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The diverse roles of integrin α3β1 in cancer: Lessons learned from skin and breast carcinogenesis

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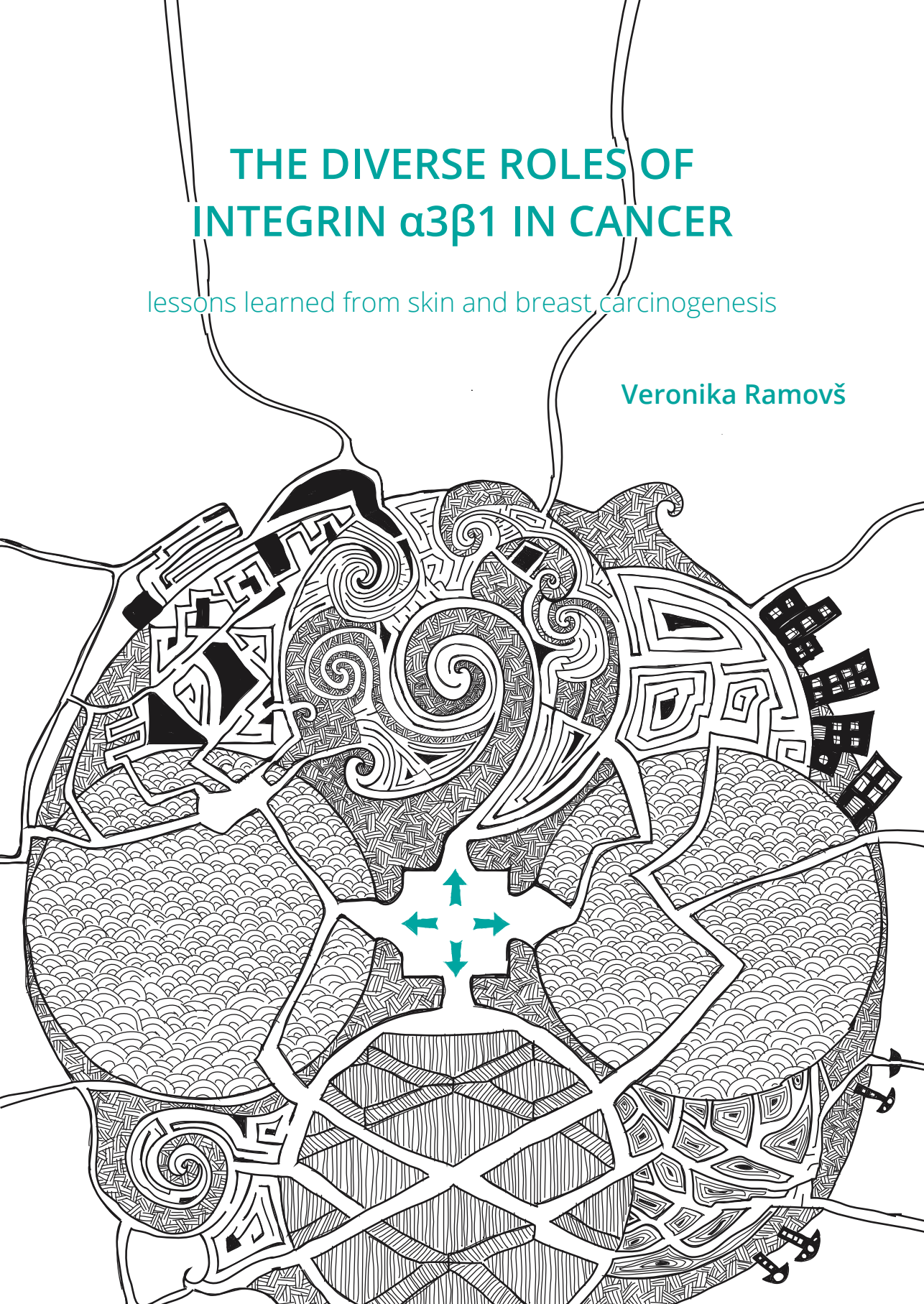
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THE DIVERSE ROLES OF INTEGRIN α3β1 IN CANCER

lessons learned from skin and breast carcinogenesis

Veronika Ramovš



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From the perspective of the researcher, the role of α3β1 in cancer depends on "time" and "place"; the stage and the type of cancer investigated. Thus, conducting such research sometimes feels like being dropped in the middle of a maze - depending on the first turn you take, you might end up following different paths. Adding a few dead ends, it is also a somewhat appropriate metaphor for the progression of my PhD. At least sometimes.

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THE DIVERSE ROLES OF INTEGRIN α3β1 IN CANCER

lessons learned from skin and breast carcinogenesis

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INTRODUCTION AND SCOPE OF THE THESIS

Half of the century has passed since we discerned that the survival of normal epithelial cells depends on their adhesion to the extracellular matrix and that this limitation can be overcome by transformed cells during the progression of carcinogenesis [1,2]. Moving two decades forward, integrins, a family of transmembrane proteins that mediate cell-extracellular matrix adhesion, emerged as the main regulators of this process [3]. Since then, our knowledge on integrin-mediated adhesion and signaling in normal and cancer cells has grown by leaps and bounds with novel roles of integrins beyond cell-matrix adhesion emerging [4,5].

The groundwork for this thesis was laid by former graduate student Norman Sachs and by postdoctoral researcher Pablo Secades, both working as my predecessors in the research group of prof. dr. Arnoud Sonnenberg. They were the first to observe the dramatic effect of the epithelial deletion of the laminin-binding integrin α3β1 on skin carcinogenesis: mice, lacking α3β1 in the skin were almost completely protected against tumor formation induced by two-stage chemical carcinogenesis protocol. Their research also reinforced the notion that the roles of integrin α3β1 in cancer can be diverse and even opposing at different stages of the disease: when α3β1 was absent from the epidermis during the progression of cutaneous carcinogenesis, the invasive potential and the malignant grade of carcinomas increased [6].

In the light of the crucial but often diverse roles that integrin α3β1 can have in cancer (discussed in depth in chapter 2), we reasoned that there is a need for a better understanding of the function of this integrin in well-defined histopathological types and stages of cancer. This research thesis was launched with the goal to uncover the mechanism behind the previously demonstrated essential role of integrin α3β1 during the initiation of non-melanoma skin tumorigenesis. I was fortunate enough to expand our objective to HER2-driven breast carcinogenesis, common epithelial cancer in which the role of α3β1 has not been thoroughly investigated before. Here, we briefly introduce both cancer types and present the scope of this thesis.

NON-MELANOMA SKIN CANCER

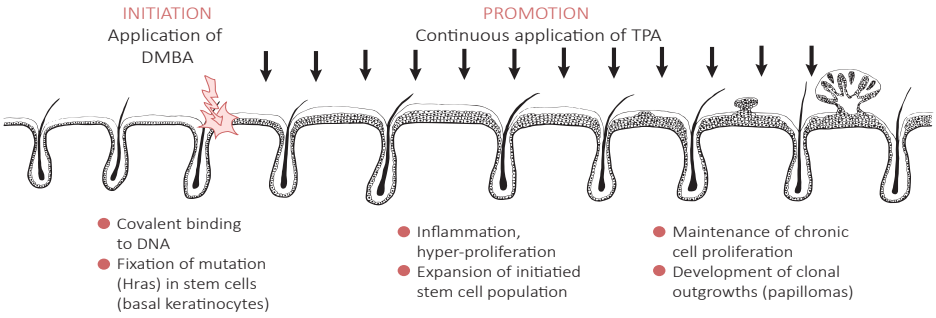


Figure 1: Two-stage chemical carcinogenesis model (DMBA/TPA treatment).

