Reduced Susceptibility to Two-Stage Skin Carcinogenesis in Mice with Epidermis-Specific Deletion of *Cd151*

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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 after treatment with 7,12-dimethylbenzanthracene (DMBA)/12-*O*-tetradecanoylphorbol-13-acetate (TPA). Furthermore, *Cd151*-null epidermis showed a reduced hyperproliferative response to short-term treatment with TPA as compared with wild-type skin, whereas epidermal turnover was increased. Tumors were formed in equal numbers after 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 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.

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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 (Sincock *et al.*, 1997; Kazarov *et al.*, 2002; Sterk *et al.*, 2002). Patients carrying a nonsense mutation in *CD151* display skin blistering of the pretibia and kidney dysfunction, defects that are partially recapitulated in patients with mutations in *ITGA3*, *ITGA6*, and *ITGB4* encoding the integrin subunits α3, α6, and β4, respectively (Vidal *et al.*, 1995; Ruzzi *et al.*, 1997; Karamatic-Crew *et al.*, 2004; Has *et al.*, 2012; Nicolaou *et al.*, 2012). Mice carrying null mutations for the corresponding genes show phenotypes similar to those of human patients (Georges-Labouesse *et al.*, 1996; Kreidberg *et al.*, 1996; van der Neut *et al.*, 1996; Wright *et al.*, 2004; Sachs *et al.*, 2006).

Although being a component of hemidesmosomes (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; Margadant *et al.*, 2009; Has *et al.*, 2012). Furthermore, a role of CD151 and α3β1 has been suggested in cell migration during wound healing (Wright *et al.*, 2004; Cowin *et al.*, 2006; Geary *et al.*, 2008; Reynolds *et al.*, 2008; Margadant *et al.*, 2009). Finally, both proteins are involved in skin tumorigenesis: loss of α3β1 decreases skin tumor formation, whereas it increases progression of squamous cell carcinomas (SCCs) (Sachs *et al.*, 2012b), and expression of *CD151* in oral SCCs 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).

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Abbreviations: DMBA, 7,12-dimethylbenzanthracene; HF, hair follicle; LRC, label-retaining cell; MK, mouse keratinocyte; PBS, phosphate-buffered saline; SCC, squamous cell carcinoma; TPA, 12-O-tetradecanoylphorbol-13-acetate

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While this work was in progress, Li *et al.* (2012) published a study in which they used *Cd151* knockout mice to evaluate the role of CD151 in mouse skin carcinogenesis. Their results indicate that CD151 contributes to skin carcinogenesis by reducing apoptosis in 7,12-dimethylbenzanthracene (DMBA)–initiated cells and stimulating proliferation of keratinocytes in response to 12-*O*-tetradecanoylphorbol-13-acetate (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 DMBA and promoted with TPA twice per week (Abel *et al.*, 2009). The average tumor volume was considerably lower in *Cd151* eKO than in wild-type mice, with the average number of tumors also being slightly lower (Figure 1a and 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). As 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 (Supplementary Figure S1 online). 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 with *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 because of increased proliferation. However, the epidermis of *Cd151* eKO mice was significantly thinner because of a lower proliferation rate (Figure 2a). It was unlikely that DMBA-induced apoptosis contributed to this effect as very few interfollicular epidermis cells died 24 hours 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 was the same in wild-type and *Cd151* eKO mice (Supplementary Figure S2 online). 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 (MKs) 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) (Supplementary Figure S3 online), 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 because of increased migration

Long-lived, slow-cycling label-retaining cells (LRCs) in hair follicles (HFs) and the interfollicular epidermis 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). In addition, 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 interfollicular epidermis 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

