Integrin α3β1 Is a Key Regulator of Several Protumorigenic Pathways during Skin Carcinogenesis

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Integrin α3β1 plays a crucial role in tumor formation in the two-stage chemical carcinogenesis model (DMBA and TPA treatment). However, the mechanisms whereby the expression of α3β1 influences key oncogenic drivers of this established model are not known yet. Using an in vivo mouse model with epidermal deletion of α3β1 and in vitro Matrigel cultures of transformed keratinocytes, we demonstrate the central role of α3β1 in promoting the activation of several protumorigenic signaling pathways during the initiation of DMBA/TPA-driven tumorigenesis. In transformed keratinocytes, α3β1-mediated focal adhesion kinase/Src activation leads to in vitro growth of spheroids and to strong Akt and STAT 3 activation when the α3β1-binding partner tetraspanin CD151 is present to stabilize cell–cell adhesion and promote Smad2 phosphorylation. Remarkably, α3β1 and CD151 can support Akt and STAT 3 activity independently of α3β1 ligation by laminin-332 and as such control the essential survival signals required for suprabasal keratin-10 expression during keratinocyte differentiation. These data demonstrate that α3β1 together with CD151 regulate the signaling pathways that control the survival of differentiating keratinocytes and provide a mechanistic understanding of the essential role of α3β1 in early stages of skin cancer development.


INTRODUCTION

Over the past few decades, the two-stage chemical carcinogenesis mouse model (DMBA/TPA treatment) has enabled a better understanding of key players and complex processes occurring during different stages of cutaneous cancer. This established model consists of a single application of carcinogen DMBA, causing an activating mutation in Hras1 gene, followed by biweekly applications of the phorbol ester TPA, stimulating increased production of GFs and inflammatory cytokines. This leads to sustained epidermal hyperplasia and the development of benign tumors papillomas, which can progress to invasive carcinomas upon further TPA treatment (Abel et al., 2009). The most prominent factors leading to papilloma development are stromal and inflammatory responses and activation of several GF signaling pathways (Huang and Balmain, 2014; Rundhau and Fischer, 2010). In addition to the Raf/MAPK/ERK cascade (Bourcier et al., 2006; Scholl et al., 2009), resulting from the activating Hras1 mutation and TPA treatment, FAK/Src (Matsumoto et al., 2003; McLean et al., 2004; Serrels et al., 2009), PI3K/Akt (Segrelles et al., 2007; Suzuki et al., 2003), Jak/STAT3 (Ancri et al., 2007; Chan et al., 2004; Kim et al., 2009a), and TGFβ/Smad (Li et al., 2004; Pérez-Lorenzo et al., 2010) signaling pathways play a central role in tumor development.

We previously showed that integrin α3β1 is also required for the development of papillomas upon DMBA/TPA treatment (Sachs et al., 2012). Yet, how α3β1 affects known key drivers of this model has not been elucidated. Integrins constitute a family of transmembrane glycoproteins that mediate cell-matrix adhesion but also function as bidirectional transducers of mechanical and biochemical signals. They play diverse roles in tumorigenesis and tumor progression (Hamidi and Ivaska, 2018). Integrin α3β1 often exerts opposing functions in cancer, switching from a tumor-promoting to -suppressing mechanism depending on the cancer type and driving mechanism, cell environment, and stage of the disease (Ramovs et al., 2017; Stipp, 2010). Together with integrin α6β4, α3β1 mediates cell-matrix adhesion of basal keratinocytes (KC) by binding the extracellular matrix proteins laminin-332 and laminin-511. Furthermore, α3β1 can stabilize E-cadherin–based cell–cell junctions, especially when forming a complex with the tetraspanin CD151 (Stipp, 2010). In this study, we investigate the impact of α3β1 on the activation of several key signaling pathways during the initiation phase of mouse skin tumorigenesis. Our study uncovers the crucial role of α3β1 in the activation of FAK, Akt, and STAT3 in transformed KCs and...
RESULTS AND DISCUSSION

Integrin α3β1 is required for efficient activation of STAT3, Akt, and FAK signaling during the initiation phase of DMBA/TPA tumorigenesis in vivo

To assess the impact of α3β1 on DMBA/TPA-mediated protumorigenic signaling, we subjected mice with an epidermis-specific deletion of α3β1 (Itga3-knockout [KO] mice) and wild-type (WT) mice (Itga3 WT mice) to topical short-term DMBA/TPA treatment, when protumorigenic pathways are switched on, but tumors have not yet been formed (Figure 1a). Signaling through the IL-6/Jak/STAT3 and PI3K/Akt/mTOR pathways is critical for the initiation and progression of papillomas (Ancrile et al., 2007; Carr et al., 2015; Chan et al., 2004; Segrelles et al., 2007; Suzuki et al., 2003). Epidermal depletion of α3β1 compromises both pathways, as judged by the strong reduction of the activity of STAT3 and Akt in the epidermis of Itga3-KO compared with that of Itga3-WT mice (Figure 1b and c). We also observed a reduction of phosphorylated FAK in the short-term DMBA/TPA-treated skin of Itga3-KO mice (Figure 1d). However, the loss of α3β1 did not affect phosphorylated ERK1/2 levels (Figure 1d), which is consistent with the unperturbed phosphorylation of ERK1/2 in Stat3-KO mice (Chan et al., 2004). The reduction of active FAK upon the deletion of α3β1 is in line with the role of integrin-mediated adhesion in FAK/Src signaling (Hamidi and Ivaska, 2018) and the requirement of FAK for development of papillomas in mice (McLean et al., 2004).

Together, these data demonstrate that α3β1 is required for full activation of the STAT3, Akt, and FAK protumorigenic signaling pathways during the early stage of skin tumorigenesis.