Non-melanoma skin cancer is the 5th most common cancer, with a rough estimate of one million diagnoses worldwide in 2018. Although the first stages of the disease can be successfully treated, late detection of invasive non-melanoma skin cancer often offers poor prognosis [7]. To better understand the molecular changes, driving different stages of the disease, a mouse skin model of multi-stage chemical carcinogenesis (also called DMBA/TPA treatment) has been developed over 60 years ago and since then extensively studied on various transgenic mouse models [8]. The first stage of the model, i.e. initiation, consists of a single application of carcinogen 7,12-dimethylbenz[a]-anthracene (DMBA), which causes an activating mutation in *Hras* gene. Although this event is irreversible, second, promotion stage needs to take place for the outgrowth of benign tumors called papillomas. The common promotion agent is phorbol ester, 12-Otetradecanoylphorbol-13-acetate (TPA), which is applied bi-weekly for 20 weeks for full two-stage chemical carcinogenesis protocol (**Fig. 1**). If the TPA-treatment is continued, some of the papillomas eventually will progress into invasive squamous carcinomas and metastasize [8]. Pro-inflammatory TPA-treatment causes activation of several growth factor signaling pathways, leading to hyperproliferation of the skin and the expansion of DMBA-initiated keratinocytes into papillomas. Decades of the research on transgenic mouse models helped us to identify the main signaling pathways that play a central role in this tumorigenesis (**Fig. 2**).

As DMBA-initiated keratinocytes have to persist in epidermis sufficiently long to accumulate additional mutations before they can outgrow into clonal papillomas, the target cell population for tumor initiation has long been determined as epidermal stem cells: slow-cycling keratinocytes, located in the basal layer of epidermis [11]. However, the contributions of specific epidermal stem cell populations to DMBA/TPA-initiated

tumorigenesis has remained a controversial topic, with hair bulge stem cells often suggested to be the main reservoir for tumor-initiating cells [12].

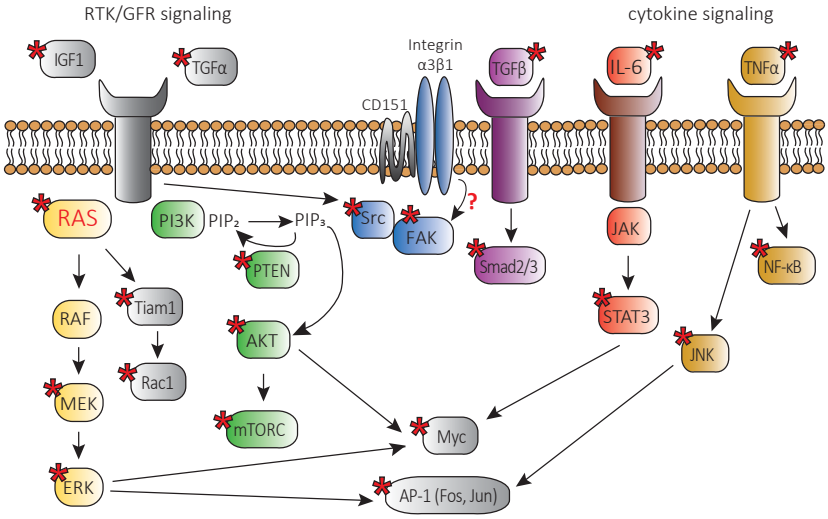


Figure 2: Simplified scheme of the main signaling pathways that have been shown to play a central promoting role in the DMBA/TPA-driven tumor formation.
Asterisk: signaling components have been directly investigated for their *in vivo* functions during skin tumor development using transgenic mouse models. RTK: receptor tyrosine kinase. GFR: growth factor receptor. Adjusted from: [9,10].

Even though our group has demonstrated the indispensable role of integrin α3β1 during the initiation of DMBA/TPA-induced tumorigenesis, the mechanism behind it remains largely speculative and fails to resolve the relation of α3β1 with known key oncogenic signaling pathways of this model. The mice with epidermal deletion of α3β1 exhibited an increased epidermal turnover and the loss of slow-cycling keratinocytes, residing in the hair follicles and in interfollicular epidermis. These observations coincided with the miss-localization of keratinocytes expressing keratin 15, a marker of hair bulge stem cells. Thus, the hair bulge stem cells were suggested to be the main reservoir for tumor-initiating cells in our mouse model and the mechanism behind the absence of tumor formation upon α3β1 deletion was proposed to be their loss by premature efflux and terminal differentiation [6].

HER2-DRIVEN BREAST CANCER

Breast cancer is a heterogeneous disease, which can be categorized into several subtypes. Majority of breast cancers are carcinomas, i.e. they arise from epithelial cells, and can be further divided into at least six distinct "intrinsic" subtypes based on global gene expression analyses: luminal A, luminal B, HER2-enriched, basal-like (i.e. triple-negative) and claudin-low tumors, as well as a normal breast-like group [13]. HER2-enriched subtype is defined by gene amplification and/or overexpression of a member of the epidermal growth factor receptor family, epidermal growth factor receptor 2 (HER2), leading to activation of PI3K/Akt and MAPK/ERK signaling pathways [14]. Roughly 20-25% of breast cancers are classified as HER2-enriched and even though their treatment strategy has come far with several HER2-targeting therapies available, HER2-positive advanced cancer still remains an aggressive disease, associated with a poor prognosis and survival outcome [15].

Consistent with the heterogeneity of the breast cancer, the role of α3β1 in this disease strongly varies depending on the study and/or the disease model (described in depth in chapters 2 and 5). Whereas the pro-survival and pro-proliferative role of α3β1 in basal-like breast cancer types has been quite established [16,17], the role of α3β1 in HER2-enriched mammary cancer remains to be investigated *in vivo*.

SCOPE OF THE THESIS

In this thesis, we aim to shed light on the diverse and often opposing roles of integrin α3β1 in cancer. In **chapter 2**, we provide an overview of current literature on two major laminin-binding integrins in epidermal cells: α3β1 and α6β4, both of which are known to act as promoters and suppressors of tumorigenesis and tumor progression, depending on the cell type and context. We speculate the conditions that define the nature of their role in cancer and we establish the importance of determining their function in well-defined tumor types and cancer stages. In **chapters 3 and 4**, we focus on the role of integrin α3β1 in the first stages of non-melanoma skin cancer using the two-stage chemical carcinogenesis model. In **chapter 3**, we uncover that α3β1 in hair bulge stem cells contributes only moderately to the formation of papillomas and that this contribution is indirect, via the promotion of a tumor permissive environment. We refute the original hypothesis that the dramatic effect of epidermal *Itga3* deletion on tumor formation is due to an efflux of hair bulge stem cells, thus reopening the question of the mechanism behind the essential role of α3β1 in DMBA/TPA-driven tumorigenesis. We address this in **chapter 4**, where we uncover that α3β1 plays a central role in promoting the activation of several pro-tumorigenic signaling pathways and together

with the tetraspanin CD151 regulates signaling molecules that control the survival of differentiating keratinocytes. In **chapter 5**, we extended our research to breast cancer and uncovered that the downregulation of α3β1 in HER2-driven mouse model and in HER2-enriched human mammary carcinoma cells promotes tumor progression and invasiveness of the cells. We show that the role of α3β1 in cell invasion depends on environmental factors and reaffirm that this role is specific for HER2-enriched cell-type. In **chapter 6**, we discuss the remaining questions, address the potential future research and provide future perspectives through the preliminary data based on human skin biopsies and human colorectal organoids.

