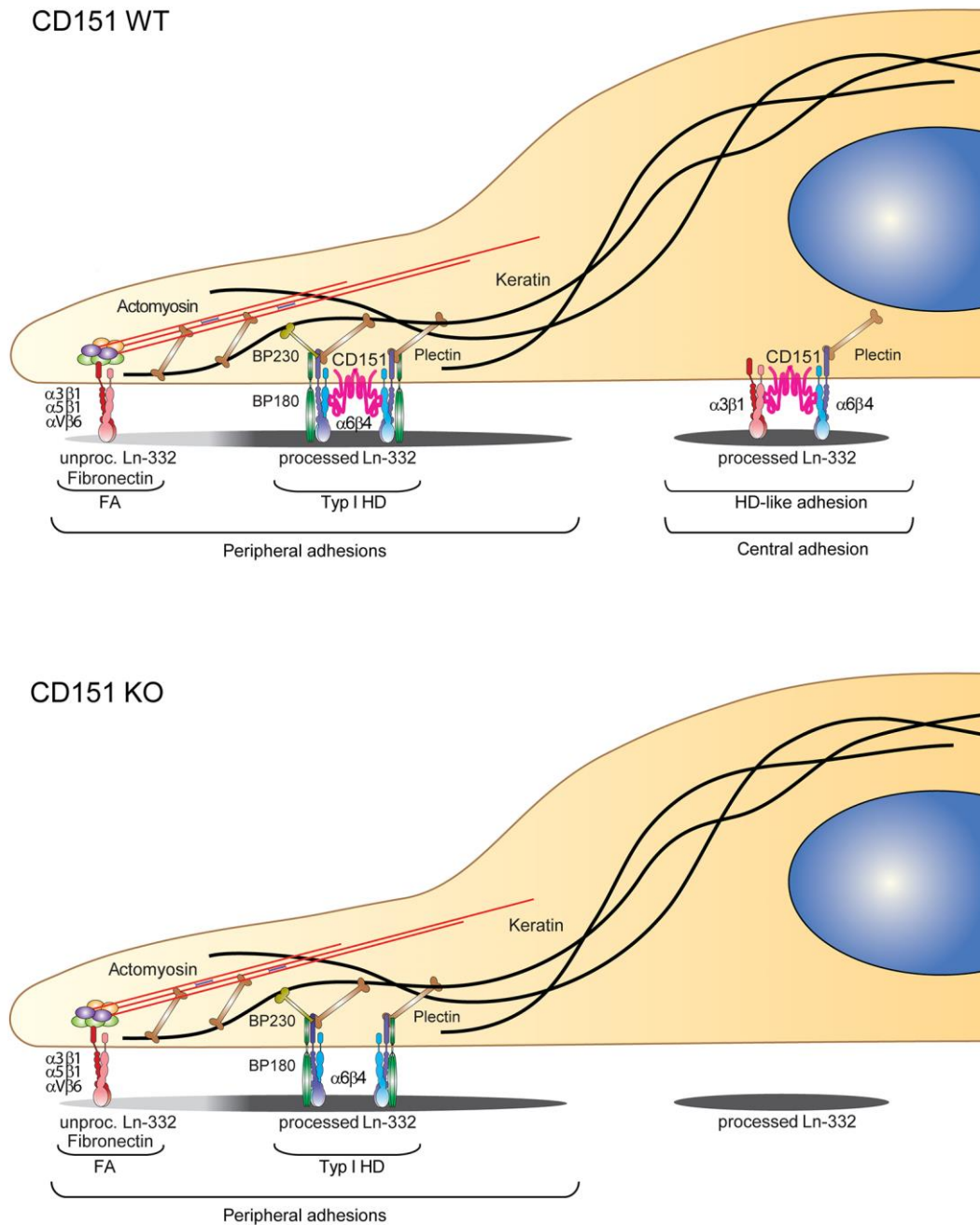
(A) FACS analyses of  $\beta 4$  surface expression on WT/ $\beta 4$ -AD/ $\beta 4$ -RW expressing PA-JEB keratinocytes. (B) Quantification of the central  $\beta 4$  clusters (score 0-3) in WT (39 images; 3 exp.),  $\beta 4$ -AD (40 images; 3 exp.) and  $\beta 4$ -RW (42 images; 3 exp.). Graph: Mean+s.d.; P values: \*\* < 0.01, \*\*\*\* < 0.0001 (Mann-Whitney U test). (C) Confocal images of  $\beta 4$  in WT and  $\beta 4$ -AD/ $\beta 4$ -RW PA-JEB/ $\beta 4$  keratinocytes. Scale bars are 10  $\mu$ m. (D) Confocal images of  $\beta 4$  in WT PA-JEB/ $\beta 4$  keratinocytes grown on coverslips for two days in the absence (-) or presence (+) of  $\beta 4$  blocking antibody ASC8. Scale bars are 10  $\mu$ m. (E) Quantification of the presence of central  $\beta 4$  clusters (score 0-3) in WT PA-JEB/ $\beta 4$  treated without (59 images; 3 exp.) or with (60 images; 3 exp.) ASC8 and HaCaT keratinocytes treated without (60 images; 3 exp.) or with (60 images; 3 exp.) ASC8. Graph: Mean+s.d.; P value: \*\*\*\* < 0.0001 (Mann-Whitney U test). (F) Quantification of the presence of central  $\beta 4$  clusters (score 0-3) in WT (110 images; 6 exp.) and plectin-KO (102 images; 6 exp.) PA-JEB/ $\beta 4$  keratinocytes. Graph: Mean+s.d.; P value: \*\*\*\* < 0.0001 (Mann-Whitney U test). (G) Quantification of the mean intensity of  $\beta 4$  clustered in the peripheral adhesions in WT (73 images; 4 exp.) and plectin-KO (71 images; 4 exp.) PA-JEB/ $\beta 4$  cells. Graph: boxplots dividing the data in quartiles; P value: \*\*\*\* < 0.0001 (Mann-Whitney U test). (H) Confocal images of  $\beta 4$  (magenta) and plectin (cyan) in WT and plectin-KO PA-JEB/ $\beta 4$  keratinocytes. Scale bars are 10  $\mu$ m.



