Reduced susceptibility to two-stage skin carcinogenesis in mice with epidermis-specific deletion of *Cd151*

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Abstract

Altered expression of the tetraspanin CD151 is associated with skin tumorigenesis; however, whether CD151 is causally involved in the tumorigenic process is not known. To evaluate its role in tumor formation, we subjected epidermis-specific *Cd151* knockout mice to chemical skin carcinogenesis. Mice lacking epidermal *Cd151* developed fewer and smaller tumors than wild-type mice following DMBA/TPA treatment. Furthermore, *Cd151*-null epidermis showed a reduced hyperproliferative response to short-term treatment with TPA compared to that of wild-type skin, while epidermal turnover was increased. Tumors were formed in equal numbers following DMBA only treatment. We suggest that DMBA-initiated keratinocytes lacking *Cd151* leave their niches in the epidermis and hair follicles in response to TPA treatment and subsequently are lost by differentiation. Because genetic ablation of *Itga3* also reduced skin tumor formation, we tested whether reduced expression of α3 could further suppress tumor formation in epidermis-specific *Cd151* knockout mice. Although the response to DMBA/TPA-induced formation of skin tumors was similar in compound heterozygotes for *Cd151* and *Itga3* to that in wild-type mice, heterozygosity for *Itga3* on a *Cd151*-null background diminished tumorigenesis suggesting genetic interaction between the two genes. We thus identify CD151 as a critical factor in TPA-dependent skin carcinogenesis.

Keywords

integrin; tetraspanin; skin carcinogenesis; DMBA; TPA

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Introduction

The tetraspanin CD151 is highly expressed in a variety of cell types in which it primarily associates with the laminin-binding integrins α3β1 and α6β4 (Kazarov *et al.*, 2002;Sincock *et al.*, 1997;Sterk *et al.*, 2002). Patients carrying a nonsense mutation in *CD151* display skin blistering of the pre-tibia and kidney dysfunction, defects which partially are recapitulated in patients with mutations in *ITGA3*, *ITGA6*, and *ITGB4* encoding the integrin subunits α3, α6, and β4, respectively (Has *et al.*, 2012; Karamatic-Crew *et al.*, 2004; Nicolaou *et al.*, 2012; Ruzzi *et al.*, 1997; Vidal *et al.*, 1995). Mice carrying null mutations for the corresponding genes show phenotypes similar to those of human patients (Sachs *et al.*, 2006; Wright *et al.*, 2004; Kreidberg *et al.*, 1996; van der Neut *et al.*, 1996; Georges-Labouesse *et al.*, 1996).

Although being a component of hemidesmosomes (HDs, stable adhesion plaques anchoring basal keratinocytes to the underlying basement membrane) (Sterk *et al.*, 2000), the absence of CD151 does not cause the severe form of epidermolysis bullosa observed when the hemidesmosomal integrin α6β4 is deleted. Instead, the mild skin blistering phenotype resembles that of mice with an epidermis-specific deletion of *Itga3*, which develop minor skin defects soon after birth (Dipersio *et al.*, 1997; Has *et al.*, 2012; Margadant *et al.*, 2009). Furthermore, a role of CD151 and α3β1 has been suggested in cell migration during wound healing (Cowin *et al.*, 2006; Geary *et al.*, 2008; Margadant *et al.*, 2009; Reynolds *et al.*, 2008; Wright *et al.*, 2004). Finally, both proteins are involved in skin tumorigenesis: loss of α3β1 decreases skin tumor formation while it increases progression of squamous cell carcinomas (SCCs) (Sachs *et al.*, 2012b), and expression of *CD151* in oral SSCs correlates with a decreased disease-free survival of patients (Romanska *et al.*, 2012). Expression of α3β1 in the suprabasal epidermis suppresses malignant conversion (Owens and Watt, 2001), whereas increased expression of CD151 in SCCs in humans is correlated with tumor aggressiveness (Suzuki *et al.*, 2011; Li *et al.*, 2012).