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THE OPPOSING ROLES OF LAMININ-BINDING INTEGRINS IN CANCER

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ABSTRACT

Integrins play an important role in cell adhesion by linking the cytoskeleton of cells to components in the extracellular matrix. In this capacity, integrins cooperate with different cell surface receptors, including growth factor receptors and G-protein coupled receptors, to regulate intracellular signaling pathways that control cell polarization, spreading, migration, survival, and gene expression. A distinct subfamily of molecules in the integrin family of adhesion receptors is formed by receptors that mediate cell adhesion to laminins, major components of the basement membrane that lie under clusters of cells or surround them, separating them from other cells and/or adjacent connective tissue. During the past decades, many studies have provided evidence for a role of laminin-binding integrins in tumorigenesis, and both tumor-promoting and suppressive activities have been identified. In this review we discuss the dual role of the laminin-binding integrins α3β1 and α6β4 in tumor development and progression, and examine the factors and mechanisms involved in these opposing effects.

LAMININ-BINDING INTEGRINS, WHAT THEY ARE AND WHAT THEY DO

Laminins are large heterotrimeric extracellular matrix (ECM) glycoproteins that contain an α, a β, and a γ chain. They are major components of the basement membrane (BM) that separates the nervous system, epithelial, endothelial, fat and muscle cells from adjacent connective tissue [1]. The BM, however, is not just a physical barrier; it also contributes to the adhesion, proliferation, migration and survival of cells. Integrins are heterodimeric transmembrane glycoproteins that function as adhesion receptors for ligands in the extracellular matrix (ECM) and transduce mechanical signals from the ECM into biochemical signals within the cell. Four integrins recognize laminins as their extracellular ligands: α3β1, α6β1, α7β1 and α6β4 (reviewed in [2]). Their specificity and affinity for binding to various laminin isoforms differ considerably [3–5] (**Table 1**). Alternative mRNAs splicing of the α3, α6 and α7 subunits further increases the functional diversity of these laminin-binding integrins by generating evolutionary conserved isoforms with different affinities for ligand binding and signaling activities [6]. The α6 and α7 subunits have distinct isoforms that differ in both their extracellular (X1 and X2) and cytoplasmic domains (A and B), while the α3 subunit only exists as two distinct cytoplasmic variants [7–14]. The expression of these isoforms is tissue specific and developmentally regulated [15–18], however a full understanding of their role is still lacking.

Integrin α6β4 is expressed at the base of most epithelial cells, but also by a subset of endothelial cells [19] and by perineural fibroblasts and Schwann cells in peripheral nerves [20,21]. It mediates cell adhesion to laminins and plays a crucial role in the formation of specific cell-matrix complexes, *i.e.* hemidesmosomes (HDs) [22]. Hemidesmosomal dysfunction is associated with a group of inherited disorders called epidermolysis bullosa, symptoms of which are severe blistering of the skin and mucosal membranes [23]. Mice lacking either the integrin α6 or β4 subunit display very similar defects in skin and mucosal membranes, and die perinatally [24–26].

Despite sharing the common β1 subunit, the integrins α3β1, α7β1 and α6β1 have unique functions and distinct distribution patterns. Integrin α3β1 is most abundant in skin, kidneys, lungs, intestine, bladder and stomach. In these tissues, it mediates adhesion of epithelial cells to laminin-332 and -511 in the BM, and plays a role in the maintenance of cell-cell contacts. Recently, mutations in the gene encoding the α3 subunit (*ITGA3*) have been identified in patients suffering from a congenital nephrotic syndrome, interstitial lung disease and a mild form of epidermolysis bullosa [27–29]. Similar symptoms have been previously described in genetically engineered mice lacking α3β1 [30]. Notably, the skin defects observed in the absence of α3β1 occur

early in life and are associated with micro-blisters and a disorganized BM. Later in life, these defects are no longer observed [31,32].

LAMININ ISOFORM SPECIFICITY		GENE	MOUSE PHENOTYPE		HUMAN DISEASE
-332 -511/521	α6β 4	***Itgb4***	LETHAL, Perinatal	Severe skin blistering	Epidermolysis bullosa
-111, -332, -511/521 -211/221, -411	α6β1	***Itga6***	LETHAL Birth	Severe skin blistering, defects in cerebral cortex and retina	Epidermolysis bullosa
-511/521, -332 -211/221	α3β1	***Itgb1***	LETHAL E 5.5	Inner cell mass deterioration	Lethal
		Itga3	LETHAL Birth	Defects in kidneys, lungs, skin, and cerebral cortex. Disorganization of the BM	Congenital nephrotic syndrome, interstitial lung disease, and epidermolysis bullosa
-211/221 -111, -511/521	α7β1	***Itga7***	VIABLE Fertile	Muscular dystrophy	Congenital myopathy

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

Integrin α7β1 is most prominently expressed in cardiac and skeletal muscles, where it connects muscle fibers to laminin-211/221 in the BM of the myotendinous junction. In line with its function, patients with a loss-of-function mutation in the gene encoding the α7 subunit (*ITGA7*) suffer from congenital myopathy [33], and mice lacking α7β1 develop muscular dystrophy [34].

Finally, integrin α6β1 is predominantly expressed on platelets, leukocytes, gametes and some epithelia. It binds to a wide range of laminin isoforms, with the highest affinity to laminin-111, -511 and -332 [5]. Apart from a defect in laminar organization of the developing cerebral cortex and retina, seen in the α6-deficient mice (but not in β4-deficient mice), no other defects are associated with the absence of this integrin α subunit in mice [26,35]. The β1 subunit is ubiquitously expressed and can bind to as many as 12 different α subunits (reviewed in [2]). Therefore, it is not surprising that its depletion causes a failure of embryonic development [36].

Laminin-binding integrins can be found in two different adhesion complexes, focal adhesions (FAs) and HDs. FAs are dynamic protein complexes that form mechanical links between the ECM and the actomyosin cytoskeleton [37]. The dynamic regulation of FAs and the reorganization of the associated actin cytoskeleton are important determinants for cell migration. HDs are more stable adhesion structures that act as

anchoring sites for intermediate filaments (reviewed in [38–41]). These adhesions need to be disassembled during migration and several mechanisms have been suggested to contribute to the disassembly of HDs, including endocytosis of HD proteins [42,43], laminin chain processing [44], cleavage of the β4 subunit by calpain or caspases [45,46], and phosphorylation of HD proteins [47–52]. Upon dissociation of HDs, α6β4 has been reported to be redistributed to actin-rich filopodia and lamellae [53,54], where it plays a role in the regulation of cell migration. However, the mechanism responsible is poorly understood.

In addition to their role in maintaining structural integrity of tissues, the laminin-binding β1 integrins also function as bidirectional signaling molecules. "Inside-out" signaling regulates the binding affinity and/or avidity of the integrin to its ECM ligand, while "outside-in" signaling is triggered upon adhesion and results in the transduction of signals into the cell (reviewed in [55–58]). As integrins lack intrinsic enzymatic activity, they signal through direct or indirect interactions of their cytoplasmic domains with numerous intracellular effector molecules (reviewed in [59–61]). Classical integrin outside-in signaling triggers autophosphorylation of focal adhesion kinase (FAK) [62]. Consequently, the FAK/Src complex is activated, resulting in the stimulation of multiple downstream signaling pathways, leading to the activation of effectors such as mitogen-activated protein kinases (MAPKs) ERK1/2 and JNK, as well as the Rho-family of small GTPases Cdc42 and Rac1. Through these effector molecules, the laminin-binding β1 integrins regulate cell polarization, spreading, migration, survival, and gene expression of cells [59]. Interestingly, compared to the β1 integrins that bind to fibronectin, the laminin-binding integrins support strong activation of Rac1 and Cdc42, and a minimal activation of RhoA. It has recently been pointed out by Stipp in his expert review [63] that this particular signaling results in the formation of smaller focal contacts on a laminin matrix as well as in dynamic actin cytoskeleton remodeling and rapid cell migration. Although α6β4 is reported to be involved in the activation of many of the kinases mentioned above, α6β4 and β1-integrins use different signaling mechanisms and the current understanding is that α6β4 needs to be dissociated from HDs to fulfill its role in signaling [64]. Whether α6β4 needs to adhere to laminin-332 in order to signal is unclear, since both adhesion-dependent and adhesion-independent signaling have been reported [65–68]. Data suggests that in transformed cells the β4 cytoplasmic domain is phosphorylated on specific tyrosine residues that serve as a docking platform for various signaling molecules to amplify the signaling output of growth factor receptors [69–71].