**Fig. 7. CD151 contributes to  $\alpha3\beta1$ -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/cm<sup>2</sup>). Graph: The line depicts the mean; no significant differences (unpaired T-tests corrected for multiple comparisons). (B) CD151 surface expression of WT,  $\alpha3$ -KO,  $\beta4$ -KO and double  $\alpha3$ +CD151 and  $\beta4$ +CD151 KO HaCaT keratinocytes analyzed by FACS. (C) Expression of  $\alpha3$ ,  $\beta4$  and  $\alpha$ -tubulin (loading control) in whole cell lysates from WT, CD151-KO and CD151-RESC,  $\alpha3$ -KO,  $\beta4$ -KO and double  $\alpha3$ +CD151 and  $\beta4$ +CD151 KO HaCaT keratinocytes analyzed by WB. (D,E) Percentage of WT (squares; n=16),  $\alpha3$ -KO (triangles; n=11) and double  $\alpha3$ +CD151 KO (diamonds; n=11) HaCaT keratinocytes (D) and of WT (squares; n=16),  $\beta4$ -KO (triangles; n=9) and double  $\beta4$ +CD151 KO (diamonds; n=10) HaCaT keratinocytes (E) that remained adherent after being submitted to increasing conditions of shear stresses (in dynes/cm<sup>2</sup>). Graph: The line depicts the mean; P values: \*\*\*<0.001, \*\*\*\*< 0.0001 (unpaired T-tests corrected for multiple comparisons). (F)  $\tau50$  calculated for  $\beta4$ -KO and double  $\beta4$ +CD151 KO HaCaT cells from the same dataset shown in (E). Graph: Mean+s.d.; P value: \*\*\*< 0.001 (Mann-Whitney U test).