While this work was in progress, Li *et al*. published a study in which they used *Cd151* knockout mice to evaluate the role of CD151 in mouse skin carcinogenesis (Li *et al.*, 2012). Their results indicate that CD151 contributes to skin carcinogenesis by reducing apoptosis in DMBA-initiated cells and stimulating proliferation of keratinocytes in response to TPA treatment. However, because in this study total *Cd151* knockout mice were used, *Cd151*-deletion in tissues other than epidermis may have influenced the development and progression of tumors. Furthermore, it was suggested that CD151 controls keratinocyte proliferation, survival, and tumorigenesis through the activation of signaling pathways downstream of the integrin α6β4 (Li *et al.*, 2012). A similar mechanism has been proposed to explain why CD151 increases mammary tumorigenesis (Deng *et al.*, 2012). However, CD151 binds most strongly to the integrin α3β1 (Yauch *et al.*, 1998). We recently showed that epidermal expression of α3β1 is essential for chemically induced skin carcinogenesis by retaining slow-cycling cells in their epidermal niches allowing them to accumulate a sufficient number of mutations for inducing tumorigenesis (Sachs *et al.*, 2012b). We wondered whether epidermal expression of *CD151* influences this process through a similar mechanism. We therefore subjected epidermis-specific *Cd151* knockout mice to chemically-induced skin carcinogenesis and tested whether there is a genetic interaction between *Cd151* and *Itga3*.

Results

Reduced two-stage skin carcinogenesis in the absence of epidermal Cd151

We first subjected epidermis-specific *Cd151* knockout mice (*Cd151*^{fl/fl}; K14-Cre+ (FVB), referred to as *Cd151* eKO) and wild-type littermates (*Cd151*^{fl/fl}; K14-Cre- (FVB), referred to as wild-type) to the two-stage protocol of skin carcinogenesis. Tumors were initiated with a single dose of 7,12-dimethylbenzanthracene (DMBA) and promoted with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) twice per week (Abel *et al.*, 2009). The average tumor volume was considerably lower in *Cd151* eKO than in wild-type mice, the average number of tumors also being slightly lower (**Figure 1A-B**). Large tumors appeared later and less frequently in *Cd151* eKO mice (**Figure 1C**). Apart from their size, we observed no obvious differences in the histological structure of benign and malignant tumors (**Figure 1D**). Since the deletion of *Itga3* in the epidermis also decreases tumorigenesis (Sachs *et al.*, 2012b) and CD151 forms a stable complex with α3β1 (Yauch *et al.*, 1998), we wondered whether the complex is essential for the effects described above. We therefore decided to investigate whether there is a genetic interaction between *Cd151* and *Itga3* and subjected compound heterozygote mice (*Cd151*^{fl/+}; *Itga3*^{fl/+}; K14-Cre+, referred to as *Cd151* eHET; *Itga3* eHET) to DMBA/TPA-induced tumorigenesis. No differences were found between *Cd151* eHET; *Itga3* eHET and wild-type mice (*Cd151*^{+/+}; *Itga3*^{+/+}; K14-Cre+) with respect to the number and volume of tumors (**Figure S1**). However, we detected an additional effect of *Itga3* heterozygosity in the complete absence of *Cd151* (*Cd151*^{fl/fl}; *Itga3*^{fl/+}; K14-Cre+, referred to as *Cd151* eKO; *Itga3* eHET). Reduced tumor volume (after 18 weeks of tumor promotion) and number (after 10 weeks of tumor promotion) compared to *Cd151* eKO mice indicated a genetic interaction under these circumstances (**Figure 1B**).

Impaired proliferation of transformed keratinocytes in the absence of Cd151

To explain the difference in volume of the tumors in wild-type and *Cd151* eKO mice we examined the proliferative capacity of epidermal cells in these mice. We therefore treated their back skin with either single doses of TPA, a single dose of DMBA followed by four doses of TPA, or respective vehicle controls. As shown in **Figure 2A** these short term treatments caused epidermal thickening likely due to increased proliferation. However, the epidermis of *Cd151* eKO mice was significantly thinner due to a lower proliferation rate (**Figure 2A**). It was unlikely that DMBA-induced apoptosis contributed to this effect as very few IFE cells died 24h after a single DMBA dose and differences in the thickness of the epidermis between wild-type and *Cd151* eKO mice were not significant (**Figure 2B**). TPA induced apoptosis seems negligible and were the same in wild-type and *Cd151* eKO mice (**Figure S2**). Papillomas originating from DMBA/TPA-treated *Cd151* eKO mice showed significantly less Ki67 labeling than those in their respective wild-type littermates (**Figure 2C**). Furthermore, the proliferative rate of papillomas produced by *Cd151* eKO; *Itga3* eHET mice was even further decreased, indicating genetic interaction (**Figure 2C**). We next generated mouse keratinocytes (MK) from a newborn *Cd151*^{fl/fl} mouse, deleted *Cd151*, rescued expression with either CD151[WT] or CD151[QRD*] (the latter being incapable of binding α3β1 (Kazarov *et al.*, 2002)) (**Figure S3**) and determined their proliferative rates.