Integrin-mediated signaling can be additionally enhanced or modulated through interaction of integrins with integrin-associated proteins (IAPs), e.g. tetraspanins [72],

urokinase-type plasminogen activator receptor (uPAR) [73] and several growth factor receptors [74]. Therefore, extensive crosstalk takes place between pathways activated by integrins and other receptors, especially receptor tyrosine kinases (RTKs).

LAMININ-BINDING INTEGRINS AND CANCER

Over the last decades evidence for a role of laminins in cancer has accumulated and been addressed in several excellent reviews [75–77]. Laminin-332, -511 and -111 are reported to be particularly important in carcinogenesis and the motility of tumor cells [77]. Accordingly, α3β1, α6β1 and α6β4 have all been implicated in the development and progression of cancer, but there is little evidence for such role of α7β1 in tumorigenesis and cancer progression [78–81]. In the limited amount of available literature its tumor suppressing function in a variety of tumor cell types, as well as in a reduction of metastatic potential in melanoma cells, is described (reviewed in [63]). Recently it was reported that α7β1 is upregulated in biopsies of hepatocellular carcinoma, while *in vitro* its downregulation caused decreased invasion and migration, indicating that α7β1 can also act as a tumor promoter [82]. Such dual role in cancer is not restricted to α7β1; α6β4 and especially α3β1 can have both, disease suppressive and promoting roles. This does not seem to be the case for α6β1, which has been predominantly characterized as a tumor promoter, contributing to the spreading and invasion of tumors as well as mediating dissemination and the formation of metastases in areas rich in laminin, such as bone matrix and the space surrounding the nerve of the prostate gland. The role of α6β1 in cancer progression has been recently reviewed by others [63,83].

In this review we will focus on laminin-binding integrins with both an inhibitory and a promoting role in cancer, i.e. α3β1 and α6β4, and try to elucidate the factors and mechanisms involved in these opposing effects. Cancer is a disease with several different stages of development, which can be correlated to specific processes that are essential for its progress and development, i.e. hallmarks of cancer [84]. During primary tumorigenesis cancer cells exhibit sustained proliferation and avoidance of apoptosis. With growing tumor mass, a switch of metabolism and angiogenesis become important for the further development of the disease. During later stages of tumor progression, cancer cells acquire invasive properties in order to spread to distant tissues and form metastases. Integrins represent an important link between tumors and their environment as well as between different tumor cells within the tumor mass. Their role in each stage of tumorigenesis therefore will depend on external influences, for example, the molecular composition of the microenvironment and juxtacrine signaling from neighboring cells. Also oncogenic insults can have an effect on the function of integrins in different stages of cancer progression.

INETGRIN α3β1 AND CANCER

The opposing role of laminin-binding integrins in cancer is especially evident when considering α3β1-mediated tumorigenesis events (**Table 2**). From a number of studies it is clear that different stages of cancer can be influenced by the presence or absence of α3β1 and, conversely, that transformed cells can modulate the function of α3β1 by regulating its expression or post-translational modifications, through IAPs and via the induction of α3β1-mediated signaling. Furthermore, the expression of α3β1 in transformed cells can be influenced by oncogenic stimuli, such as the activation of K-Ras, and, vice versa, α3β1 can regulate the expression of a large number of genes in immortalized keratinocytes [85,86]. This suggests that transformed cells can be dependent on α3β1 for sustaining signaling pathways and cellular processes.

TISSUE	UPREGULATED/ CANCER PROMOTER		DOWNREGULATED/ CANCER SUPRESSOR	
	PRIMARY TUMORS	METASTASIS/ INVASION	PRIMARY TUMORS	METASTASIS/ INVASION
SKIN	[87–89]	[87]	[90]	[89]
BRAIN	[91,92]	[93,94]		[95]
ORAL CAVITY	[96]	[97–101]	[102]	[103]
HEAD AND NECK	[104]	[105]		
LUNG	[106,107]	[108,109]	[90,110]	[111]
BREAST	[112–118]	[117,119]	[118]	[118,120–122]
REPRODUCTIVE SYSTEM	[123]			[124–130]
STOMACH AND INTESTINE	[131]	[132–134]		[135,136]
PANCREAS	[137]			
LIVER	[138]	[138]		
BLADDER AND KIDNEYS	[139,140]			[141]
BONE			[142]	

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

Role of α3β1 in supporting sustained proliferation and avoidance of apoptosis

One of the most fundamental traits of cancer is the ability of tumor cells to maintain sustained proliferation. Integrins regulate cell proliferation through adhesion to the ECM [143]. Although the adhesion-dependent control of cell proliferation is generally downregulated in tumors, several studies have shown that proliferation of transformed cells can still be affected by integrin-mediated adhesion. Two recent studies, investigating the role of α3β1 in tumorigenesis of the epithelium of the skin and

mammary gland, showed that α3β1 is essential for the initiation of tumors and efficient proliferation of tumor cells [89,116]. The impaired proliferation in mammary epithelia was associated with the downregulation of activated FAK, resulting in a reduction of active Rac1 and its effector serine/threonine-protein kinase PAK1, and therefore in reduced activation of ERK1/2 and JNK [116]. This is consistent with the results of an earlier study, showing that the engagement of laminin-332 by α3β1 is essential for growth factor-stimulated cell proliferation, mediated through activation of the MAPK signaling pathway [144]. Both studies mentioned above observed that transformed cells deposit laminin into the matrix; therefore, their proliferation may still be dependent on signals, derived from integrin-mediated cell adhesion. In line with this, both α3β1 and laminin are required for an efficient proliferation of various types of tumors and the upregulation of laminin-511 together with α3β1 was shown to be a marker of poor prognosis in breast cancer [117,145,146].

It is therefore evident that α3β1 can promote proliferation of tumors that are adherent to the pre-existing or newly deposited laminin matrix. However, in later stages of carcinogenesis, when tumors rely less on adherent-mediated proliferation, loss of α3β1 may destabilize E-cadherin-mediated cell-cell adhesion, resulting in epithelial to mesenchymal transition (EMT)-like events and consequently increased tumor progression and metastatic growth at distant sites [128,147]. Loss of α3β1 can also contribute to metastatic growth through interactions of tumors with the metastatic environment. It was shown that *in vitro* the adhesion and proliferation of α3-deficient prostate carcinoma cells on laminin-332 was impaired, but the growth of the tumor was increased when injected into mice. Increased growth of α3-depleted tumor cells was also observed *in vitro* when these cells were co- cultured with stromal cells or grown in fibroblast-conditioned medium [129]. These observations further suggest that the role of α3β1 in modulating cancer cell proliferation is dual, depending on the stage of tumor progression (**Fig. 1**).

Integrins also contribute to tumorigenesis by regulating cell survival; ligated integrins can prevent pro- apoptotic signaling cascades initiated by anoikis (cell death by loss of adhesion) and relay survival signals. Studies of transformed cells depleted of α3β1 showed increased activation of caspase-3/7, reduced cell survival and increased radiosensitivity [116,148,149]. In all cases, α3β1 supported survival through adhesion to laminin and initiation of the FAK/ERK signaling pathway, indicating the importance of this mechanism.