**Fig. 8. CD151 is essential for the maintenance of central HD-like adhesions but not the formation of peripheral FAs and type I HDs.**

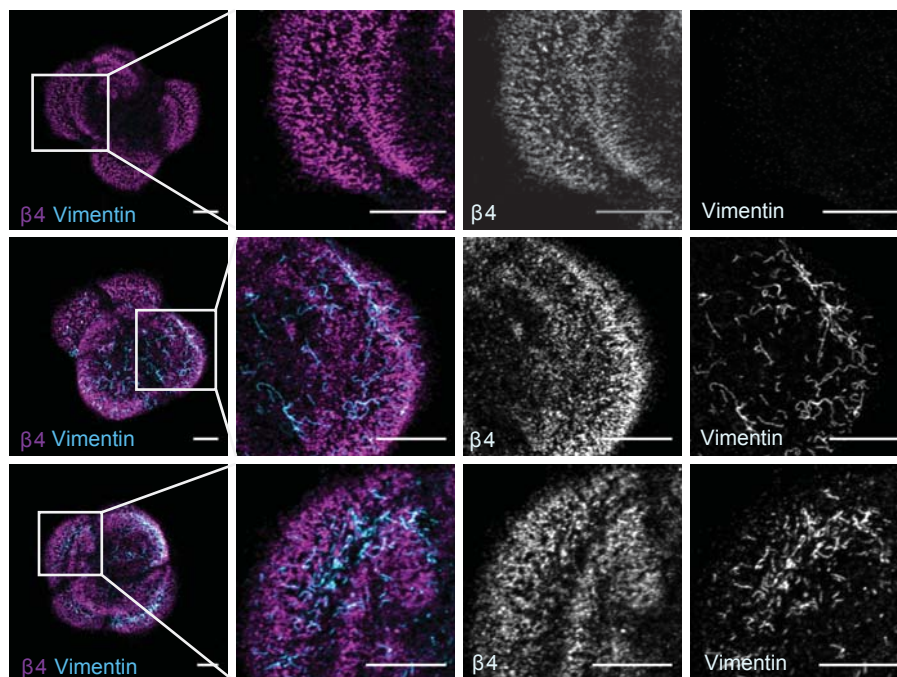
In the presence of CD151 (top panel),  $\alpha3\beta1$  and  $\alpha6\beta4$  are present together with CD151 in central HD-like adhesions. Additionally,  $\alpha3\beta1$  is present in FAs in the periphery of the cell island preceding the type I HDs in which  $\alpha6\beta4$  is present also together 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.

| Target            | Antibody                 | Application   | Source/kind gift from            |
|-------------------|--------------------------|---|----------------------------------|
| $\alpha$ -tubulin | Mouse mAb                | WB (1:10000)  | Sigma #t5168                     |
| BP230             | 5E, human mAb            | IF (1:400)  | Takashi Hashimoto                |
| CD151             | 5C11, mouse mAb          | IF (1:1), IP (1:5), WB (1:50)   | Fedor Berditchevski              |
| Coll. XVII        | VK14, mouse mAb          | IF (1:5)  | Hendri Pas                       |
| CD151             | 11G5 (IgG1), mouse mAb   | IP ( $1 \mu\text{g ml}^{-1}$ ), FACS (1:400), WB (1:2000)                                       | Jonathan Humphries               |
| CD151             | 11B1.G4 (p48), mouse mAb | WB (1:2500)   | Leonie Ashman                    |
| GAPDH             | Rabbit pAb               | WB (1:2000)   | Cell Signaling #5174             |
| GFP               | Mouse mAb                | WB (1:5000)   | Covance                          |
| Itg. $\alpha$ 3   | J143, mouse mAb          | IF (1:250), IP ( $1 \mu\text{g ml}^{-1}$ ), FACS (1:400), blocking ( $30 \mu\text{g ml}^{-1}$ ) | American Type Culture Collection |
| Itg. $\alpha$ 3   | A-3, mouse (IgG2a) mAb   | IF (1:100)  | Santa Cruz #sc-374242            |
| Itg. $\alpha$ 3A  | Rabbit pAb               | WB (1:1000)   | Homemade                         |
| Itg. $\alpha$ 6   | AA6NT, rabbit pAb        | WB (1:500)  | Anne Cress                       |
| Itg. $\alpha$ 6   | GoH3, rat mAb            | IP ( $1 \mu\text{g ml}^{-1}$ )  | Homemade                         |
| Itg. $\beta$ 1    | Rabbit pAb               | WB (1:2000)   | Reinhard Fässler                 |
| Itg. $\beta$ 4    | 439-9B, rat mAb          | IF (1:200)  | BD Bioscience #555719            |
| Itg. $\beta$ 4    | PE-Rat anti-human-CD104  | FACS (1:400)  | BD Pharmingen #555720            |
| Itg. $\beta$ 4    | ASC8, mouse, mAb         | Blocking (1:5)  | Amy Skubitz                      |
| Itg. $\beta$ 4    | Rabbit pAb               | WB (1:2000)   | Homemade                         |
| Keratin 14        | Rabbit pAb               | IF (1:1000)   | Covance #PRB-155P                |
| Laminin-332       | R14, rabbit pAb          | IF (1:500)  | Monique Aumailley                |
| pPaxillin Y31     | Rabbit pAb               | IF (1:200)  | Biosource #44-720G               |
| Plectin           | P2, guinea pig pAb       | IF (1:400)  | Harald Herrmann                  |