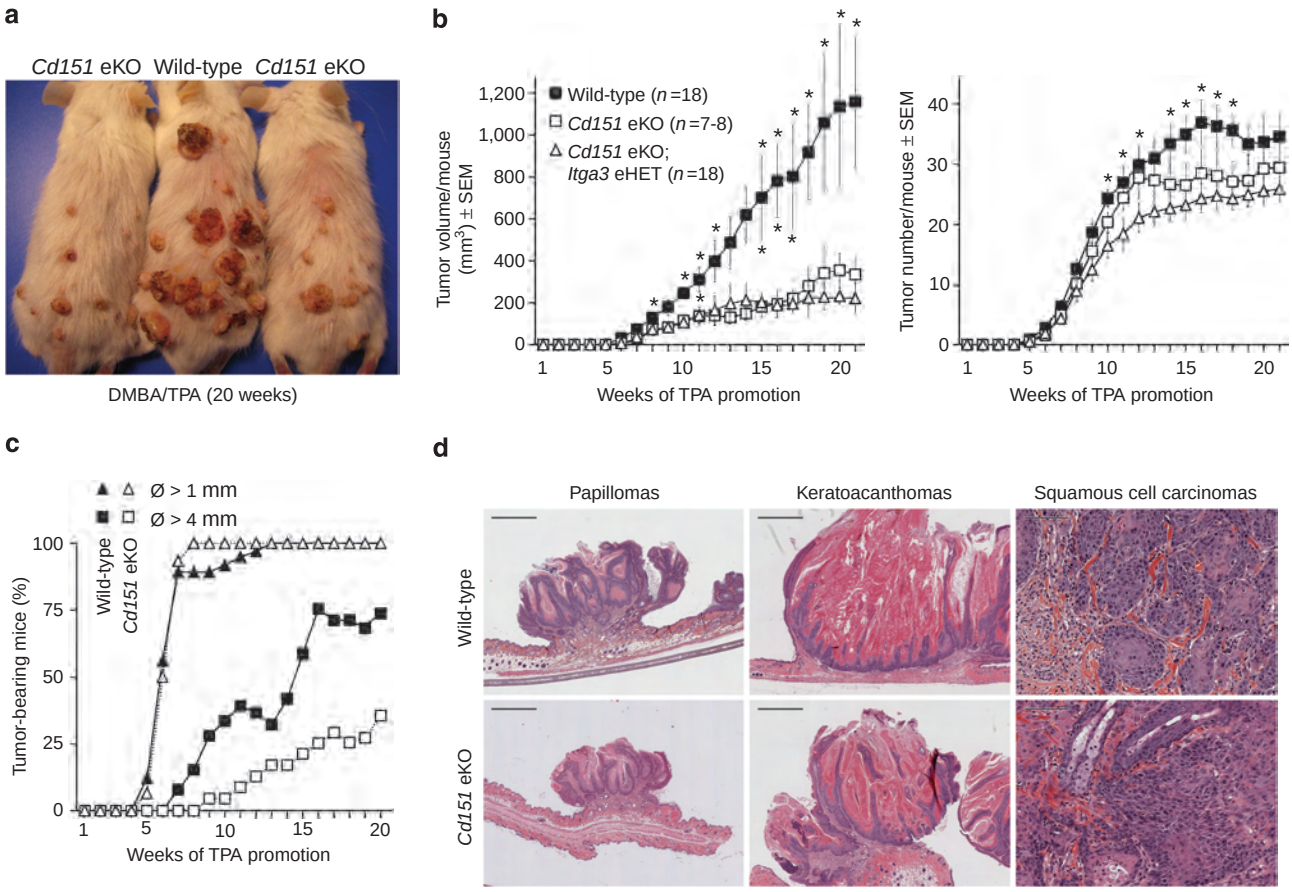

Figure 1. Impaired tumor formation in *Cd151* eKO mice after 7,12-dimethylbenzanthracene (DMBA)/12-*O*-tetradecanoylphorbol-13-acetate (TPA) carcinogenesis. (**a**) Tumor burden of wild-type and *Cd151* eKO littermates 20 weeks into the DMBA/TPA protocol. (**b**) Tumor volume and number are diminished in *Cd151* eKO mice compared with those 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 with *Cd151* eKO; *Itga3* eHET group; * below the wild-type group represents $P<0.05$ compared with *Cd151* eKO group as determined by one-way analysis of variance (ANOVA) and Bonferroni). (**c**) The incidence of tumors with a diameter of at least 1 mm is equal in the two groups. However, tumors >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 squamous cell carcinomas (SCCs) are regularly found in both groups (scale bars = 100 μm).

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 and 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 and d and Supplementary Figure S4 online).