Role of α3β1 in tumor-associated angiogenesis

The potential role of α3β1 in regulating angiogenesis has received relatively little attention and is not yet fully understood. In *Itga3* knockout mice the capillary loops in the kidneys are dilated and their number is reduced [30]. Furthermore, conditional deletion of *Itga3* in the epidermis of mice caused impaired cutaneous wound healing, due to a defect in angiogenesis and failure of α3-negative keratinocytes to promote the expression of the pro-angiogenic factor MRP3 (mitogen-regulated protein 3) [150]. In cancer-induced angiogenesis, α3β1 seems to act as both a promoter and suppressor of angiogenesis, and it influences vascular formation when expressed by tumor or endothelial cells (**Fig. 1**). Studies investigating the effect of α3β1 on endothelial cells mostly reported its suppressive function in angiogenesis, due to the inhibition of cyclooxygenase-2 (COX-2)-dependent angiogenic signaling, the regulation of vascular endothelial growth factor (VEGF), or the inhibition of endothelial cell proliferation, migration, and tubule formation [90,110,151,152]. In contrast, when expressed on tumor cells, α3β1 is predominantly associated with the promotion of angiogenesis. The expression of COX-2 and α3β1 is positively correlated in invasive ductal carcinoma, resulting in higher blood vessel density [115]. In MDA-MB-231 breast cancer cells, α3β1 controls the expression of COX-2 and influences endothelial cell function and invasion of tumor cells [112]. A possible explanation for the role of α3β1 in COX-2-mediated angiogenesis and stimulation of the tumor's microenvironment was recently provided by Subbram *et al.* [153], who showed that α3β1 can directly influence COX-2 expression by stabilizing its mRNA. Furthermore, there is data suggesting that the association of α3β1 with non-conventional ligands, such as the noncollagenous domain of the α3 chain of type IV collagen [α3(IV)NC1] [110], a tissue inhibitor of metalloproteinases (TIMP-2) [151,152,154] and thrombospondin-1 (TSP-1) [155,156], has an impact on tumor-associated angiogenesis.

Integrin α3β1-dependent regulation of angiogenesis can be also mediated via its lateral association with tetraspanin CD151, an established IAP (see below). An increased expression of CD151 is correlated with increased vascularity in breast cancer, and *in vitro* experiments in three-dimensional (3D) extracellular matrices showed that CD151 modulates the response of endothelial cells to cancer cells through its association with both α3β1 and α6β4 [157].

Role of α3β1 in invasion and metastasis

The literature describing the role of α3β1 in later stages of tumor progression pays almost equal amount of attention to its cancer promoting and suppressing functions. While in numerous clinical studies a positive correlation between α3β1 expression and tumor invasiveness or poor prognosis has been observed, opposite findings have also

been reported (**Table 2**). A similar trend was observed in *in vitro* studies investigating the invasive and migratory phenotype of cells from transformed cell lines. While an increased expression of α3β1 in a head and neck carcinoma cell line is correlated with a more invasive phenotype [105], a low expression of α3β1 has been associated with reduced migration and invasiveness of many different types of tumor cells [158–160]. On the contrary, loss or inhibition of α3β1 function can result in enhanced migration and invasion of tumor cells [89,124,135,161].

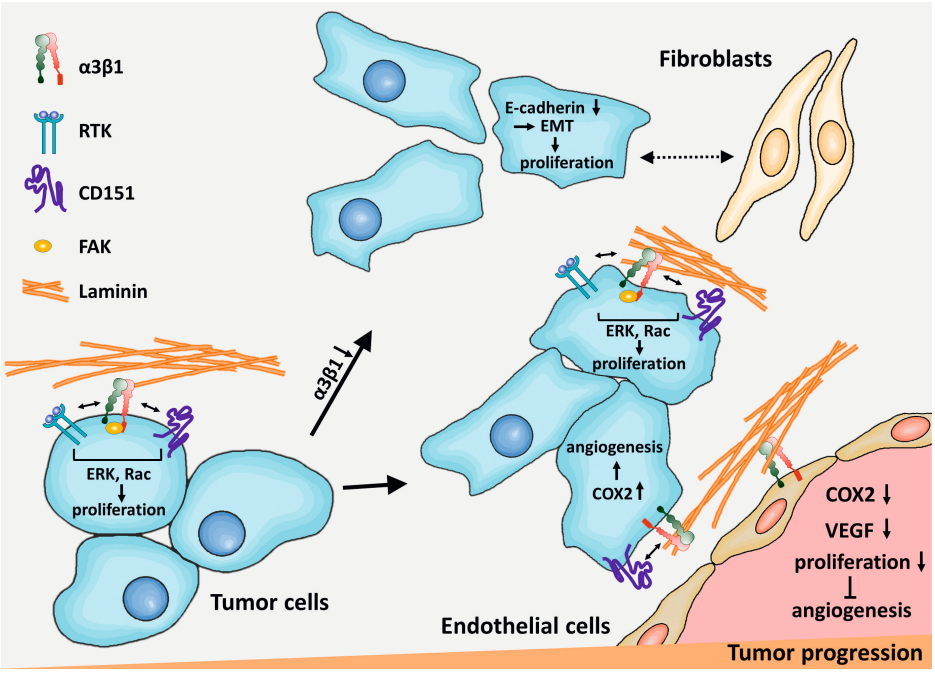


Figure 1: The role of α3β1 in tumor cell proliferation and angiogenesis. Ligation of α3β1 by laminins can result in the initiation of survival and growth signals via activation of FAK signaling, leading to the activation of the MAPK signaling pathway, which can additionally be supported through crosstalk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

Different transcription factors have been reported that can either positively or negatively regulate the expression of α3β1 in tumor cells in order to acquire a more aggressive phenotype [142,162]. This suggests that the presence of α3β1 can have either beneficial or unfavorable effect on tumor cell invasion, and that it is important enough for tumor cells to develop mechanisms for regulating its expression. A remaining question is: what determines whether tumor cells will require either the presence or absence of α3β1 for successful invasion and the formation of metastases?

Laminin-dependent ligation of α3β1

Ligation of α3β1 by laminin-511 or -332 is one of the major events through which this integrin mediates cell adhesion and migration. This makes laminins of key importance for α3β1-mediated effects on cancer progression and invasion. Even more so since, as already discussed, laminins are implicated in the progression and spreading of cancer. In an early study it was shown that α3β1 is essential for the migration of keratinocytes on laminin-332 during wound healing [163]. A decade later Choma *et al*. [164] demonstrated that persistent keratinocyte migration is driven via the interaction of α3β1 with laminin-332, which induces FAK/Src kinase activity, thereby promoting Rac1 activation and polarized lamellipodium extension.

There is a strong positive correlation between the degree of invasiveness of glioma cells and α3β1-mediated migration. As it has been pointed out in a recent review, the attachment of the glioma cells to the ECM must be transient for them to be able to invade [165]. Furthermore, ECM rich in laminin-332 and -511 contributes strongly to the migration of highly invasive gliomas [93,166,167], while downregulation of α3β1 in glioma cells led to decreased migration and invasiveness [93], which was correlated with decreased phosphorylation of ERK1/2 [94]. In invasive protrusions of glioblastomas, α3β1 was found to be co-localized with the Ephrin A2 (EphA2), a known promoter of cancer invasiveness, making it plausible that the cross-talk between EphA2 and α3β1 additionally contributes to the adhesion-dependent signaling that leads to a more invasive phenotype [168,169]. Integrin α3β1-mediated cell adhesion in areas of the brain that are rich in laminin not only drives the invasion of glioma cells, but also plays a role in the formation of brain metastases of non-small cell lung carcinoma [108].