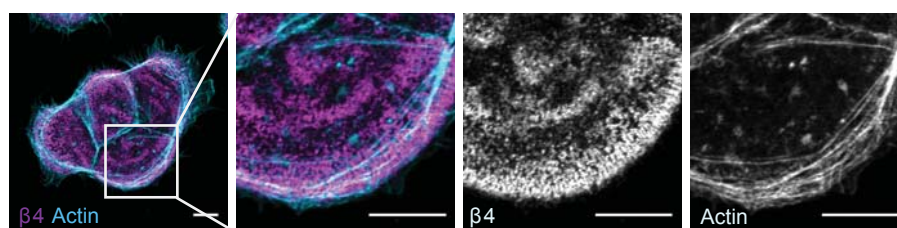
**Table S1. Primary antibodies used in various techniques**

Abbreviations used: Immune fluorescence (IF), Immune precipitation (IP), fluorescence-activated cell sorting (FACS) and Western blot (WB).

**A**

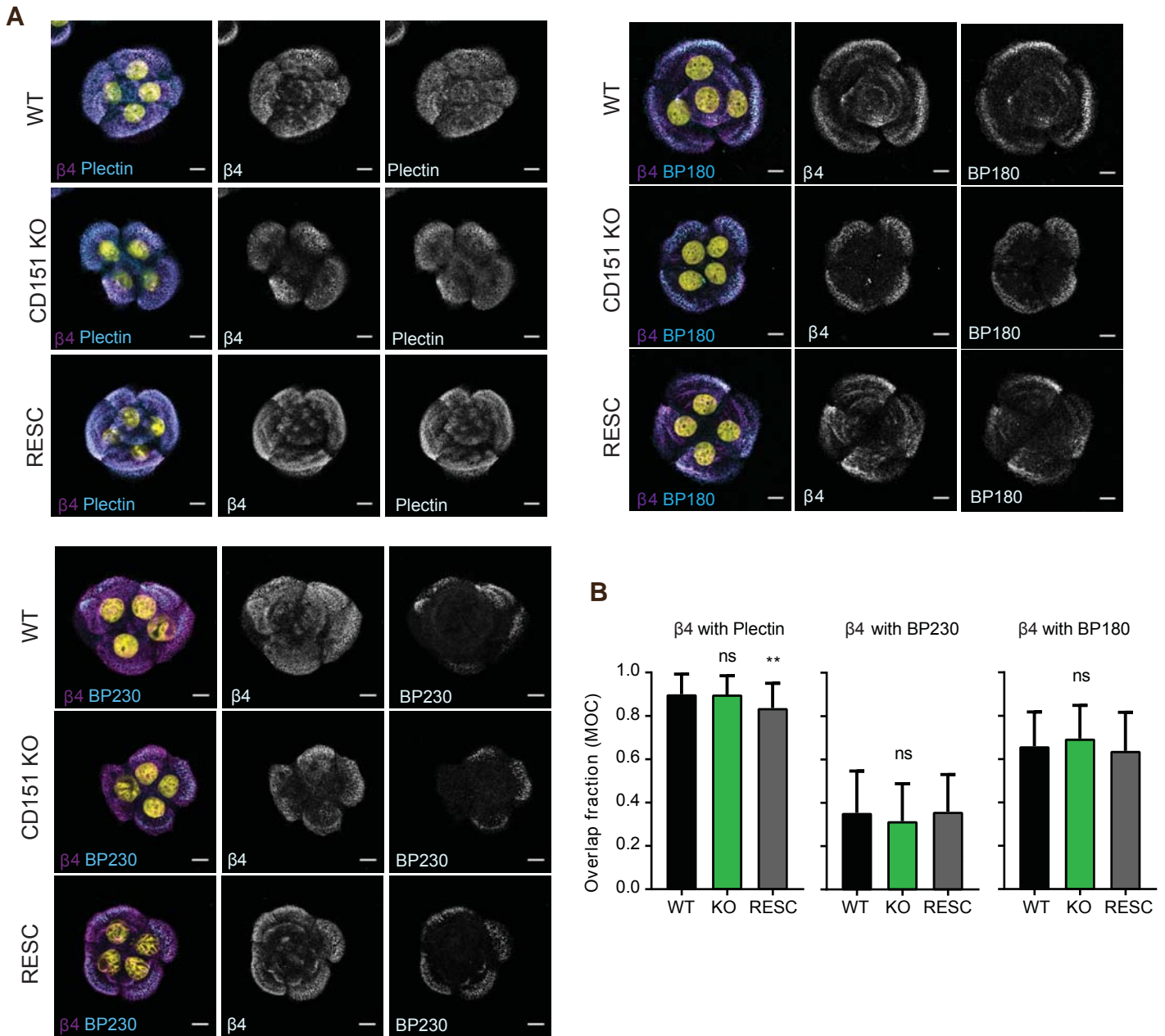


**B**



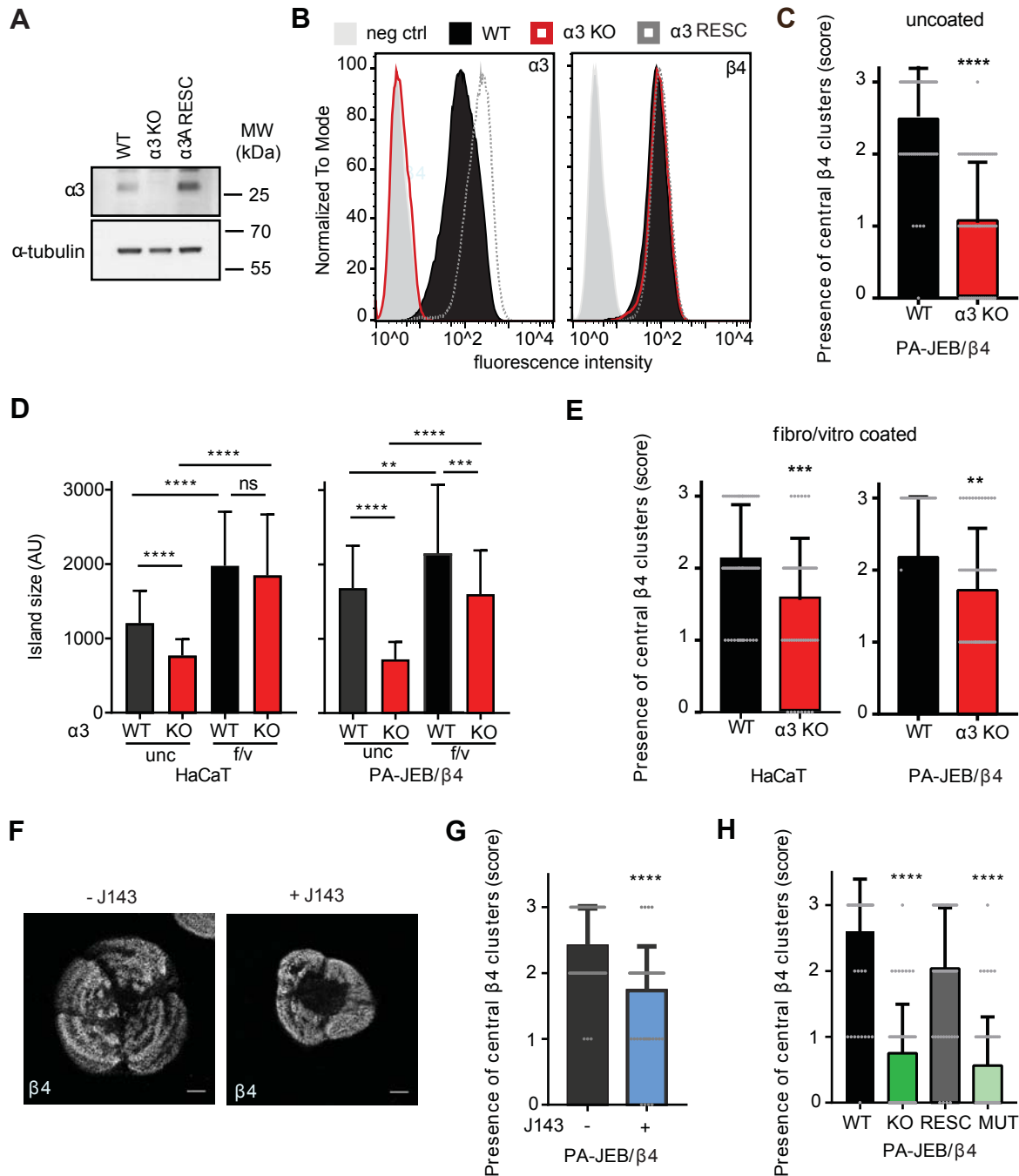
**Fig. S1  $\alpha 6\beta 4$  in central HD-like adhesions is not associated with actin or vimentin.**