α3β1-mediated activation of STAT3, Akt, and FAK/Src is crucial for three-dimensional growth of transformed KCs in vitro

To further investigate the role of α3β1 in protumorigenic signaling, we made use of Hras1-transformed KCs isolated
Figure 2. α3β1-mediated activation of STAT3, Akt, and FAK/Src is crucial for 3D growth of transformed KCs in vitro. (a–c) Analysis of Itga3-WT spheroids treated with 2 μM niclosamide, 10 μM MK2206, or 500nM AZD8055 and Itga3-KO spheroids transfected with WT α3 WT, CA-STAT3, or Myr-Akt. (a) Bright-field images and quantifications (n = 80–95) of the spheroid area. (b) IF images of z-slice and quantifications (n = 29–30) of pSTAT3 (Y705)-positive nuclei. (c) IF images of maximum intensity projection and quantifications (n = 30) of pAkt (S473)-positive area. (d) WB and quantification showing reduced pFAK (Y397) and pSrc (Y416) protein levels in Itga3-KO compared with WT spheroids. (e) Representative bright field and IF images and quantifications of the area (n = 80), pSTAT3 (Y705)-positive nuclei (n = 20), and pAkt (S473)-positive area (n = 20) of Itga3-KO, WT, and Itga3-KO spheroids transfected with CA-FAK construct. (f–h) Representative bright field and IF images and quantifications of the area (n = 80), pSTAT3 (Y705)-positive nuclei (n = 21–26), and pAkt (S473)-positive area (n = 20–26) of nontreated, 10 μM saracatinib–treated, and 5μM VS-4718–treated Itga3-WT spheroids. Bar = 20 μm (IF) and 50 μm (bright field). Statistics: mean ± SD; (d, g, h) unpaired t-test; (a–c, e) Sidak’s multiple comparisons test. *P < 0.05, **P < 0.005, ****P < 0.0001. 3D, three-dimensional; Akt, protein kinase B; AU,
from Itga3-WT mice that underwent full DMBA/TPA treatment (Itga3-KO and Itga3-WT mouse squamous carcinoma cells (MSCCs)) (Sachs et al., 2012). Consistent with the lack of tumorigenesis in the model of two-stage skin carcinogenesis (Sachs et al., 2012) and the reduced protumorigenic signaling in Itga3-KO mice, the growth of MSCCs in three-dimensional (3D) Matrigel depended on the presence of α3β1 and its ability to support STAT3 and Akt signaling (Figure 2a–c). The ability of α3β1 to support the growth of transformed KCs is in line with the high-level expression of α3β1 (Supplementary Figure S1a) and the activity of STAT3 (Chan et al., 2004) and Akt (Segrelles et al., 2002) in papillomas as well as with Akt- and STAT3-dependent progression of the cell cycle through the activation of cyclin D during DMBA/TPA treatment (Chan et al., 2004; Segrelles et al., 2007). Interestingly, MSCC spheroids exhibited codependent activation of STAT3 and Akt; whereas treatment with Akt (MK2206) or mTOR (AZD8055) inhibitors ablated STAT3 activity (Figure 2a), treatment with STAT3 inhibitors (niclosamide and stattic) strongly reduced the phosphorylation of Akt (Figure 2a and Supplementary Figure S1b). In line with this, the expression of constitutively activated STAT3 in Itga3-KO KCs restored Akt activation and vice versa; the expression of myristoylated Akt, which renders Akt constitutively active, activated STAT3 (Figure 2a and b). Such crosstalk between the PI3K/Akt and STAT3 pathways has been demonstrated before in different

Figure 3. α3β1 supports STAT3 and Akt signaling independently of its ligation by laminin matrix. (a) Quantifications (dot = mouse, averaged eight images) and representative images of the percentage of pSTAT3-positive nuclei and pAkt-positive area located suprabasally in the short-term DMBA/TPA-treated IFE of Itga3-KO and -WT mice. Dotted line delineates suprabasal KCs. High pAkt expression is commonly observed in suprabasal KCs (arrows). (b) IF image of short-term DMBA/TPA-treated Itga3-WT epidermis. α3β1 colocalizes with laminin-332 in the basement membrane but is also expressed in cell–cell contacts suprabasally. Bar: 50 μm. (c) IF images of Itga3-KO spheroids transfected with WT α3 (α3WT) or laminin-binding mutant G163A (α3G163A). α3β1 colocalizes with laminin-332 at the outer layer of spheroids and with E-cadherin in cell–cell contacts. Bar = 20 μm. (d–e) Representative bright-field and IF images and quantifications of the area (n = 80–115), pSTAT3 (Y705)-positive nuclei (n = 30), and pAkt (S473)-positive area (n = 26–30) of Itga3-KO spheroids and Itga3-KO spheroids expressing either α3WT or α3G163A. Bar = 20 μm (IF) and 50 μm (bright field). (f) WB and quantification showing the levels of pFAK (Y397) and pSrc (Y416) in MSCC-α3WT and -α3G163A spheroids. Statistics: mean ± SD; Sidak's multiple comparisons test; *p < 0.05, **p < 0.0005, ***p < 0.0001. Akt, protein kinase B; AU, arbitrary unit; DMBA, 7,12-dimethylbenz[a]anthracene; FAK, focal adhesion kinase; IF, immunofluorescence; IFE, interfollicular epidermis; KC, keratinocyte; KO, knockout; MSCC, mouse squamous carcinoma cell; pAkt, phosphorylated Akt; pFAK, phosphorylated FAK; pSrc, phosphorylated Src; pSTAT3, phosphorylated STAT3; STAT3, STAT, signal transducer and activator of transcription; TPA, 12-O-tetradecanoylphorbol-13-acetate; WB, western blot; WT, wild type.
types of tumors (Blando et al., 2011; Han et al., 2010; Kim et al., 2013). Furthermore, several functional links have been established between STAT3 and Akt signaling pathways, such as TEC kinases, which activate STAT3 downstream of PI3K (Vogt and Hart, 2011), and phosphoinositide-dependent kinase 1, which is a master regulator of Akt and has been shown to be a direct transcriptional target of STAT3 in melanomas (Picco et al., 2019).