A pattern is now emerging of how laminin-332 and -511 promote the spreading of cancer cells via α3β1. Firstly, they facilitate α3β1-mediated tumor cell migration and invasion from the primary tumor site, which, in addition to the cases mentioned above, was observed in numerous other types of cancers [79,170–176]. Secondly, ligation of α3β1 by laminin-332 can increase the secretion of matrix metalloproteinase-9 (MMP-9), which then further promotes the invasion and migration through the dense ECM

[119,173,177]. Thirdly, laminins deposited by endothelial cells, can mediate α3β1-driven migration of tumor cells and stimulate trans-endothelial tumor cell invasion, thereby promoting tumor cell dissemination through the vasculature [178,179]. Fourthly, α3β1 expressed by endothelial cells may strengthen the adhesion of circulating tumor cells to the endothelium by stabilizing the binding of endothelium-expressed galectin-3 and cancer-associated carbohydrate Thomsen-Friedenreich antigen (TF-Ag) [180]. Lastly, α3β1 can mediate the initiation of new metastases in laminin-rich environment. Several studies have established a role of α3β1 in haptotatic migration and invasion toward laminin-511, suggesting that α3β1 plays an active role in the colonization of laminin-rich tissues by tumor cells [119,181–183]. This was confirmed in *in vivo* mouse studies, observing that α3β1 drives the formation of metastasis to the lung, lymph nodes and peritoneum [109,117,133,177,184].

Ligation of α3β1 by laminin, however, does not always clearly promote the spreading and invasion of cancer cells. For example, in highly invasive and metastatic prostate carcinoma cells the expression of α3β1 was decreased and they failed to spread when grown *in vitro* [124]. Furthermore, a recent study of patient samples of squamous cell carcinomas of the lower lip showed the absence of α3β1 at the invasive front, where the expression of laminin-332 was often detected [102]. One possible explanation for the anti-invasive-effect of ligated α3β1 is that the integrin suppresses the formation of invadopodia, actin-linked structures with putative adhesion properties, which are frequently observed to mediate BM degradation in epithelial tumors. It was proposed that a balance of focal contacts and invadopodia is necessary for cells to migrate and invade the BM [185]. In fact, Liu *et al*. [186] recently showed that depletion of either laminin-332 or α3β1 resulted in an increased number of invadopodia in bladder carcinoma cells. They proposed a mechanism, by which laminin-332-α3β1 interaction acts as a potent upstream inhibitor of cell invasion via mediating focal contacts that in turn limit the availability of active Src, necessary for inducing the formation of invadopodia.

Association of α3β1 with tetraspanins

The ability of α3β1 to interact with several IAPs offers further explanation for its dual role in cancer invasion and its progression in general. Tetraspanins, multispanning membrane proteins that cluster into tetraspanin-enriched microdomains (TEMs) on the plasma membrane, are one of the most prominent proteins that can interact with laminin-binding integrins, thereby influencing their localization and function [63,72]. Several tetraspanins, including CD9, CD81 and CD63 have been suggested to associate with α3β1 and to influence the migration and invasiveness of tumor cells. With a few exceptions, these complexes are mainly associated with reduced migration and low

metastatic potential, and thus a better prognosis [98,126,135,187–189]. Recently, the tetraspanin CO-029 was found to form a complex with α3β1 and rictor in malignant gliomas, and thus to mediate migration of glioma cells via mammalian target of rapamycin (mTOR) complex 2 (mTORC2), of which rictor is a key component [190]. However, a clear understanding of how these tetraspanins associate with α3β1 and regulate α3β1-mediated migration and invasiveness is still lacking.

A direct and stable association has only been shown for CD151 and α3β1 [191,192]. The interaction of CD151 and α3β1 influences the distribution of α3β1 and shifts it from FAs into TEMs [193]. Furthermore, it strengthens α3β1-mediated cell adhesion and promotes the proliferation and migration of different types of tumors cells on laminin-332 [194–196]. Two major mechanisms may account for the CD151-dependent regulation of tumor cell behavior by α3β1. Firstly, CD151, which contains a YXXφ endocytosis motif in its C-terminal cytoplasmic domain, may stimulate cell migration by facilitating α3β1 recycling [197]. Secondly, CD151 may contribute to pro-migratory signaling of α3β1 by suppressing RhoA activity and formation of stress fibers [63,164,198,199]. Additionally, the signaling properties of α3β1 may be influenced by phosphatidylinositol 4-kinase (PI4K) that is associated with CD151 [192] (**Fig. 2**).

Recent data suggests that CD151 can also control cell migration independently of its association with α3β1, and that the balance between integrin-free CD151 and CD151-α3β1 complexes is important with regard to tumor invasion [130,200,201]. Scales *et al.* [199] demonstrated that the ligation of α3β1 by laminin promoted the association between α3β1 and CD151 and that cells lacking α3β1 exhibited increased formation of CD151 homodimers. This suggests that α3β1-mediated cell adhesion to laminin skews the balance from CD151-CD151 homodimers towards CD151-α3β1 complexes. The balance can also be altered by changes in the expression of either α3β1 or CD151, which is not uncommon in cancer [63]. Alternatively, association of α3β1 with CD151 could be regulated via α3 or β1 glycosylation, as it was shown in highly metastatic melanoma cells [202].

In breast cancer, it was shown that the complex of CD151 and α3β1 mediates malignancy through interaction with ErbB-2 (HER2) [203,204]. In invasive ductal carcinomas, the CD151-α3β1 complex is a marker of poor outcome, and experiments with ErbB-2 overexpressing breast cancer cells indicated that CD151-α3β1 complexes promote dimerization of ErbB-2 by keeping Rho activity low [114]. In contrary to invasive ductal carcinomas, in invasive lobular carcinomas poor patient survival is connected to the lack of correlation between CD151 and α3β1 [118].

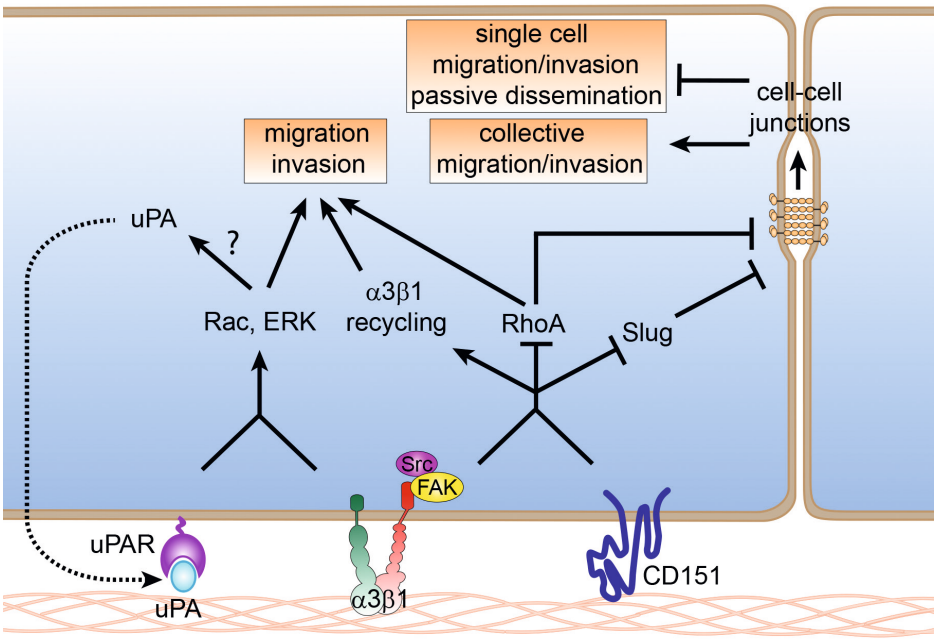


Figure 2: The interaction of integrin α3β1 with CD151 and uPAR can affect tumor cell migration and invasion. Integrin α3β1 can form complexes with several IAPs, which have an impact on the progression and development of tumors. The association of CD151 with α3β1 might be required to prevent the integrin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