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 after 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; Takeda *et al.*, 2007;

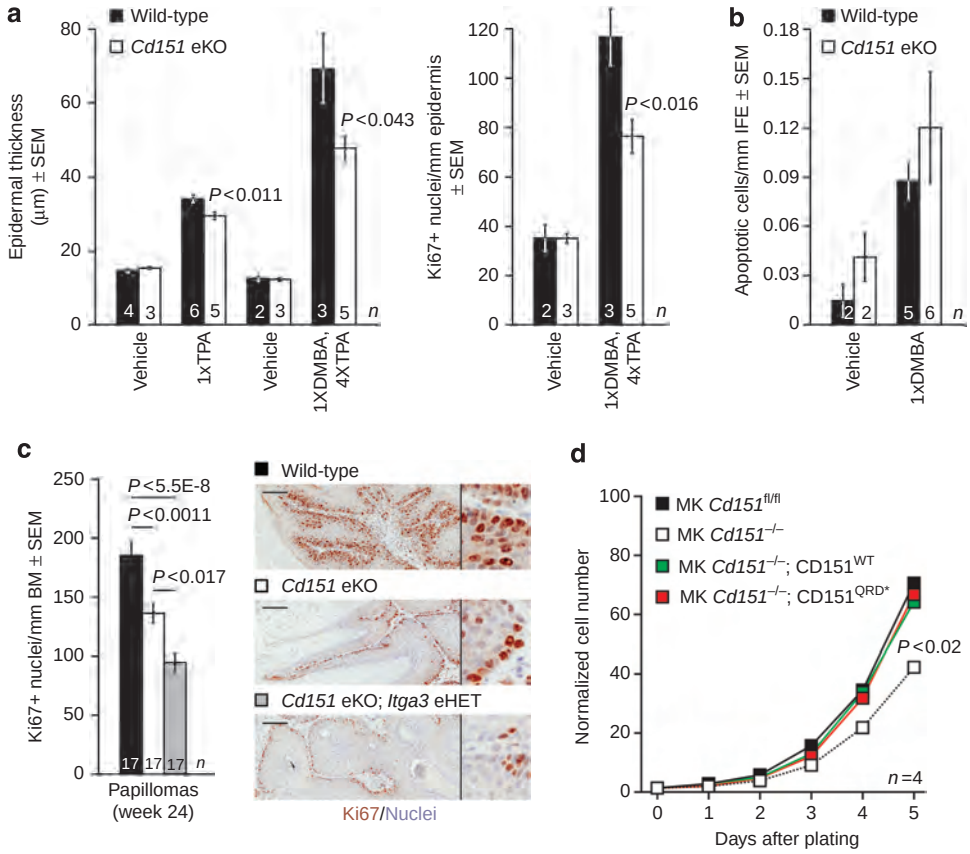

Figure 2. Decreased proliferation of (transformed) keratinocytes lacking *Cd151*. (**a**) Single and multiple doses of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) applied to *Cd151* eKO back skin result in significantly decreased hyperproliferation compared with that of skin of wild-type littermates. (**b**) The number of apoptotic cells in the interfollicular epidermis (IFE) of wild-type and *Cd151* eKO mice does not differ significantly after a single dose of 7,12-dimethylbenzanthracene (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 = 100 μm, 5 × insets). (**d**) *In vitro*, untransformed $Cd151^{-/-}$ mouse keratinocytes proliferate significantly less strongly than cells from the parental $Cd151^{fl/fl}$ mouse keratinocyte (MK) line. The proliferation defect is rescued by the expression of not only wild-type, but also the integrin-binding mutant CD151 (see Supplementary Figure S2 online for characterization of these cells).