Consistent with our in vivo data (Figure 1d), the absence of α3β1 in spheroids resulted in reduced activation of FAK as well as Src (Figure 2d). The FAK/Src complex presents a likely link between α3β1 and the PI3K/Akt and STAT3 pathways (Pylayeva et al., 2009; Visavadiya et al., 2016; Xia et al., 2004). This hypothesis is supported by our finding that Itga3-KO spheroids expressing constitutively active FAK (Supplementary Figure S1c) showed an increased STAT3

Figure 4. The deletion of CD151 reduces cell–cell contact integrity, resulting in reduced activation of STAT3 and Akt but not reduced FAK/Src signaling. (a) Flow cytometry quantification showing reduced expression of CD151 upon the deletion of α3β1 in the epidermis. Box and whisker plot, dot = mouse (gating strategy: Supplementary Figure S3a). (b) IF staining for laminin-332, α3β1, and E-cadherin of WT and Cd151-KO spheroids shows reduced cell–cell contacts (arrow) and localization of laminin-332 in inner KC layers (asterisk) in the absence of CD151. Bar = 20 μm. (c) WB with quantifications showing reduced pSmad2 (S465/467) in Cd151-KO spheroids. (d) WB showing reduced pSmad2 (S465/467) in the epidermis of short-term DMBA/TPA-treated Itga3-KO compared with WT mice (quantification: Figure S3e). (e) Representative bright-field and IF images and quantifications of the area (n = 80) of pSTAT3 (Y705)-positive nuclei (n = 22–25), and pAkt (S473)-positive area (n = 28) of Itga3-KO and WT spheroids and Itga3-WT spheroids with deletion of Cd151, obtained with two distinct CRISPR/Cas9 gRNAs (G1 and G2). Bar = 20 μm (IF) and 50 μm (bright field). (f) WB showing comparable levels of pSrc (Y416) and pFAK (Y397) in WT and Cd151-KO spheroids (quantification: Figure S3e). (g, h) Representative bright-field images and quantifications (n = 85) of the size of Cd151-KO spheroids with deletion of Cd151 treated with (g) 10 μM saracatinib and (h) 5 μM VS-4718. Bar = 50 μm. (i) Bright-field images and (j) quantifications (n = 85) of the size of MSCC-α3G163A spheroids and MSCC-α3WT and -α3G163A spheroids with deletion of Cd151. Bar = 50 μm. Statistics: mean ± SD; (a, g, h) unpaired t-test; (c, e, j) Sidak’s multiple comparisons test. *P < 0.05, **P < 0.005, ****P < 0.0001. Akt, protein kinase B; AU, arbitrary unit; DMBA, 7,12-dimethylbenz[a]-anthracene; FAK, focal adhesion kinase; gRNA, guide RNA; IF, immunofluorescence; KO, knockout; MSCC, mouse squamous carcinoma cell; pAkt, phosphorylated Akt; pFAK, phosphorylated FAK; pSmad2, phosphorylated Smad2; pSrc, phosphorylated Src; pSTAT3, phosphorylated STAT3; STAT3, STAT, signal transducer and activator of transcription; TPA, 12-O-tetradecanoylphorbol-13-acetate; WB, western blot; WT, wild type.
activation and, albeit moderately, increased Akt activation and 3D growth (Figure 2e). Likewise, FAK and Src inhibition by VS-4718 and saracatinib (AZD0530), respectively, ablated the activation of Akt and STAT3 and inhibited 3D growth of transformed KCs (Figure 2f).

Our results strongly suggest that α3β1 promotes the growth of papillomas through FAK/Src signaling, which supports codependent activation of the PI3K/Akt and STAT3 signaling pathways.

**Integrin α3β1 can support STAT3 and Akt signaling independently of its ligation by laminin**

In hyperplastic DMBA/TPA-treated skin, phosphorylated STAT3- and phosphorylated Akt-positive KCs can be observed in both basal and suprabasal cell layers of the epidermis (Figures 1b and c and 3a). In fact, suprabasal KCs often exhibit elevated levels of phosphorylated Akt compared with basal cells (Figure 3a), which is consistent with previous observations (Calautti et al., 2005). Such high suprabasal activity of Akt and STAT3 is surprising considering the fact that laminin-332 and laminin-511, the ligands of α3β1, are only found in the basement membrane underlying the basal KCs (Figure 3b). Because α3β1 is also expressed by suprabasal, differentiating KCs in hyperplastic mouse skin (Figure 3b), unconventionally, α3β1 may support protumorigenic STAT3 and Akt signaling independently of its ligation by laminin-332. To test this hypothesis, we reconstituted Itga3-KO MSCC with either WT human α3 (α3WT) or an α3 mutant (α3G163A), which is unable to bind to laminin-332 (Zhang et al., 1999) (Supplementary Figure S2a and b). The MSCCs-α3WT and -α3G163A grown in spheroids exhibited tight cell–cell adhesion with laminin-332 deposited only by cells forming the outermost basal cell layer (Figure 3c). In these cells, α3β1 localizes at the cell–extracellular matrix interface. In addition, α3β1 is present in the inner cell layers at cell–cell contacts (Figure 3c). Consistent with our in vivo observations, ligation of α3β1 to laminin-332 was not required for strong activation of STAT3 and Akt (Figure 3d and e). The expression of α3G163A also almost completely restored the growth of spheroids (Figure 3d and e). This was further confirmed by inhibiting α3β1 ligation to laminin-332 using function-blocking J143 antibody, which did not have any
effect on Akt or STAT3 activation or on 3D KC growth (Supplementary Figure S2c). Integrin signaling has been conventionally linked to the ligation of integrins by extracellular matrix proteins, leading to their association with actomyosin cytoskeleton and activation of FAK and Src family kinases (Sun et al., 2016). Even though the expression of z3G163A leads to reduced FAK activation and a small decrease in Src phosphorylation (Figure 3b), Itgα3-KO spheroids exhibit a much more prominent reduction of FAK/Src signaling (Figure 2d). Thus, z3β1 can support FAK/Src activation at the level, sufficient to drive STAT3 and Akt signaling, independently of its ligation by laminin-332. Nonligated z3β1 may mediate the phosphorylation of FAK directly if clustered by other integrins or integrin-associated proteins. Alternatively, z3β1 may support the activation of other integrins, which promote FAK phosphorylation.