Figure 2D shows that CD151, but not its integrin-binding function, is required for efficient proliferation of untransformed keratinocytes *in vitro*.

Label-retaining cells lacking CD151 exit their niche possibly due to increased migration

Long-lived, slow-cycling label-retaining cells (LRCs) in hair follicles (HFs) and the interfollicular epidermis (IFE) are thought to be the primary source of chemically induced skin tumors (Morris *et al.*, 1986). DMBA-initiated cells persist and can be efficiently promoted to tumors even after extended periods of time (Berenblum and Shubik, 1949; Stenback *et al.*, 1981). Furthermore, HFs of adult *Itga3* eKO mice contain fewer LRCs than wild-type ones (Sachs *et al.*, 2012b). We therefore quantified the number of LRCs in the HFs of *Cd151* eKO mice and wild-type littermates 8 weeks after 6 BrdU pulses given between 5 and 7 days after birth. As expected, *Cd151* eKO HFs contained significantly fewer BrdU positive LRCs than wild-type HFs (**Figure 3A**). Additionally, the HF bulge marker Keratin 15 was not limited to HF keratinocytes (wild-type situation), but was expressed in many keratinocytes in the infundibulum and the IFE of *Cd151* eKO mice (**Figure 3B**). To test whether these observations are correlated with an increased epidermal turnover, we fluorescently labeled the cornified layer of wild-type and *Cd151* eKO mice with dansyl chloride and quantified the remaining fluorescence after 4 days of daily treatments with TPA. Interestingly, the rate of dansyl chloride clearance was almost twice as fast in *Cd151* eKO mice as in wild-type mice (**Figure 3C**). Furthermore, short term TPA-exposure leads to a significant increase in Keratin 15-positive keratinocytes in the suprabasal layer of the *Cd151*-null epidermis (**Figure 3D**).

Loss of Cd151 mildly decreases tumor progression

To investigate whether the observed tumor phenotype was dependent on the action of TPA, we subjected wild-type and *Cd151* eKO (FVB) mice to the complete carcinogenesis protocol of weekly DMBA applications. Under these conditions, both mouse strains developed a similar number of SCCs (**Figure 4A-B**). Furthermore, histological analysis showed that the grades of differentiation of SCCs in *Cd151* eKO and wild-type mice were similar, although there was a slight tendency of SCCs to be more poorly differentiated in wild-type mice (**Figure 4C-D, S4**).

In summary, our findings indicate a strong requirement for CD151 in skin tumor initiation and growth, whereas its influence on SCC differentiation status is weak.

Discussion

In this study we subjected mice lacking *Cd151* in the epidermis to chemically induced skin carcinogenesis and we show that efficient tumor formation and growth depends on epidermal expression of this tetraspanin. Consistent with a recent report (Li *et al.*, 2012), we found that *Cd151* eKO mice are less susceptible to two-stage skin carcinogenesis as shown by the number and size of the tumors formed. Especially the development of large tumors following DMBA/TPA treatment depends on CD151. Importantly, tumor growth is dependent on a sufficient blood supply through angiogenesis (Folkman, 1974), which might be directly affected in total *Cd151*-knockout mice (Wright *et al.*, 2004; Zhang *et al.*, 2011;

Takeda *et al.*, 2007). By using *Cd151* eKO mice we circumvented possible indirect effects of the *Cd151*-deficient vasculature on skin-tumorigenesis.