Zhang *et al.*, 2011). 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, transforming growth factor-β, and hepatocyte growth factor (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 because of 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 with that in wild-type mice. Loss of *Cd151* has recently been shown to increase apoptosis in response to DMBA

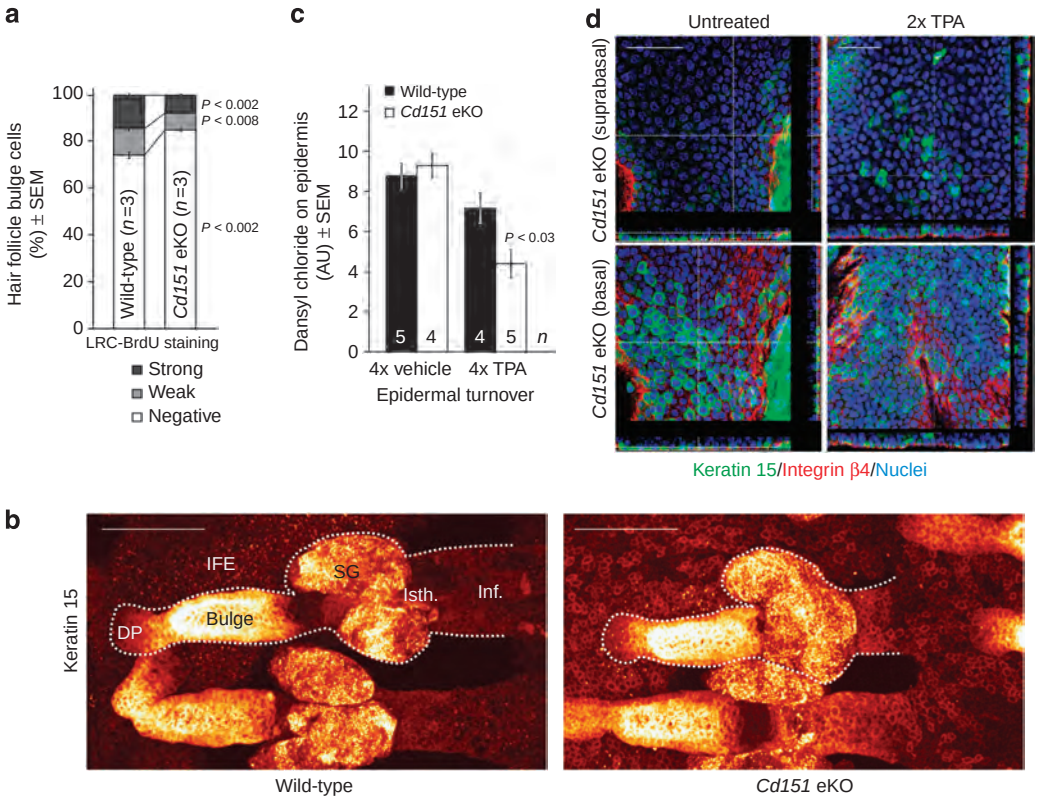

Figure 3. Loss of label-retaining cells (LRCs) lacking *Cd151*. (**a**) The number of BrdU LRCs is significantly reduced in the back skin hair follicles (HFs) of 8-week-old *Cd151* eKO mice compared with HFs of wild-type littermates. (**b**) Krt15$^+$ keratinocytes are confined to the HFs of wild-type mouse tails, but present in HFs and interfollicular epidermis (IFE) of *Cd151* eKO mouse tails (dotted lines outline HF; scale bars = 100 μm). DP, dermal papilla; Inf., infundibulum; Isth., isthmus; SG, sebaceous gland (stained aspecifically). (**c**) The 12-*O*-tetradecanoylphorbol-13-acetate (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 = 50 μm.

(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; Stenback *et al.*, 1981; Morris *et al.*, 1986). 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 with 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 (Chometon *et al.*, 2006; Sachs *et al.*, 2012a). Deletion of *Cd151* delays epidermal re-epithelialization and keratinocyte migration following skin wounding (Cowin *et al.*, 2006; Geary *et al.*, 2008) and a mAb against CD151 immobilizes tumor cells *in vivo* (Zijlstra *et al.*, 2008). In keratinocytes, the integrin α6β4–based hemidesmosomes render α3β1–CD151 adhesions less important. Disassembly of hemidesmosomes through TPA-mediated phosphorylation of β4 causes decreased keratinocyte adhesion and increased migration (Frijns *et al.*, 2010). The simultaneous weakening of two main keratinocyte adhesion structures (TPA treatment increases hemidesmosome dynamics, whereas deletion of *Cd151* weakens α3β1-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