We conclude that z3β1 is present in cell–cell contacts in suprabasal KCs, where it supports Akt and STAT3 signaling independently of its ligation by laminin-332.

Deletion of CD151 impairs cell–cell contact integrity, resulting in reduced activation of STAT3 and Akt but not reduced FAK/Src signaling

The role of integrins in cell–cell contacts is poorly understood. Integrin z3β1 is known to stabilize cell–cell contacts (Zhang et al., 2003) and to associate with E-cadherin (Kim et al., 2009b). The ability of z3β1 to control cell–cell junction stability is dependent on its binding to tetraspanin CD151 (Chattopadhyay et al., 2003; Johnson et al., 2009; Shigeta et al., 2003; Zevian et al., 2015), a tumor promoter in DMBATPA carcinogenesis model (Li et al., 2013; Sachs et al., 2014). Furthermore, CD151 has been shown to promote TPA-induced STAT3 phosphorylation in mouse and human KCs (Li et al., 2013). We thus wondered whether z3β1-CD151 complexes play a central role in protumorigenic signaling, especially in suprabasal cells. Interestingly, we observed reduced expression of CD151 in the epidermis of Itgα3-KO compared with that of Itgα3-WT mice (Figure 4a and Supplementary Figure S3a and b), which is in line with the observations in leukemic K562 cells (Sterk et al., 2002; Yauh et al., 1998). Because it was shown that the suppression of z3β1 expression by RNA interference affects the clustering of CD151 and promotes its homodimerization (Palmer et al., 2014; Scales et al., 2013), our data may suggest that the stability of CD151 in mouse epidermis depends on its ability to form a complex with z3β1. As expected, deletion of CD151 in MSCCs using CRISPR/Cas9 technology with two distinct guide RNAs (Cd151-KO G1 and G2) destabilized cell–cell contacts in spheroids, as observed by staining for E-cadherin and the presence of a laminin-332 matrix between KCs in the inner cell layers of the spheroids (Figure 4b). Furthermore, CD151-KO MSCCs exhibited decreased Smad2 phosphorylation, which is induced by TGFβ and promoted by the association of z3β1 and E-cadherin (Kim et al., 2009b) (Figure 4c). We observed reduced phosphorylated Smad2 also in the epidermis of Itgα3-KO mice (Figure 4d and Supplementary Figure S3c), demonstrating a role of z3β1-CD151 in sustaining the protumorigenic TGFβ/Smad2/3 pathway (Li et al., 2004; Pérez-Lorenzo et al., 2010). The deletion of Cd151 in MSCCs resulted in strongly reduced levels of phosphorylated Akt and impaired nuclear translocation of phosphorylated STAT3 (Figure 4e). Remarkably, the reduced activity of Akt and STAT3 did not affect the 3D growth of Cd151-KO KCs (Figure 4e). A similar trend was observed when spheroids were grown in the presence of the E-cadherin function–blocking antibody DECMA-1 (Supplementary Figure S3d), further supporting a role of CD151 and cell–cell junctions in promoting STAT3 and Akt activity. Because Cd151-KO and Cd151-WT spheroids showed similar levels of active Src and FAK (Figure 4f and Supplementary Figure S3d), we wondered whether 3D growth of these spheroids depends primarily on FAK/Src
signaling. Inhibition of FAK and Src impaired the growth of CD151-KO spheroids (Figure 4g, and h), thus demonstrating that FAK/Src signaling supports the proliferation of these cells, despite their low Akt and STAT3 activity. Because \( \alpha_3 \beta_1 \)-mediated adhesion to laminin-332 increases FAK activation (Figure 3f), the presence of laminin-332 and \( \alpha_3 \beta_1 \) in the inner layers of CD151-KO spheroids (Figure 4b) could promote extracellular matrix-adhesion–supported prosurvival signaling and thus explain their sole dependency on FAK/Src signaling for growth. Indeed, the deletion of CD151 in MSCCs-\( \alpha_3 G163A \) and the treatment of CD151-KO MSCC-\( \alpha_3 WT \) spheroids with function-blocking J143 antibody resulted in strongly reduced spheroid size (Figure 4i and j and Supplementary Figure S3f and g). However, it should be noted that the levels of phosphorylated FAK and phosphorylated Src were comparable in CD151-KO MSCC-\( \alpha_3 G163A \) and CD151-KO MSCC-\( \alpha_3 G163A \) spheroids (Figure S3h) but higher than those observed in \( \alpha_3 \)KO spheroids (Figures 2d and 3f). Therefore, \( \alpha_3 \beta_1 \) can still support some FAK/Src activity independently of laminin or CD151 association.

Together, our data show that even though FAK/Src activation is required, it is not sufficient to support Akt and STAT3 signaling in our model. In transformed KCs, efficient Akt and STAT3 activation also depends on the cell–cell contact-stabilizing function of CD151, a binding partner of \( \alpha_3 \beta_1 \).

\( \alpha_3 \beta_1 \) and CD151 are needed for 3D growth of differentiating KCs

Previous studies showed that Akt and STAT3 play important roles in the differentiation of KCs (Saeki et al., 2012; Segrelles et al., 2007). Whereas STAT3 signaling prevents terminal differentiation of KCs through the regulation of the transcription factor activator protein-1 (Saeki et al., 2012), activation of Akt promotes the survival of differentiating KCs (Calautti et al., 2005; Peng et al., 2003). The activation of PI3K/Akt in primary mouse KCs occurs concomitantly with the expression of KC differentiation markers and, as in \( \alpha_3 \)KO-WT MSCC spheroids, depends on the E-cadherin–mediated adhesion and the activity of the Src family kinases (Calautti et al., 2005). We therefore wondered whether \( \alpha_3 \beta_1 \) and CD151 play a role in the differentiation of MSCCs through the regulation of Akt and STAT3 signaling. In \( \alpha_3 \)KO-WT MSCC spheroids, we could observe a single outer layer of keratin (K) 14–positive basal KCs that adhere to laminin-332 and express integrin \( \alpha_6 \beta_4 \), and a variable number of inner, postmitotic suprabasal cells that express K10 (Figure 5a and Supplementary Figure S4a). The \( \alpha_3 \)KO spheroids were small, containing only a few \( \alpha_6 \beta_4 \)-expressing KCs and no K10-positive cells. Interestingly, several of the KCs in the outer cell layer of MSCC-\( \alpha_3 G163A \) spheroids exhibited the expression of K10 together with laminin-332. Because this was not found in WT spheroids, it is likely that \( \alpha_3 \)β1–laminin-332 ligation prevents aberrant differentiation (Figure 5a). Strikingly, none of the CD151-KO KCs expressed K10, whereas laminin-332 colocalized with \( \alpha_6 \beta_4 \) in addition to \( \alpha_3 \)β1 in the outer and inner layers of the spheroids (Figures 4a and 5a). Furthermore, consistent with the roles of Akt and STAT3 in promoting the survival of differentiating KCs, activated Akt and STAT3 could be observed in the K10-positive differentiating \( \alpha_3 \)WT- and \( \alpha_3 G163A \)-transformed KCs (Figure 5b).