In agreement with the smaller size of papillomas in *Cd151* eKO mice, we observed that the numbers of Ki67^{+} nuclei in these mice are decreased, indicative of an impaired proliferative capacity. CD151 has indeed been shown to increase the proliferation of (transformed) cells by enhancing several signaling pathways including those activated by EGF, TGFβ and HGF (Franco *et al.*, 2010; Sadej *et al.*, 2010; Li *et al.*, 2012). In line with the proposed role of CD151 in modulating the function of the integrin α3β1 (Nishiuchi *et al.*, 2005), we observed a similar phenotype in *Itga3* eKO mice (Sachs *et al.*, 2012b). To prove shared functionality of the two proteins, we generated compound heterozygotes and subjected them to the two-stage carcinogenesis protocol. However, tumorigenesis is as efficient in these mice as in wild-type littermates and single heterozygotes. Apparently, a reduction of the two proteins by 50% is not enough to impair skin carcinogenesis possibly due to the still effective formation of functional α3β1-CD151 complexes (Yauch *et al.*, 1998). However, deletion of one *Itga3*-allele in *Cd151* eKO mice shows that there is genetic interaction between *Itga3* and *Cd151* with respect to tumor size and proliferation in transformed keratinocytes. In contrast to the expression of α3β1, expression of CD151 in epidermal keratinocytes renders these cells responsive to TPA-induced proliferation. Consistent with this observation, CD151 confers a proliferative advantage over untransformed keratinocytes *in vitro* also when not bound to integrins. Proliferation of untransformed keratinocytes therefore is independent of CD151-α3β1 complexes, whereas for proliferation of transformed keratinocytes both proteins are needed. These experiments also explain why normal keratinocytes proliferate equally well with or without α3β1 (Margadant *et al.*, 2009).

The number of DMBA/TPA-induced tumors is decreased mildly in the absence of epidermal CD151 as compared to that in wild-type mice. Loss of *Cd151* has recently been shown to increase apoptosis in response to DMBA (Li *et al.*, 2012). Even though we failed to reproduce the statistical significance of these results, we cannot exclude that fewer DMBA-initiated cells survive in the absence of *Cd151*. In fact, we did observe a trend for higher DMBA-sensitivity in the absence of *Cd151*, similar to that seen in the absence of α3β1 (Sachs *et al.*, 2012b). Given the very low number of apoptotic cells following DMBA exposure we focused on the fate of slow-cycling LRCs as the proposed cells from which tumors are formed (Berenblum and Shubik, 1949; Morris *et al.*, 1986; Stenback *et al.*, 1981). We found a strong association between the decrease in the number of tumors and the absence of slow-cycling LRCs in the HFs of the *Cd151* eKO mice. Interestingly, a similar association was observed in mice lacking epidermal α3β1 (Sachs *et al.*, 2012b). Deletion of one *Itga3*-allele in *Cd151* eKO mice further decreases the number of tumors. However, epidermal turnover is only increased in *Cd151* eKO mice as compared to that in wild-type mice following exposure to TPA. Whereas this increased turnover in *Cd151* eKO mice is dependent on the treatment with TPA, it is not in *Itga3* eKO mice (Sachs *et al.*, 2012b). Furthermore, because the proliferation of epidermal keratinocytes is decreased in *Cd151* eKO mice, it is likely that their differentiation is increased.

Functionally, CD151 forms tight complexes with α3β1 (Yauch *et al.*, 1998) that increase cell adhesion and decrease cell migration (Sachs *et al.*, 2012a; Chometon *et al.*, 2006).

Deletion of *Cd151* delays epidermal re-epithelialization and keratinocyte migration following skin wounding (Cowin *et al.*, 2006; Geary *et al.*, 2008) and a monoclonal antibody against CD151 immobilizes tumor cells *in vivo* (Zijlstra *et al.*, 2008). In keratinocytes, the integrin $\alpha6\beta4$-based HDs render $\alpha3\beta1$-CD151 adhesions less important. Disassembly of HDs through TPA-mediated phosphorylation of $\beta4$ causes decreased keratinocyte adhesion and increased migration (Frijns *et al.*, 2010). The simultaneous weakening of two main keratinocyte adhesion structures (TPA treatment increases HD dynamics, deletion of *Cd151* weakens $\alpha3\beta1$-mediated cell adhesion) may thus result in a similar phenotype as produced by deletion of *Itga3*, namely increased epidermal turnover and fewer tumors. The TPA dependence of suppressing tumorigenesis in the *Cd151* eKO mice is apparent in the DMBA only model of complete carcinogenesis. In contrast to *Itga3* eKO mice (Sachs *et al.*, 2012b), *Cd151* eKO mice develop the same number of SCCs as wild-type littermates. SCCs lacking CD151 show a higher degree of differentiation which is consistent with the strongly positive effect of CD151 on proliferation and its correlation with SCC-aggressiveness in men (Suzuki *et al.*, 2011). Together, our studies identify CD151 as an essential factor in chemically induced skin carcinogenesis, and show that it supports tumorigenesis through mechanisms that are both dependent and independent of its association with the integrin $\alpha3\beta1$.