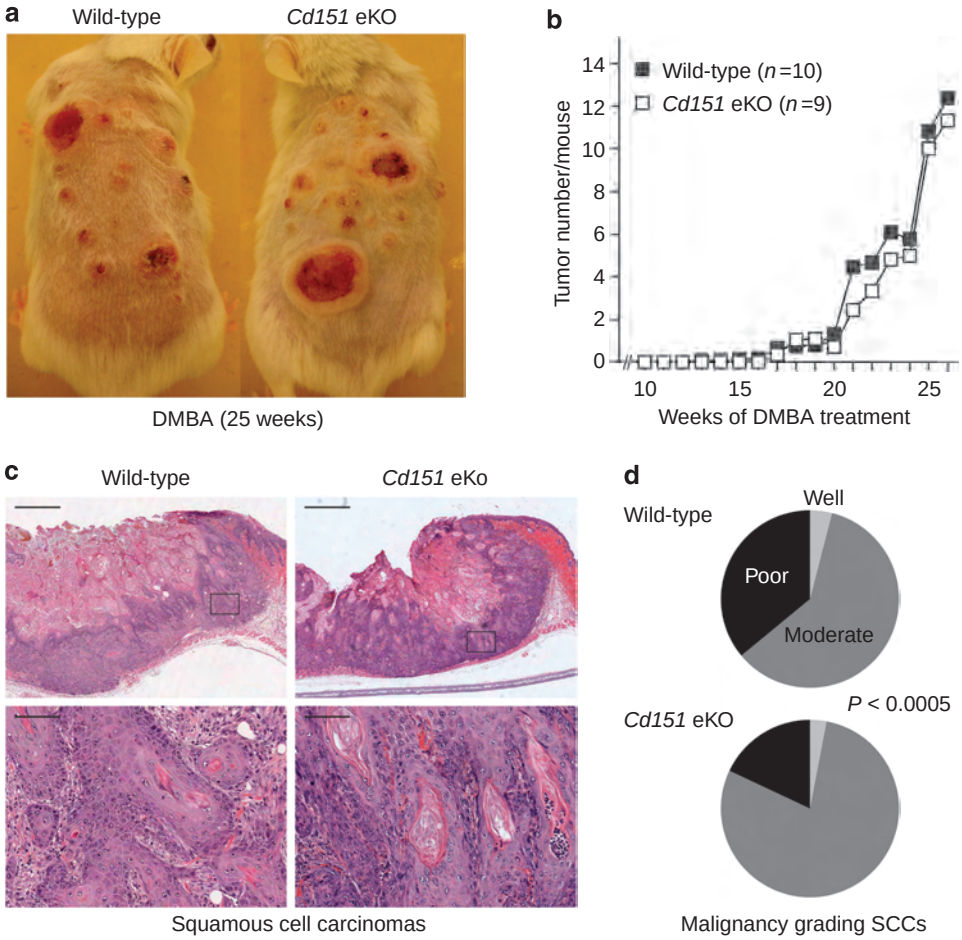

Figure 4. Complete carcinogenesis in wild-type and *Cd151* eKO mice. (**a**) Macroscopic image of two littermates following a 25-week regimen of 7,12-dimethylbenzanthracene (DMBA)-only carcinogenesis with (**b**) corresponding quantification of the entire cohort. *Cd151* eKO and wild-type mice develop the same number of tumors. (**c**) Representative histological examples of squamous cell carcinomas (SCCs) found in wild-type and Cd151 eKO mice after complete carcinogenesis (scale bars = 1 mm (overview) and 100 μm (detail)). (**d**) Pie chart of SCC differentiation showing mild increase of poorly differentiated SCCs in the wild-type group ($P<0.0005$; χ^2 test; see Supplementary Figure S4 online for total tumor numbers).

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 that 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 α3β1.