Thus, whereas sustained 3D growth of basal-like CD151-KO KCs primarily depends on FAK/Src signaling and less on the strong activity of Akt and STAT3, the growth of spheroids that similarly to papillomas contain differentiating KC layers requires FAK/Src, STAT3, and Akt activation (Figure 2a, b, and g).

Final remarks

We previously showed that \( \alpha_3 \)KO and CD151-KO mice displayed increased epidermal turnover and loss of slow-cycling stem cells (Ramovs et al., 2020; Sachs et al., 2014, 2012). However, skin thickness and a number of proliferative KCs were similar between \( \alpha_3 \)KO and WT mice during DMBA/TPA treatment (Supplementary Figure S4b) (Sachs et al., 2012), which suggests that the increased rate of proliferation is coupled with enhanced differentiation to maintain the faster turnover of KCs in the \( \alpha_3 \)KO mice. The question, therefore, arises on how to relate these observations to our new findings. In \( \alpha_3 \)KO mice, decreased STAT3 and Akt activity may accelerate the terminal differentiation of the suprabasal KCs, leading to an increased rate of proliferation of basal KCs and the loss of slow-cycling cells. Because it is generally accepted that DMBA-initiated basal KCs must persist long enough in hyperplastic skin to acquire additional mutations leading to the outgrowth of clonal papillomas, eventual tumor outgrowth could depend on the Akt- and STAT3-regulated survival of suprabasal KCs. However, the deletion of \( \alpha_3 \)β1 results also in the increased epidermal turnover of the nontreated mouse skin, which consists of much thinner epidermis and where \( \alpha_3 \)β1 is mostly restricted to the basal KCs. Thus, the loss of stem cells in DMBA/TPA-primed skin of \( \alpha_3 \)KO mice is likely caused by a combination of reduced adhesion strength of \( \alpha_3 \)KO basal KCs, reflected in reduced FAK/Src activation, and an increased rate of terminal differentiation, promoted by decreased Akt and STAT3 activation in \( \alpha_3 \)β1-depleted suprabasal KCs.

In conclusion, \( \alpha_3 \)β1 is a key regulator of several protumorigenic pathways during the initiation of DMBA/TPA-mediated tumorigenesis. In transformed KCs, \( \alpha_3 \)β1-mediated FAK/Src activation leads to strong Akt and STAT3 signaling when CD151 is present, promoting cell–cell contact stability and Smad2 phosphorylation. Remarkably, \( \alpha_3 \)β1-CD151 can support Akt and STAT3 activity independently of \( \alpha_3 \)β1 ligation by laminin-332 and as such promote prosurvival signaling in suprabasal-differentiating KCs, which likely delays epidermal turnover and enables eventual papilloma formation. For tumor outgrowth, transformed KCs depend on CD151-independent \( \alpha_3 \)β1-mediated FAK/Src activation, which promotes the proliferation of basal KCs, and on \( \alpha_3 \)β1-CD151–mediated STAT3 and Akt activation in suprabasal layers, which is required for the 3D growth of differentiating transformed KCs (Figure 6).

MATERIALS AND METHODS

Animal experiments

Epidermis-specific K14tm1(cre)Wbm; \( \alpha_3 G163A \) (\( \alpha_3 \)KO mice) and K14tm1(cre)Wbm (\( \alpha_3 \)WT mice) on FVB/N background have been previously described (Sachs et al., 2012). DMBA/TPA
tumorigenesis has been done before (Sachs et al., 2012). Briefly, the backs of 6-week-old mice were shaved, and after a week, they were topically treated with 30 μg (in 200 μl acetone) of DMBA (Sigma, St. Louis, MO: D3254), followed by biweekly topical applications of 12.34 μg (in 200 μl acetone) of TPA (Sigma; P1585) for 20 weeks. For short-term DMBA/TPA treatment, mice were treated with four doses of TPA for over 2 weeks after DMBA treatment. Thereafter, the mice were killed, and the skin was isolated and processed for immunofluorescence and immunohistochemistry analysis and/or protein lysate preparation. All animal studies were performed according to the Dutch guidelines for care and use of laboratory animals and were approved by the animal welfare committee of the Netherlands Cancer Institute.

Cell culture and generation of stable cell lines
Itga3-WT and Itga3-KO MSCCs were generated as described (Sachs et al., 2012). A detailed description of the generation of other cells can be found in the Supplementary Materials and Methods. Cell lines were cultured in DMEM with 10% heat-inactivated fetal calf serum and antibiotics at 37 °C in a humidified, 5% carbon dioxide atmosphere. For 3D cell culture, 70 μl of GF-reduced Matrigel Basement Membrane Matrix (Corning Technology, Corning, NY; 354230) was pipetted per well of 96-well plate and incubated for 30 minutes at 37 °C. A total of 1,000 cells in cold DMEM containing 10% fetal calf serum and 2% Matrigel were seeded on top of the Matrigel layer and grown for 7 days with or without inhibitors and/or function-blocking antibodies (detailed explanation is in the Supplementary Materials and Methods).