Materials and Methods

Animal experiments

According to Mouse Genome Informatics (The Jackson Laboratory, Bar Harbor, ME, USA) the names of *Itga3* eKO and *Cd151* eKO mice are *Itga3*[tm1Son/tm1Son]; *Krt14*[tm1(cre)Wbm] on FVB(N6), and *Cd151*[tm2Son/tm2Son]; *Krt14*[tm1(cre)Wbm] on FVB(N10), respectively (Huelsken *et al.*, 2001;Sachs *et al.*, 2006;Sachs *et al.*, 2012a). Compound heterozygotes were produced by crossing the above mice mentioned above. For DMBA/TPA-induced carcinogenesis, the backs of 7-week-old mice were shaved and treated with a single dose of DMBA (30 μg in 200 μl acetone, Sigma) followed by biweekly applications of TPA (12.34 μg in 200 μl acetone, Sigma) for 20 weeks. For DMBA only carcinogenesis, the backs of 7-week-old mice were shaved and treated with weekly doses of DMBA (30 μg in 200 μl acetone) for up to 25 weeks. Number and size of arising tumors were measured weekly. For short-term treatments, mice were treated with a daily dose of 12.34 μg TPA (1 day, Figure 2A; 2 or 4 days, Figure 3C-D) and sacrificed 24 h later, with a single dose of 30 μg DMBA and sacrificed 24 h later (Figure 2B), or with a single dose of 30 μg DMBA followed by 4 semiweekly doses of 12.34 μg TPA and sacrificed 3 days later (Figure 2A, Figure S2A). For LRC tracing, mice were injected i.p. with 6×50 μg BrdU every 12h from day 3 (Cotsarelis *et al.*, 1990) and chased for 14 days. We dissected four ˜1 cm long skin strips per mouse and counted BrdU+ and Brdu- cells of the bulges of HFs whose dermal papilla and isthmus were present in the histological sections (at least 25 per mouse). All animal studies were performed according to Dutch guidelines for care and use of laboratory animals and were approved by the animal welfare committee of the Netherlands Cancer Institute.

Histology

Tissues were excised, fixed for one day in formaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Images were taken with PL APO objectives (10×/ 0.25 NA, 40×/0.95 NA, and 63×/1.4 NA oil) on an Axiovert S100/AxioCam HR color system using AxioVision 4 software (Carl Zeiss MicroImaging, Inc., Oberkochen, Germany) or with a 20×/0.75 NA PL APO objective ± a 2× optical mag changer on a ScanScope XT system using ImageScope v10 software (Aperio Technologies, Inc., Vista, CA). Tumor classification and grading were performed blindly by a mouse pathologist according to the degree of differentiation of the tumor cells, mitotic activities of the cells, organization and demarcation of the tumor, necrosis, hemorrhages, and stromal reaction.

Immunohistochemistry and Immunofluorescence

Skin was excised and embedded in cryoprotectant (Tissue-Tek O.C.T.). Cryosections were prepared, fixed in ice-cold acetone and blocked with 2% BSA in PBS. To prepare epidermal whole mounts, tail skin was cut into 0.5 cm wide pieces and incubated in 5 mM EDTA in PBS at 37°C for 4 h. An intact sheet of epidermis was gently peeled away from the dermis and fixed in 4% paraformaldehyde in PBS for 2 h at room temperature. Fixed epidermal sheets were permeabilized and blocked in PB buffer (20 mM HEPES buffer pH 7.2 containing 0.5% TritonX-100, 0.5% skim milk powder, and 0.25% fish skin gelatin) and incubated with 2 M HCl at 37°C for 25 min when indicated (anti-BrdU stainings) . Tissues were incubated with the indicated primary antibodies in 2% BSA in PBS (whole mounts in PB buffer) for 60 min (whole mounts o/n), followed by incubation with secondary antibodies diluted 1:200 for 60 min (o/n). The following antibodies were used: mouse anti-BrdU mAb (MO744, DakoCytomation), mouse anti-human CD151 (11B1.G4) (Ashman et al., 1997), rabbit anti-cleaved caspase3 (9661L, Cell Signaling), rabbit anti-mouse CD151 (Sachs et al., 2006), mouse anti-FLAG (M2, Sigma), rabbit anti-FLAG (Sc-807, Santa-Cruz), mouse anti-mouse integrin ⍺3 (29A3) (de Melker et al., 1997), rat anti-mouse integrin ⍺4 (346-11A, BD Pharmingen), mouse anti-mouse Ki67 (PSX1028, Monosan), and mouse anti-mouse Keratin 15 (MA1-90929, Thermo scientific). Samples were analyzed at 37°C using a 63×/1.4 HCX PL APO CS oil objective on a TCS SP2 AOBS confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). Images were acquired using LCS 2.61 (Leica Microsystems GmbH, Wetzlar, Germany) and processed using Adobe Photoshop CS4 or ImageJ.