Example overview and zoom-in confocal images of PA-JEB/ $\beta 4$  keratinocytes stained for  $\beta 4$  (magenta) and vimentin (A) or actin (B) (cyan). Top panel of A represents the ~ 50% of PA-JEB/ $\beta 4$  keratinocyt cell islands in which vimentin is absent. Scale bars are 10  $\mu$ m.



**Fig. S2 Type I HDs are formed in the absence of CD151.**

(A) Confocal images of WT, CD151-KO and CD151-RESC PA-JEB/ $\beta$ 4 keratinocytes stained for plectin, BP180 or BP230 (cyan),  $\beta$ 4 (magenta) and DAPI (yellow). Scale bars are 10  $\mu$ m. B. Quantification of the colocalization (MOC) of  $\beta$ 4 with BP180 (WT: 52 images, KO: 47 images, RESC: 48 images; 2 exp. each), BP230 (WT: 97 images, KO: 90 images, RESC: 89 images; 5 exp. each) and plectin (WT: 46 images, KO: 42 images, RESC: 41 images; 3 exp. each) in WT, CD151-KO and CD151-RESC PA-JEB/ $\beta$ 4 keratinocytes. Graphs show the mean+s.d.; P value: \*\*< 0.01 (Mann-Whitney U test), ns>0.05.