Immunofluorescence, immunohistochemistry, western blotting, flow cytometry, adhesion assay, and statistical analysis
We applied a standard methodology that can be found in the Supplementary Materials and Methods.

Data availability statement
No large datasets were generated or analyzed during this study.

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CONFLICT OF INTEREST
The authors state no conflicts of interest.

ACKNOWLEDGMENTS
We would like to thank Yoshikazu Takada, Daniel Peeper, and Rene Bernards for sharing their reagents, Coert Margadant, and Roy Zent for useful discussions and for proofreading the manuscript. This work was supported by a grant from the Dutch Cancer Society.

AUTHOR CONTRIBUTIONS
Conceptualization: VR, AS; Formal Analysis: VR; Investigation: VR, AKG; Methodology: VR, MK; Supervision: AS; Visualization: VR; Writing - Original Draft Preparation: VR; Writing - Review and Editing: AS

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.07.024.

REFERENCES


SUPPLEMENTARY MATERIALS AND METHODS

Generation of stable cell lines

To obtain *Itga3*-knockout (KO) mouse squamous carcinoma cells (MSCCs), stably expressing wild-type *z3* (*Itga3-KO MSCC-z3*WT), full-length human *z3A* cDNA was ligated into pUC18-z3. After digestion with SpHh, *z3A* cDNA was ligated into LZRS-IREs-zeo, a modified LZRS retroviral vector conferring resistance to zeocin (Kinsella and Nolan, 1996). The retroviral vector was introduced into the Phoenix packaging cells by the calcium phosphate precipitation method, and virus-containing supernatant was collected (Kinsella and Nolan, 1996). *Itga3*-KO MSCCs were infected with the recombinant virus by the 1,2-dioleoyl-3-trimethylammonium-propane method (Boehringer Ingelheim, Ingelheim am Rhein, Germany). After infection overnight at 37 °C, infected cells were selected with 0.2 mg/ml zeocin (Invitrogen, Carlsbad, CA). Cells expressing *z3B1* were obtained by FACS. To obtain *Itga3*-KO MSCCs, stably expressing laminin-binding mutant *z3G163A* (Zhang et al., 1999) (MSCC-z3G163A), *z3G163A* in pBJ-1 expression vector (a kind gift from R. Bernards, The Netherlands) or active myristoylated protein kinase B (Akt) (Huang et al., 2012) (a kind gift from M. Aumailey), rabbit anti-integrin *a3* (Santa Cruz, CA; sc-374242, 1:500). For immunofluorescence, we used primary antibodies: goat anti-integrin *a3* (R&D Systems, Minneapolis, MI; AF2787, 1:50), mouse anti-human integrin *a3* (Santa Cruz, CA; sc-374242, 1:500). For western blot, we used primary antibodies: rabbit anti-phosphorylated STAT3 Y705 (Cell Signaling, Danvers, MA; #9131S, 1:100), rabbit anti-pSmad2 1984, 1:100), rabbit anti-pStat3 Y705 (Cell Signaling; 9131S, 1:100), rabbit anti-phosphorylated Akt (Cell Signaling; 9271, 1:100), rabbit anti-pSrc Y416 (Cell signaling, #2101, 1:1,000), rabbit anti-integrin *a3* (Santa Cruz, CA; sc-374242, 1:500). For immunohistochemistry, primary antibodies were used: rabbit anti-phosphorylated STAT3 Y705 (Cell Signaling, Danvers, MA; #9145, 1:100), rabbit anti-Ki67 (Abcam, Cambridge, United Kingdom; ab15580, 1:3,000), and mouse anti-integrin *a3* (Santa Cruz, CA; sc-374242, 1:500). For immunofluorescence, we used primary antibodies: goat anti-integrin *a3* (R&D Systems, Minneapolis, MI; AF2787, 1:50), mouse anti-human integrin *a3* (Santa Cruz, CA; sc-374242, 1:500). For western blot, we used primary antibodies: rabbit anti-phosphorylated STAT3 Y705 (Cell Signaling, Danvers, MA; #9131S, 1:100), rabbit anti-phosphorylated Akt (Cell Signaling; 9271, 1:100), rat anti-integrin β4 (BD Pharamceutical, Franklin, NJ; 346.11A, 1:50), rabbit anti-laminin-332 R14 (1:400, kind gift from M. Aumailey), rabbit anti-keratin 14 (Covance, Princeton, NJ), rabbit anti-keratin 10 (non-diluted supernatant, kind gift from D. Ivanji [Ivanji et al., 1989]). For secondary antibodies, we used 1:200 of goat anti-rabbit Alexa Fluor 488, donkey anti-rabbit Alexa Fluor 594, donkey anti-goat Alexa Fluor 488, donkey anti-goat Alexa Fluor 647, donkey anti-rat Alexa Fluor 488, donkey anti-rat Alexa Fluor 647, donkey anti-mouse Alexa Fluor 488, and goat anti-mouse Alexa Fluor 647 and goat anti-mouse Alexa Fluor 568 (Invitrogen). For flow cytometry, we used primary rat anti-CD151 (R&D Systems; MAB4609, 1:50) and mouse anti-human integrin *a3* J143 ([Fradet et al., 1984], 1:100) antibodies. For secondary antibodies, donkey anti-rat PE (Biolegend, San Diego, CA; #406421; 1:200 dilution) and donkey anti-mouse PE (Biolegend; #406421; 1:200 dilution) were used. For western blot, we used primary antibodies: rabbit anti-phosphorylated FAK Y397 (Invitrogen; 44-624G, 1:1,000), mouse anti-FAK (BD transduction laboratories; Franklin, NJ; #610087, 1:1,000), rabbit anti-pERK1/2 T202/Y204 (Cell Signaling; #4376s, 1:1,000), rabbit anti-ERK1/2 (Cell signaling; #9102, 1:1,000), rabbit anti-integrin *a3* (homemade, 1:2,000), rabbit anti-pSrc Y416 (Cell signaling, #2101, 1:1,000), rabbit anti-Src (Cell signaling, #2123, 1:1,000), rabbit anti-CD151 140190 (Sachs et al., 2006; 1:500), rabbit anti-pSmad2 S465/467 (Cell signaling; #31085, 1:1,000), mouse anti-GAPDH (Calbiochem, San Diego, CA; CB1001, 1:1,000), and mouse anti-tubulin (Sigma, St. Louis, MO; B-5-1-2, 1:5,000). As secondary antibodies, we used stabilized goat
anti-mouse HRP-conjugated and stabilized goat anti-rabbit HRP-conjugated (Bio-Rad Laboratories, Hercules, CA).