The biological differences between the two diseases were related to differences in cell-cell and cell-matrix interactions, the loss of E-cadherin being the most prominent characteristic of invasive lobular carcinoma [205]. As previously mentioned, α3β1 does not only mediate adhesion and migration on laminin substrates, but also plays a role in regulating the stability of cell-cell contacts. Although a precise mechanism has not yet been defined, it has been suggested that the CD151-α3β1 complex controls the stability of E-cadherin mediated cell-cell adhesion by regulating the expression of the membrane protein tyrosine phosphatase PTPm and its association with the E-cadherin-catenin complex in embryonic kidney cells [206]. Stipp and colleagues [207,208], however,

showed that this mechanism is not operative in A431 epidermoid carcinoma cells. In these cells, CD151 promotes the stability of cell-cell junctions by reducing α3β1-dependent activation of RhoA. In the absence of CD151, Rho activation is increased, which resulted in reduced collective migration in two-dimensional (2D) *in vitro* assay. However, in 3D matrices, the level of Rho activation, although being disruptive for junctional stability, did not prevent their formation and cells still invaded in a collective manner. Furthermore, there is evidence that the CD151-α3β1 complex plays a role in maintaining the integrity of ovarian carcinomas by repressing Slug-mediated EMT and canonical Wnt signaling [128]. Integrin α3β1-mediated cell-cell cohesion could hinder metastasis also in the context of passive tumor cell shedding to the blood stream. In line with this, tumor cells that have been released into the circulation of mice that contained induced (primary) renal tumors exhibited reduced levels of α3β1 [141]. Thus, the role of the CD151-α3β1 complex in carcinoma progression appears to be context-dependent and to depend on the mode of invasion and the phenotype of the tumor.

Association of α3β1 with uPAR

uPAR, the receptor for urokinase (uPA), is a glycosylphosphatidyl inositol-anchored protein expressed by many cell types. It forms complexes with several integrins, including α3β1, and has been implicated in tumor progression (**Fig. 2**). In oral squamous cell carcinomas, increased expression of α3β1 and uPAR correlates with a poor prognosis. *In vitro* and *in vivo* studies have shown that α3β1 clustering induces the recruitment of uPAR and the formation of α3β1-uPAR complexes that promote invasive cell behavior via Src and ERK1/2 signaling as well as via enhanced uPA expression [96,209]. In an independent study, it was shown that $p130^{Cas}$ is phosphorylated by Src in response to uPAR-α3β1-laminin-332 engagement, and that this led to enhanced cell motility through activation of Cdc42 and actin reorganization [210]. Complexes of uPAR and α3β1 have also been implicated in the fibroblast associated protein α (FAPα)-stimulated migration of ovarian cancer cells via activation of Rac1 [211]. In fact, an early study had already described that this complex can mediate binding to vitronectin [212]. Recently, an interesting novel mechanism was reported by Ferraris *et al*. [213], who showed that uPAR-mediated cell adhesion to vitronectin triggers integrin signaling independently of integrin-matrix engagement, by increasing the membrane tension. The same group also proposed that, in integrin ligand-independent conditions, the frictional membrane resistance participates in establishing adequate lamellipodial tension, which predominantly depends on coupling of the C-terminal talin-actin binding site to actomyosin-driven retrograde actin flow force [214]. This mechanism could provide an explanation for the role of uPAR-binding integrins, such as α3β1, in migration in an environment lacking conventional integrin ligands.

Altered glycosylation of α3β1

The α3 and β1 subunits contain 14 and 12 potential N-glycosylation sites, respectively, and it has become increasingly clear that malignant transformation is associated with aberrant glycosylation of α3β1, which can modulate its function, signaling and lateral associations with IAPs [215,216]. In bladder carcinomas, the overexpression of aberrantly glycosylated α3β1 is correlated with poor clinical outcome, and a monoclonal antibody that recognizes the aberrantly glycosylated epitope on α3β1 has potent anti-tumor activity in bladder cancer *in vivo*. *In vitro* experiments revealed that aberrant glycosylation of α3β1, conferred by the glycosyltransferase GALNT1, initiates FAK signaling, resulting in c-Jun phosphorylation and increased cell cycle progression, and proliferation through upregulation of cyclin D1 and activation of CDK4 [139]. Aberrant N-glycosylation of α3β1 also influences the motility and invasiveness of cancer cells [217,218]. The presence of high mannose and sialylated tri- or tetra-antennary complex type N-glycans on α3β1 is associated with a reduced adhesion to laminin and an increased invasive behavior of bladder cancer cells [219,220]. Baldwin *et al.* [221] has shown that α3β1-mediated cell migration can also be influenced by N-glycosylation of α3β1 without a detectable loss of cell adhesion to laminin-332. They found that the changes to N-glycosylation of α3β1, induced by its binding to CD151 during biosynthesis, influenced tumor cell migration toward laminin-332 [221]. The exact mechanism underlying the effect of α3β1 glycosylation on cell migration is unknown, but may involve galectin-3-mediated clustering of α3β1 and subsequent activation of Rac1 signaling [222]. Recently, a study was published proposing a role of N-glycosylation modifications in the efficient translocation of α3β1 to the plasma membrane [223]. Glycosylation of α3β1 was observed to be suppressed by hypoxia, resulting in decreased levels of α3β1 at the plasma membrane, which facilitated the invasion of epidermoid carcinoma cell line A431. There is therefore strong evidence that glycosylation of α3β1 can play a role in promoting cell migration and invasiveness, as well as contribute to increased tumorigenesis.

Role of α3β1 in gene regulation

As already mentioned, in transformed cells the expression of α3β1 is often regulated to modulate its function. One of the examples of such regulation is mediated by transforming growth factor β (TGF-β). In invasive hepatocellular carcinoma cells, TGF-β stimulates the expression of α3β1 by transcriptional upregulation via Ets transcription factors, resulting in a pro-invasive phenotype on laminin-332 [138,162]. Similar observations were reported in bladder cancer and in oral squamous carcinoma cells [224,225]. However, there is also an increasing amount of evidence for the role of α3β1 in gene regulation and for the consequences this brings to the development and progression of tumors. α3β1-mediated gene regulation in the context of metastasis has

been recently reviewed [79] and will therefore only be briefly mentioned here. The most striking examples of the gene regulatory role of α3β1 were observed in immortalized keratinocytes. In these cells α3β1 was shown to induce expression of MMP-9 upon transformation of cells [226], which was mediated via α3β1-dependent stabilization of MMP-9 mRNA transcripts [227]. Furthermore, the production of MMP-9 in immortalized keratinocytes was potentiated by α3β1 in response to TGF-β stimulation [228]. The mechanism behind mRNA stabilization was recently proposed by Missan *et al.* [229], who observed that a shorter, more stable mRNA was preferentially generated in immortalized keratinocytes expressing α3β1. The presence of this short transcript was dependent on active ERK/MAPK signaling. Integrin α3β1 was also shown to influence the stability of COX-2 mRNA (discussed in chapter on proliferation and angiogenesis) and to regulate the expression of fibulin-2, a matrix-associated protein that binds laminin-332 and serves as a mediator of matrix remodeling and invasion [85]. In line with the latter, a recent study reported that α3β1 mediates the stability of the BM through fibulin-2 induction, indicating the importance of α3β1-mediated gene regulation not only in cancer progression, but also in maintenance of healthy tissue [230].