Cell lines

MK $Cd151^{fl/fl}$ were generated from neonatal $Cd151^{tm2Son/tm2Son}$ mice as described (Margadant *et al.*, 2009) and grown in keratinocyte serum-free medium (Gibco) supplemented with 50 μg/ml bovine pituitary extract, 5 ng/ml EGF, 100 U/ml penicillin and 100 U/ml streptomycin. Adeno-Cre obtained from F. Graham (Anton and Graham, 1995) was used to delete $Cd151$ and generate MK $Cd151^{-/-}$. Retroviral expression constructs carrying wild-type and 194QRD-INF196 CD151 from M. Hemler (Kazarov *et al.*, 2002) were used to rescue expression of CD151 in MK $Cd151^{-/-}$. Cells were seeded at 5000 cells per well of a standard 12-well plate and counted daily in duplicate to measure proliferation.

Immunoblotting and immunoprecipitations

For biochemical assays, cells were lysed in 1% (vol/vol) Nonidet P-40, 20 mM Tris-HCl, pH 7.6, 4 mM EDTA, 100 mM NaCl, supplemented with a cocktail of protease inhibitors (P8340, Sigma). Lysates were cleared by centrifugation for 20 min at $20,000 \times g$ and 4°C followed by separation of proteins on 4-12% polyacrylamide gels under nonreducing conditions (NuPage) and transferred to Immobilon PVDF membranes (EMD Millipore). For immunoprecipitations, lysates were incubated overnight with mAb 29A3 coupled to gamma-bind sepharose (GE Healthcare) or mAb M2-coupled agarose (A2220, Sigma). Beads were spun down at $500 \times g$, washed with lysis buffer and PBS and processed by SDS-PAGE, as above. After western blotting, membranes were blocked and blots were developed with the indicated antibodies using an ECL detection kit (GE Healthcare) according to the manufacturer's protocol.