Immunohistochemistry

Tumors and skin were isolated, fixed in ethanol glacial acetic acid mixture (3:1), containing 2% of formaldehyde and/or formaldehyde, embedded in paraffin, sectioned, and stained. Images were taken with the Aperio ScanScope (Aperio, Vista, CA), using ImageScope software, version 12.0.0 (Aperio). Count of phosphorylated-STAT3-positive nuclei was performed blindly. Image analysis was performed using ImageJ (Rueden et al., 2017; Schindelin et al., 2012).

Immunofluorescence

The skin was isolated, embedded in Tissue-Tek optimal cutting temperature cryoprotectant, and frozen. Frozen skin was cryosectioned, and sections were fixed in ice-cold acetone and blocked with 2% BSA (Sigma) in PBS for 1 hour at room temperature. Isolated spheroids were mounted on Poly-L-Lysine-coated slides (Santa Cruz; 25988-63-0), fixed in 4% paraformaldehyde in PBS for 10 minutes, permeabilized with 0.2% Triton-X-100 for 5 minutes, and blocked with PBS containing 2% BSA for 1 hour at room temperature. Tissues or spheroids were incubated with primary antibodies in 2% BSA in PBS overnight, followed by incubation with secondary antibodies diluted at 1:200 for 60–90 minutes. All samples were counterstained with DAPI for 5 minutes at room temperature. Cryosections were mounted in Vectashield (Vector Laboratories, Burlingame, CA; H-1000) and skin sections in Mowiol. Samples were analyzed by Leica TCS SP5 confocal microscope (Leica, Wetzlar, Germany) with 40 and 63× (NA 1.4) oil objectives. When spheroids were analyzed, the thickness of spheroids was determined, and z-stacks with step size every 1.2–1.3 μm were acquired. All images were processed using ImageJ (Rueden et al., 2017; Schindelin et al., 2012). The number of phosphorylated-STAT3-positive nuclei was manually counted in total mouse epidermis, interfollicular mouse epidermis, and in three separate images per z-stack. In spheroids, the percentage of phosphorylated-STAT3-positive nuclei was calculated by dividing the number of phosphorylated-STAT3-positive nuclei in the image with the total number of nuclei on the basis of DAPI staining. The percentage of phosphorylated-Akt-positive area in mouse epidermis was calculated using the Analyze Particle function, with delineated total or interfollicular epidermis as a region of interest. The percentage of phosphorylated-Akt-positive area in spheroids was calculated on maximum intensity-projected z-stacks, with the delineated surface of spheroids based on DAPI staining as a region of interest. The phosphorylated-Akt-positive area was divided by the total region of interest area to define the percentage of phosphorylated-Akt-positive area.

Flow cytometry

KCIs were isolated from mouse back skin as described before (Jensen et al., 2010), or MSSC KCs were trypsinized. Cells were further washed in PBS containing 2% fetal calf serum, incubated for 1 hour at 4°C with the primary antibody in PBS 2% fetal calf serum, washed, and incubated with secondary antibody for 30 minutes at 4°C. Cells were analyzed on a Becton Dickinson FACS Calibur analyzer after addition of the indicated life/dead cell marker. For FACs, α3-positive or CD151-negative cell populations were obtained using a Becton Dickinson FACS Aria Ilu cell sorter.

Western blotting

Protein lysates were obtained from KCs, isolated from mouse back skin as described before (Jensen et al., 2010), from isolated spheroids or from subconfluent cell cultures by lysis in RIPA buffer (20 mM Tris-hydrogen chloride [pH 7.5], 100 mM sodium chloride, 4 mM EDTA [pH 7.5], 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate) and supplemented with 1.5 mM sodium orthovanadate, 15 mM sodium fluoride (Cell Signaling), and protease inhibitor cocktail (Sigma). Lysates were cleared by centrifugation at 14,000 g for 20 minutes at 4°C and eluted in sample buffer (50 mM Tris-hydrogen chloride [pH 6.8], 2% SDS, 10% glycerol, 12.5 mM EDTA, 0.02% bromophenol blue) with a final concentration of 2% β-mercaptoethanol and denatured at 95°C for 10 minutes. Proteins were separated by electrophoresis using Bolt Novex 4–12% gradient Bis-Tris gels (Invitrogen), transferred to Immobilon-P transfer membranes (Millipore Corp, Burlington, MA), and blocked for 1 hour in 2% BSA in Tris-buffered Saline Tween buffer (10 mM Tris [pH 7.5], 150 mM sodium chloride, and 0.2% Tween-20). The blocked membranes were incubated overnight at 4°C with primary antibodies in Tris-buffered Saline Tween containing 2% BSA. After washing, the membranes were incubated for 1 hour with horseradish peroxidase–conjugated goat anti-mouse IgG or goat anti-rabbit IgG (diluted at 1:5,000 in 2% BSA in Tris-buffered Saline Tween buffer). After washing, the bound antibodies were detected by enhanced chemiluminescence using Clarity Western ECL Substrate (Bio-Rad Laboratories) or Amersham ECL Western Blotting Detection Reagent (GE Healthcare, Chicago, IL) as described by the manufacturer. Signal intensities were quantified using ImageJ (Rueden et al., 2017; Schindelin et al., 2012).