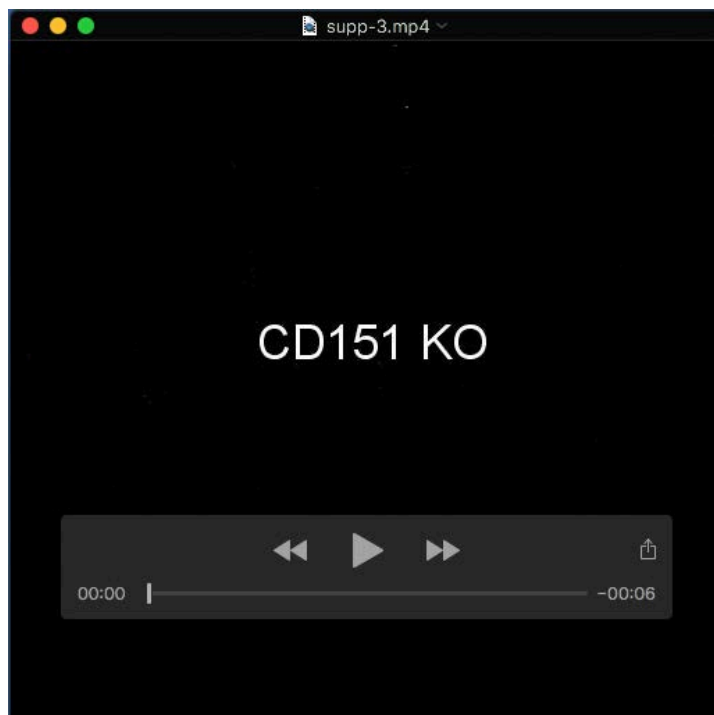
**Fig. S3 Confirmation of  $\alpha 3\beta 1$  involvement in the maintenance of central HD-like adhesions.**

(A) Whole cell lysate of WT,  $\alpha 3$ -KO and  $\alpha 3$ -RESC PA-JEB/ $\beta 4$  keratinocytes analyzed by WB for protein levels of  $\alpha 3$  and GAPDH (loading control). (B) FACS analyses of  $\alpha 3$  and  $\beta 4$  surface expression on WT,  $\alpha 3$ -KO and  $\alpha 3$ -RESC PA-JEB/ $\beta 4$  keratinocytes. (C) Quantification of the presence of central  $\beta 4$  clusters (score 0-3) in WT (61 images; 3 exp.) and  $\alpha 3$ -KO (62 images; 3 exp.) PA-JEB/ $\beta 4$  keratinocytes. (D) Island size of WT and  $\alpha 3$ -KO HaCaT and PA-JEB/ $\beta 4$  keratinocytes seeded on uncoated (unc) or fibronectin and vitronectin (f/v) coated coverslips (~60 images each; 3 exp.). (E) Quantification of the presence of central  $\beta 4$  clusters (score 0-3) in WT (60 images; 3 exp.) and  $\alpha 3$ -KO (59 images; 3 exp.) HaCaT keratinocytes, and WT (60 images; 3 exp.) and  $\alpha 3$ -KO (61 images; 3 exp.) PA-JEB/ $\beta 4$  keratinocytes seeded on fibronectin and vitronectin coated coverslips. (F) Confocal images of  $\beta 4$  in PA-JEB/ $\beta 4$  cells grown on coverslips for two days in the absence (-) or presence (+) of  $\alpha 3$  blocking antibody J143. Scale bars are 10  $\mu$ m. (G) Quantification of the presence of central  $\beta 4$  clusters (score 0-3) in WT PA-JEB/ $\beta 4$  cells grown in the absence (65 images; 3 exp.) or presence (66 images; 3 exp.) of J143. (H) Quantification of the presence of central  $\beta 4$  clusters (score 0-3) in WT (57 images; 4 exp), CD151-KO (56 images; 4 exp.), RESC (55 images; 4 exp.) and MUT (55 images; 4 exp.) PA-JEB/ $\beta 4$  keratinocytes. WT, KO and RESC data is identical to the data depicted in Fig. 2G. Graphs in C, D, E, G and H show the mean+s.d.; P values: \*\*<0.01, \*\*\*< 0.001, \*\*\*\*< 0.0001 (Mann-Whitney U test).



**Movie 1**

Movies of a time-lapse experiment using WT (movie S1) and CD151-KO (movie S2) PA-JEB/ $\beta$ 4-GFP keratinocytes. Images were taken every hour for 20h, starting from the addition of DMEM+FCS (0h timepoint, 20h after seeding).



**Movie 2**

Movies of a time-lapse experiment using WT (movie S1) and CD151-KO (movie S2) PA-JEB/ $\beta$ 4-GFP keratinocytes. Images were taken every hour for 20h, starting from the addition of DMEM+FCS (0h timepoint, 20h after seeding).