FACS

Cells were trypsinized washed with 2% FCS in PBS and stained with primary antibodies as indicated for 60 min on ice. Following washing, secondary anti-goat, -rat, and -mouse antibodies coupled to FITC were used 1:200 for 60 min on ice. Cells were strained and analyzed on a 1998 BD FACSCalibur (Becton, Dickinson and Company, Franklin Lakes, NJ) using a 488nm laser and a 530/30 FL1 filter configuration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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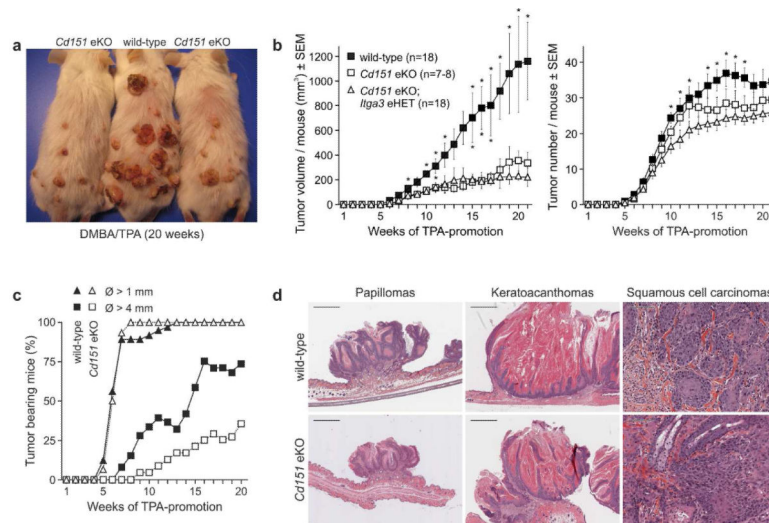**Figure 1. Impaired tumor formation in *Cd151* eKO mice following DMBA/TPA carcinogenesis**
(A) Tumor burden of wild-type and *Cd151* eKO littermates 20 weeks into the DMBA/TPA protocol. (B) Tumor volume and number are diminished in *Cd151* eKO mice compared to that in wild-type littermates after DMBA/TPA induced skin carcinogenesis. Both parameters are further reduced in the absence of one *Itga3* allele. (* on top of the wild-type group represents $P < 0.05$ compared to *Cd151* eKO; *Itga3* eHET group; * below the wild-type group represents $P < 0.05$ compared to *Cd151* eKO group as determined by one-way ANOVA, Bonferroni) (C) The incidence of tumors with a diameter of at least 1 mm is equal in the two groups. However, tumors wider than 4 mm occur less often and considerably later in *Cd151* eKO mice than in wild-type littermates. (D) Papillomas and keratoacanthomas of wild-type and *Cd151* eKO mice differ in size but not in structure. Moderately differentiated SCCs are regularly found in both groups (scale bars equal 100 μm).
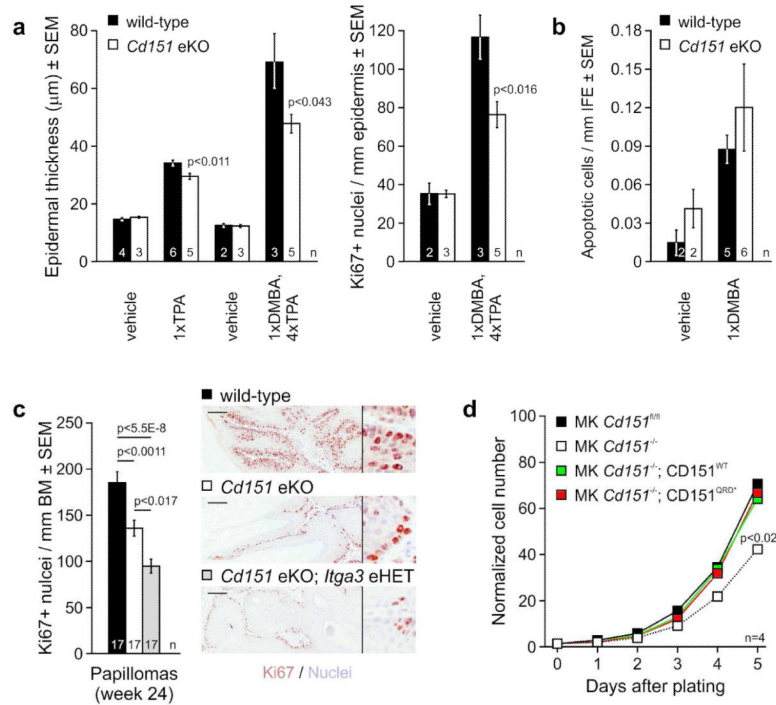
Figure 2. Decreased proliferation of (transformed) keratinocytes lacking *Cd151*