MATERIALS AND METHODS

Animal experiments

According to Mouse Genome Informatics (The Jackson Laboratory, Bar Harbor, ME) 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, 2012a). Compound heterozygotes were produced by crossing the 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-Aldrich, St Louis, MO) followed by biweekly applications of TPA (12.34 μg in 200 μl acetone; Sigma-Aldrich) 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 and d) and killed 24 hours later, with a single dose of 30 μg DMBA and killed 24 hours later (Figure 2b), or with a single dose of 30 μg DMBA followed by 4 semiweekly doses of 12.34 μg TPA and killed 3 days later (Figure 2a and Supplementary Figure S2A online). For LRC tracing, mice were injected intraperitoneally with 6 × 50 μg BrdU every 12 hours 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 1 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, 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, 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, optimum cutting temperature, Sakura Finetek, Alphen aan den Rijn, The Netherlands). Cryosections were prepared, fixed in ice-cold acetone, and blocked with 2% BSA in phosphate-buffered saline (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 hours. An intact sheet of epidermis was gently peeled away from the dermis and fixed in 4% paraformaldehyde in PBS for 2 hours at room temperature. Fixed epidermal sheets were permeabilized and blocked in PB buffer (20 mM HEPES buffer pH 7.2 containing 0.5% Triton X-100, 0.5% skim milk powder, and 0.25% fish skin gelatin) and incubated with 2 M HCl at 37 °C for 25 minutes 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 minutes (whole mounts o/n), followed by incubation with secondary antibodies diluted 1:200 for 60 minutes (o/n). The following antibodies were used: mouse anti-BrdU mAb (MO744, Dako, Glostrup, Denmark), mouse anti-human CD151 (11B1.G4) (Ashman *et al.*, 1997), rabbit anti-cleaved caspase-3 (9661L, Cell Signaling Technology, Danvers, MA), rabbit anti-mouse CD151 (Sachs *et al.*, 2006), mouse anti-FLAG (M2; Sigma-Aldrich), rabbit anti-FLAG (Sc-807; Santa Cruz Biotechnology, Dallas, TX), mouse anti-mouse integrin-α3 (29A3) (de Melker *et al.*, 1997), rat anti-mouse integrin β4 (346-11A; BD Biosciences, San Jose, CA), mouse anti-mouse Ki67 (PSX1028; Sanbio, Uden, The Netherlands), and mouse anti-mouse keratin 15 (MA1-90929; Thermo Fisher Scientific, Waltham, MA). 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) and processed using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA) or ImageJ (National Institutes of Health, Bethesda, MD).

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 (Invitrogen, Carlsbad, CA) supplemented with 50 μg ml^{-1} bovine pituitary extract, 5 ng ml^{-1} EGF, 100 U ml^{-1} penicillin, and 100 U ml^{-1} 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 5,000 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, and 100 mM NaCl, supplemented with a cocktail of protease inhibitors (P8340; Sigma-Aldrich). Lysates were cleared by centrifugation for 20 minutes at 20,000 *g* and 4 °C, followed by separation of proteins on 4–12% polyacrylamide gels under nonreducing conditions (NuPage, EMD Millipore, Billerica, MA), and transferred to Immobilon polyvinylidene difluoride membranes (EMD Millipore). For immunoprecipitations, lysates were incubated overnight with mAb 29A3 coupled to gamma-bind sepharose (GE Healthcare, Little Chalfont, UK) or mAb M2-coupled agarose (A2220; Sigma-Aldrich). Beads were spun down at 500 *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% fetal calf serum in PBS, and stained with primary antibodies as indicated for 60 minutes on ice. Following washing, secondary anti-goat, anti-rat, and anti-mouse antibodies coupled to FITC were used 1:200 for 60 minutes on ice. Cells were strained and analyzed on a 1998 BD FACSCalibur (Becton, Dickinson and Company, Franklin Lakes, NJ) using a 488 nm laser and a 530/30 FL1 filter configuration.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

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

REFERENCES

Abel EL, Angel JM, Kiguchi K *et al.* (2009) Multi-stage chemical carcinogenesis in mouse skin: fundamentals and applications. *Nat Protoc* 4:1350–62

Anton M, Graham FL (1995) Site-specific recombination mediated by an adenovirus vector expressing the Cre recombinase protein: a molecular switch for control of gene expression. *J Virol* 69:4600–6

Ashman LK, Fitter S, Sincock PM *et al.* (1997) *CD151 (PETA-3) workshop summary report.* Kishimoto T (ed). Garland Publishing: New York, pp 681–3

Berenblum I, Shubik P (1949) The persistence of latent tumour cells induced in the mouse's skin by a single application of 9:10-dimethyl-1:2-benzanthracene. *Br J Cancer* 3:384–6