Adhesion assay

For adhesion assays, 6-well plates were coated with laminin-332–rich matrix obtained by growing RAC-11P cells (Delwel et al., 1994) to complete confluence, after which the plates were washed with PBS and incubated with 20 mM EDTA in PBS overnight at 4°C. The RAC-11P cells were then removed by pipetting and washing with PBS; 1 × 10⁵ MSCCs were seeded on the laminin-332–rich matrix and left to grow overnight. Images of cells showing different ability to spread on laminin matrix were obtained with a Zeiss AxioObserver Z1 inverted microscope, utilizing 5× and 10× objectives, a Hamamatsu ORCA AG Black and White CCD camera, and a Zeiss ZEN software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 7.0c). Unpaired two-tailed t-test was used for comparisons of experimental groups with a control group. Experiments with more than two experimental groups were analyzed using one-way ANOVA. Planned comparisons were conducted using Sidak’s multiple comparison test after a global ANOVA was determined to be significant. Results with a P-value < 0.05 were considered significantly different from the null hypothesis.
SUPPLEMENTARY REFERENCES


Supplementary Figure S1. Integrin α3β1 is required for full activation of protumorigenic STAT3, Akt, and FAK signaling. (a) IHC staining for integrin α3β1 in papilloma formed after long-term DMBA/TPA treatment of Itga3-WT mice. Bar = 500 µm. (b) Representative bright-field and IF images and quantifications of the area (n = 80), pSTAT3 (Y705)-positive nuclei (n = 20), and pAkt (S473)-positive area (n = 20) of nontreated and 2 µM statict-treated Itga3-WT spheroids. Bar = 20 µm (IF) and 50 µm (bright field). Statistics: mean ± SD, unpaired t-test, *p < 0.0001. (c) Left: WB showing pFAK (Y397) expression in Itga3-WT and Itga3-KO MSCCs transfected with a construct encoding CA-FAK, grown in 2D or as spheroids. Even though the CA-FAK construct is only expressed at low levels in spheroids (upper band), this suffices for the downstream activation of Akt and STAT3 as well as for the activation of endogenous FAK (lower band). Right: quantification of total pFAK expression in the spheroids (as indicated by the dash-line box) Statistics: mean ± SD, two dimension; Akt, protein kinase B; AU, arbitrary unit; CA, constitutively active; DMBA, 7,12-dimethylbenz[a]anthracene; FAK, focal adhesion kinase; IF, immunofluorescence; IHC, immunohistochemistry; KC, keratinocyte; KO, knockout; MSCCs, mouse squamous carcinoma cells; pAkt, phosphorylated Akt; pFAK, phosphorylated FAK; pSTAT3, phosphorylated STAT3; STAT, signal transducer and activator of transcription; TPA, 12-O-tetradecanoylphorbol-13-acetate; WB, western blot; WT, wild type.
Supplementary Figure S2. a3β1 supports STAT3 and Akt signaling independently of its ligation by laminin matrix. (a) Histogram of surface levels of a3β1 in Itga3-KO MSCC spheroids and Itga3-KO MSCC spheroids with stable expression of a3WT or a3 laminin–binding mutant G163A (a3G163A). (b) Itga3-KO– and Itga3-KO MSCCs expressing a3G163A show reduced adhesion to laminin-rich matrix compared with Itga3-WT– and Itga3-KO–expressing a3 WT MSCCs. Bar = 50 μm. (c) Representative bright-field and IF images and quantifications of the area (n = 80), pSTAT3 (Y705)-positive nuclei (n = 20), and pAkt (S473)-positive area (n = 28) of Itga3-KO spheroids rescued with human a3WT construct and treated with function-blocking J143 antibody (10 μg/ml). Bar = 20 μm (IF) and 50 μm (bright field). Statistics: mean ± SD, unpaired t-test. Akt, protein kinase B; IF, immunofluorescence; KC, keratinocyte; KO, knockout; MSCC, mouse squamous carcinoma cell; pAkt, phosphorylated Akt; pFAK, phosphorylated FAK; pSTAT3, phosphorylated STAT3; STAT, signal transducer and activator of transcription; WT, wild type.
Supplementary Figure S3. The deletion of CD151 reduces cell–cell contact integrity, resulting in reduced activation of STAT3 and Akt but not reduced FAK/Src signaling. (a) Representative gating strategy of flow cytometry quantifications from Figure 4a. (b) WB showing reduced expression of CD151 upon the deletion of α3β1 in mouse epidermis. (c) Quantification of the WB from Figure 4d. (d) Representative bright-field and IF images and quantifications of the area (n = 80), pSTAT3 (Y705)-positive nuclei (n = 20), and pAkt (S473)-positive area (n = 25) of Itga3-WT spheroids treated with function-blocking DECMA-1 antibody (anti-E-cadherin antibody) (10 μg/ml). Bar = 20 μm (IF) and 50 μm (bright field). (e) Quantification of the WB from figure 4e. (f) Histogram of surface levels of CD151 in Cd151-KO MSCC, MSCC-α3G163A, and MSCC-α3WT spheroids and MSCC-α3G163A and -α3WT spheroids with Cd151 deletion. (g) Bright-field images and quantifications (n = 85) of the size of MSCC-α3WT spheroids, Cd151-KO MSCC-α3WT spheroids, and Cd151-KO MSCC-α3WT spheroids treated with α3-blocking J143 antibody (10 μg/ml). Bar = 50 μm. (h) WB and quantification of the pFAK and pSrc levels in MSCC-α3G163A and MSCC-α3WT spheroids with deletion of Cd151. Statistics: mean ± SD, (c, d, h) unpaired t-test; (e, g) Sidak’s multiple comparisons test; *P < 0.05, ****P < 0.0001. Akt, protein kinase B; AU;
Supplementary Figure S4. Basal-like KCs present the biggest population of proliferating epidermal cells in vivo and in vitro.  
(a) IF staining of Itga3-WT MSCC spheroids showing K14 expression and BrdU-positive proliferating KCs in the outer cell layer. Bar = 20 μm.  
(b) Ki67 staining of short-term DMBA/TPA-treated Itga3-WT and KO skin shows that the majority of suprabasal KCs are nonmitotic regardless of the presence of α3. Bar = 50 μm. DMBA, 7,12-dimethylbenzaanthracene; IF, immunofluorescence; K, keratin; KC, keratinocyte; KO, knockout; TPA, 12-O-tetradecanoylphorbol-13-acetate; WT, wild type.