(A) Single and multiple doses of TPA applied to *Cd151* eKO back skin result in significantly decreased hyperproliferation compared to that of skin of wild-type littermates. (B) The number of apoptotic cells in the IFE of wild-type and *Cd151* eKO mice does not differ significantly after a single dose of DMBA as assessed by cleaved caspase-3 stainings. (C) *Cd151* eKO papillomas of the DMBA/TPA protocol contain significantly less proliferating cells than wild-type papillomas, as indicated by Ki67 immunohistochemistry. *Cd151* eKO; *Itga3* eHET papillomas display an even further reduction in proliferation (scale bars equal 100 μm, 5× insets). (D) *In vitro*, untransformed *Cd151*$^{-/-}$ mouse keratinocytes proliferate significantly less strongly than cells from the parental *Cd151*$^{fl/fl}$ MK line. The proliferation defect is rescued by the expression of the wild-type, but also the integrin binding mutant CD151 (see Supplemental Fig. 2 for characterization of these cells).
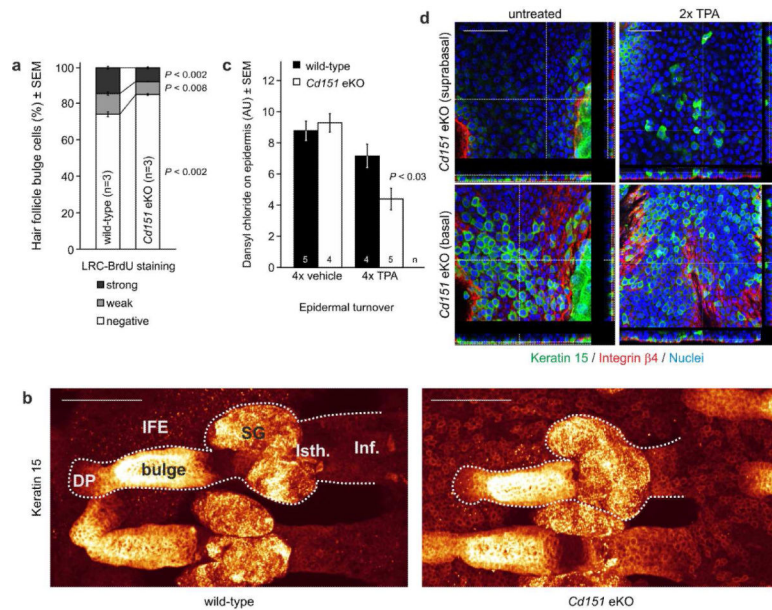
Figure 3. Loss of label-retaining cells lacking *Cd151*
(A) The number of BrdU-LRCs is significantly reduced in the back skin HFs of 8 week old *Cd151* eKO mice compared to that in HFs of wild-type littermates. (B) Krt15+ keratinocytes are confined to the HFs of wild-type mouse tails, but present in HFs and IFE of *Cd151* eKO mouse tails (dotted lines outline HF, DP, dermal papilla, SG, sebaceous gland [stained aspecifically], Isth., isthmus, Inf., infundibulum, IFE, interfollicular epidermis, scale bars equal 100 μm). (C) TPA-dependent increased epidermal turnover in *Cd151* eKO back skin is shown by accelerated loss of dansyl chloride from the epidermis after 4 days of daily TPA treatments. (D) Krt15+ keratinocytes are restricted to the basal IFE of *Cd151* eKO mouse tails (bottom row), but regularly found suprabasally after 2 days of daily TPA applications (top row). Displayed are XY projections (large image) as well as XZ and YZ projections along the indicated white lines (narrow images below and to the right of XY images). Scale bars equal 50 μm.
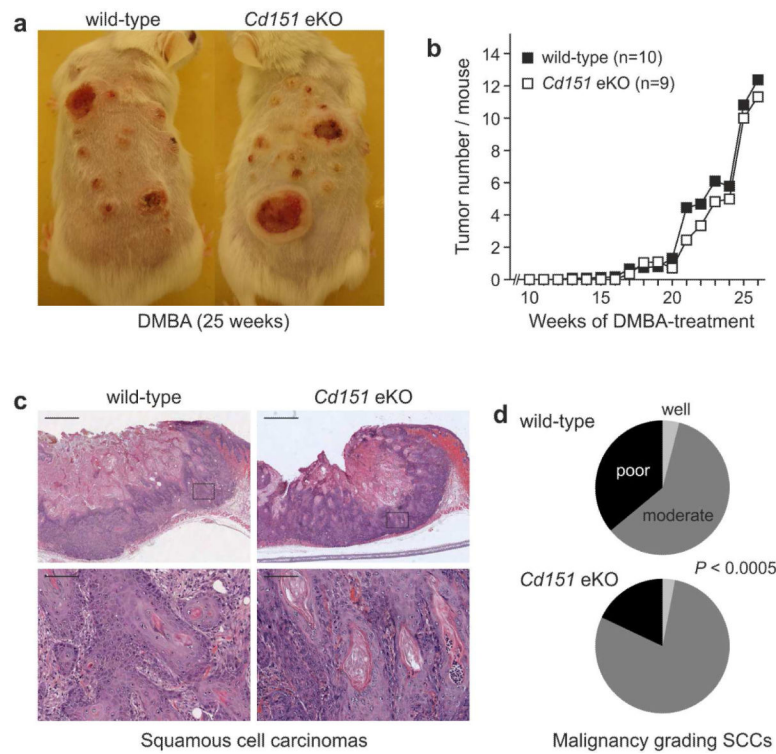
Figure 4. Complete carcinogenesis in wild-type and *Cd151* eKO mice

(A) Macroscopic image of two littermates following a 25-wk regimen of DMBA-only carcinogenesis with (B) corresponding quantification of the entire cohort. *Cd151* eKO and wild-type mice develop the same number of tumors. (C) Representative histological examples of SCCs found in wild-type and Cd151 eKO mice after complete carcinogenesis (scale bars equal 1 mm (overview) and 100 m (detail)). (D) Pie chart of SCC differentiation showing mild increase of poorly differentiated SCCs in the wild-type group (P < 0.0005; χ2 test) (see **Figure S4** for total tumor numbers).