Chometon G, Zhang ZG, Rubinstein E *et al.* (2006) Dissociation of the complex between CD151 and laminin-binding integrins permits migration of epithelial cells. *Exp Cell Res* 312:983–95

Cotsarelis G, Sun TT, Lavker RM (1990) Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell* 61:1329–37

Cowin AJ, Adams D, Geary SM *et al.* (2006) Wound healing is defective in mice lacking tetraspanin CD151. *J Invest Dermatol* 126:680–9

de Melker AA, Sterk LM, Delwel GO *et al.* (1997) The A and B variants of the alpha 3 integrin subunit: tissue distribution and functional characterization. *Lab Invest* 76:547–63

Deng X, Li Q, Hoff J *et al.* (2012) Integrin-associated CD151 drives ErbB2-evoked mammary tumor onset and metastasis. *Neoplasia* 14:678–89

Dipersio CM, Hodivala-Dilke KM, Jaenisch R *et al.* (1997) alpha3beta1 Integrin is required for normal development of the epidermal basement membrane. *J Cell Biol* 137:729–42

Folkman J (1974) Tumor angiogenesis. *Adv Cancer Res* 19:331–58

Franco M, Muratori C, Corso S *et al.* (2010) The tetraspanin CD151 is required for Met-dependent signaling and tumor cell growth. *J Biol Chem* 285:38756–64

Frijns E, Sachs N, Kreft M *et al.* (2010) EGF-induced MAPK signaling inhibits hemidesmosome formation through phosphorylation of the integrin {beta}4. *J Biol Chem* 285:37650–62

Geary SM, Cowin AJ, Copeland B *et al.* (2008) The role of the tetraspanin CD151 in primary keratinocyte and fibroblast functions: implications for wound healing. *Exp Cell Res* 314:2165–75

Georges-Labouesse E, Messaddeq N, Yehia G *et al.* (1996) Absence of integrin alpha 6 leads to epidermolysis bullosa and neonatal death in mice. *Nat Genet* 13:370–3

Has C, Sparta G, Kiritsi D *et al.* (2012) Integrin alpha3 mutations with kidney, lung, and skin disease. *N Engl J Med* 366:1508–14

Huelsken J, Vogel R, Erdmann B *et al.* (2001) beta-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. *Cell* 105:533–45

Karamatic-Crew V, Burton N, Kagan A *et al.* (2004) CD151, the first member of the tetraspanin (TM4) superfamily detected on erythrocytes, is essential for the correct assembly of human basement membranes in kidney and skin. *Blood* 104:2217–23

Kazarov AR, Yang X, Stipp CS *et al.* (2002) An extracellular site on tetraspanin CD151 determines alpha 3 and alpha 6 integrin-dependent cellular morphology. *J Cell Biol* 158:1299–309

Kreidberg JA, Donovan MJ, Goldstein SL *et al.* (1996) Alpha 3 beta 1 integrin has a crucial role in kidney and lung organogenesis. *Development* 122:3537–47

Li Q, Yang XH, Xu F *et al.* (2012) Tetraspanin CD151 plays a key role in skin squamous cell carcinoma. *Oncogene* 32:1772–83

Margadant C, Raymond K, Kreft M *et al.* (2009) Integrin alpha3beta1 inhibits directional migration and wound re-epithelialization in the skin. *J Cell Sci* 122:278–88

Morris RJ, Fischer SM, Slaga TJ (1986) Evidence that a slowly cycling subpopulation of adult murine epidermal cells retains carcinogen. *Cancer Res* 46:3061–6

Nicolaou N, Margadant C, Kevelam SH *et al.* (2012) Gain of glycosylation in integrin alpha3 causes lung disease and nephrotic syndrome. *J Clin Invest* 122:4375–87

Nishiuchi R, Sanzen N, Nada S *et al.* (2005) Potentiation of the ligand-binding activity of integrin alpha3beta1 via association with tetraspanin CD151. *Proc Natl Acad Sci USA* 102:1939–44

Owens DM, Watt FM (2001) Influence of beta1 integrins on epidermal squamous cell carcinoma formation in a transgenic mouse model: alpha3beta1, but not alpha2beta1, suppresses malignant conversion. *Cancer Res* 61:5248–54

Reynolds LE, Conti FJ, Silva R *et al.* (2008) alpha3beta1 integrin-controlled Smad7 regulates reepithelialization during wound healing in mice. *J Clin Invest* 118:965–74

Romanska HM, Potemski P, Collins SI *et al.* (2012) Loss of CD151/Tspan24 from the complex with integrin alpha3beta1 in invasive front of the tumour is a negative predictor of disease-free survival in oral squamous cell carcinoma. *Oral Oncol* 49:224–9

Ruzzi L, Gagnoux-Palacios L, Pinola M *et al.* (1997) A homozygous mutation in the integrin alpha6 gene in junctional epidermolysis bullosa with pyloric atresia. *J Clin Invest* 99:2826–31

Sachs N, Claessen N, Aten J *et al.* (2012a) Blood pressure influences end-stage renal disease of Cd151 knockout mice. *J Clin Invest* 122: 348–58

Sachs N, Kreft M, van den Bergh Weerman MA *et al.* (2006) Kidney failure in mice lacking the tetraspanin CD151. *J Cell Biol* 175:33–9

Sachs N, Secades P, van Hulst L *et al.* (2012b) Loss of integrin alpha3 prevents skin tumor formation by promoting epidermal turnover and depletion of slow-cycling cells. *Proc Natl Acad Sci USA* 109: 21468–73

Sadej R, Romanska H, Kavanagh D *et al.* (2010) Tetraspanin CD151 regulates transforming growth factor beta signaling: implication in tumor metastasis. *Cancer Res* 70:6059–70

Sincock PM, Mayrhofer G, Ashman LK (1997) Localization of the transmembrane 4 superfamily (TM4SF) member PETA-3 (CD151) in normal human tissues: comparison with CD9, CD63, and alpha5beta1 integrin. *J Histochem Cytochem* 45:515–25

Stenback F, Peto R, Shubik P (1981) Initiation and promotion at different ages and doses in 2200 mice. I. Methods, and the apparent persistence of initiated cells.. *Br J Cancer* 44:1–14

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

Sterk LM, Geuijen CA, van den Berg JG *et al.* (2002) Association of the tetraspanin CD151 with the laminin-binding integrins alpha3beta1, alpha6beta1, alpha6beta4 and alpha7beta1 in cells in culture and in vivo. *J Cell Sci* 115:1161–73

Suzuki S, Miyazaki T, Tanaka N *et al.* (2011) Prognostic significance of CD151 expression in esophageal squamous cell carcinoma with aggressive cell proliferation and invasiveness. *Ann Surg Oncol* 18:888–93

Takeda Y, Kazarov AR, Butterfield CE *et al.* (2007) Deletion of tetraspanin Cd151 results in decreased pathologic angiogenesis in vivo and in vitro. *Blood* 109:1524–32

van der Neut R, Krimpenfort P, Calafat J *et al.* (1996) Epithelial detachment due to absence of hemidesmosomes in integrin beta 4 null mice. *Nat Genet* 13:366–9

Vidal F, Aberdam D, Miquel C *et al.* (1995) Integrin beta 4 mutations associated with junctional epidermolysis bullosa with pyloric atresia. *Nat Genet* 10:229–34

Wright MD, Geary SM, Fitter S *et al.* (2004) Characterization of mice lacking the tetraspanin superfamily member CD151. *Mol Cell Biol* 24: 5978–88

Yauch RL, Berditchevski F, Harler MB *et al.* (1998) Highly stoichiometric, stable, and specific association of integrin alpha3beta1 with CD151 provides a major link to phosphatidylinositol 4-kinase, and may regulate cell migration. *Mol Biol Cell* 9:2751–65

Zhang F, Michaelson JE, Moshiach S *et al.* (2011) Tetraspanin CD151 maintains vascular stability by balancing the forces of cell adhesion and cytoskeletal tension. *Blood* 118:4274–84

Zijlstra A, Lewis J, Degryse B *et al.* (2008) The inhibition of tumor cell intravasation and subsequent metastasis via regulation of in vivo tumor cell motility by the tetraspanin CD151. *Cancer Cell* 13:221–34