INTEGRIN α6β4 AND CANCER

Early studies have identified the integrin α6β4 as a tumor antigen [231,232], whose expression is increased in squamous cell carcinomas, as well as in other types of solid cancers (reviewed in [233]). Some controversy exists regarding the stage of cancer development and progression at which β4 overexpression becomes apparent, but most studies agree that it increases with tumor grade [234,235]. In addition to increased expression of β4, changes in its distribution have also been linked to the grade of tumors [236,237]. In normal epithelial tissues, β4 is concentrated at the basal membrane of basal epithelial cells, while in many tumors, it is diffusely expressed in multiple cell layers [238–240]. Although abnormal and high expression of α6β4 in cancer is generally associated with poor patient outcome and overall survival, in certain settings α6β4 suppresses tumorigenesis. Furthermore, in tumors such as prostate carcinomas and basal cell carcinomas, the expression of α6β4 is downregulated [241–245].

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

Role of α6β4 in tumor initiation

Although α6β4 cannot induce tumorigenesis on its own [246], it has been implicated in human papilloma virus (HPV)-mediated tumor initiation. HPV plays a role in the initiation of several cancers such as anal, cervical and oropharyngeal cancer. Once the virus enters a cell, its proteins interfere with the normal cell machinery, which results in uncontrolled cell growth and avoidance of cell death. In HPV16 infected cervical cancer cells, α6 expression levels were correlated to the binding of the virus particles to the cells [247]. And in fact, it recently became clear that β4 expression and α6 processing are important for HPV entry into the basal cells [248,249]. However, viral DNA replication occurs primarily in the differentiating suprabasal cells of the epidermis. The HPV E2 protein may trigger this differentiation step by downregulating β4 expression [250,251]. These data strongly suggest that α6β4 has a dual role in different stages of tumor initiation by HPV.

Role of α6β4 in sustained proliferation and avoidance of apoptosis

The unique function of α6β4 in potentiating growth factor receptor signaling is evident from its role in supporting sustained proliferation and avoidance of apoptosis during tumor development and progression. Integrin α6β4 has been implicated in the modulation of signal transduction pathways downstream of several RTKs, including the epidermal growth factor receptor (EGFR) family members EGFR [65] and ErbB-2 [252,253], the macrophage stimulating protein (MSP) receptor (also known as Ron) [254], the hepatocyte growth factor (HGF) receptor (also called c-Met) [71,255] and the insulin-like growth factor-1 receptor [256].

Many studies attribute the synergy between α6β4 and RTK-mediated signaling to the phosphorylation of specific tyrosine residues in the cytoplasmic domain of β4 and subsequent recruitment and activation of signaling intermediates to the phosphorylated subunit (reviewed in [257]). The C-terminal segment of the β4 cytoplasmic domain that harbors the tyrosine residues is also known as the β4 signaling domain. Tyrosine phosphorylation of β4 is typically mediated by the Src family of kinases (SFKs) downstream of RTKs [254,258,259], although direct tyrosine phosphorylation of β4 by the HGF receptor c-Met has also been demonstrated [260]. Additionally, clustering of the α6β4 molecules by itself can lead to tyrosine phosphorylation of the β4 subunit [261]. Recent data suggests that members of the syndecan family of cell-surface proteoglycans may play an important role in the phosphorylation of the β4 cytoplasmic domain by positioning this domain near the plasma membrane to be phosphorylated by the SFK member Fyn downstream of EGFR and ErbB-2. Syndecans can bind directly to the cytoplasmic domain of the β4 subunit [262,263].

The signaling intermediates that are recruited by tyrosine phosphorylated β4 include the adapter proteins Shc [260,264] and IRS-1/2 [70], and the protein-tyrosine phosphatase Shp2 (also known as PTPN11) [71]. Binding of Shc by tyrosine phosphorylated β4 has been shown in squamous carcinoma cells expressing the EGFR at high levels [260], but also upon EGF treatment of cells that express normal levels of the EGFR [258]. Shc links α6β4 to the MAPK signaling pathway, which is essential for inducing cellular proliferation and transformation [264]. On the other hand, c-Met-mediated tyrosine phosphorylation of β4 has been shown to recruit Shp2, which enhances the activation of Src. Subsequently, Src induces the phosphorylation of the multi-adapter Gab1, which leads to activation of the MAPK and phosphatidylinositol 3-kinase (PI3K) signaling pathways [71,255]. Activation of MAPK signaling can also be mediated by a fraction of α6β4 that is localized in lipid rafts and associated with palmitoylated SFKs [265]. Binding of IRS-1/2 by tyrosine-phosphorylated β4 has also been implicated in the activation of PI3K downstream of α6β4 clustering [70]. Furthermore, there is data suggesting that FAK can be recruited by tyrosine phosphorylated β4 and that the subsequent activation of FAK promotes malignancy by increasing the activity of p38MAPK and Akt [259]. Tyrosine phosphorylation of β4 and subsequent activation of PI3K signaling can also promote the survival of breast cancer cells through enhanced VEGF translation and stimulation of VEGFR-mediated autocrine signaling [266] (reviewed in [267]).

Most studies agree that α6β4 supports PI3K activation by different RTKs, although the details of the mechanisms may differ between cell types. Activation of PI3K by α6β4-mediated cell adhesion was first shown by Shaw *et al.* [268], and since then, numerous other studies have reported an association between α6β4-mediated adhesion and the requirement of PI3K activation for cell survival. In breast cancer cells, blocking α6β4 function with an antibody against β4 caused a reduction in PI3K signaling, which led to increased apoptosis [269]. The induced apoptosis could be rescued by the expression of constitutively active Akt, the downstream target of PI3K [269,270]. Integrin α6β4-mediated PI3K signaling can also support cell survival through activation of the transcription factors STAT3 and c-Jun, as was shown in an ErbB-2-driven breast cancer mouse model [252].

Intriguingly, it has been reported that α6β4 can also promote cell death. *In vitro*, treatment of cells with chemical or pharmacological agents induced apoptosis via elevating the levels of β4, while the depletion of β4 promoted survival [271–275]. Furthermore, in an immunocompromised SCID mouse model of human gastric cancer, the expression of β4 at high levels promoted apoptosis [276]. Bachelder *et al.* [277] suggested that the ability of α6β4 to either promote or suppress apoptosis depends on the p53 status of the cells. Integrin α6β4 stimulates p53-transactivating function

and promotes p53-dependent apoptosis in carcinoma cells that express wild-type p53, but not in p53-deficient carcinoma cells, in which it promotes survival in a PI3K/Akt dependent manner. Interestingly, in the same colon carcinoma cells depletion of p53 associates also with enhanced β4 transcription through the p53 family members p63 and p73, thereby further augmenting the survival function of α6β4 [278]. Although the inhibition of α6β4-mediated survival signaling by p53 activation has so far been only conclusively shown in RKO colon carcinoma cells [279], the fact that p53 mutations and overexpression of α6β4 are positively correlated in a number of human malignancies [233] suggests a general mechanism by which the activity of p53 in carcinoma cells is regulated by the signaling function of this integrin. Interestingly, p53 has also been suggested to regulate adhesion of cancer cells via α6β4 [280].

In addition to supporting cell proliferation and survival by providing an additional platform for RTK signaling, α6β4 may be needed to secure attachment during oncogenic transformation when the adhesive function of integrins that are linked to the actin cytoskeleton is compromised, while oncogenic signaling is still dependent on the structural integrity of the actin cytoskeleton. This might be responsible for the requirement of the presence of α6β4 and its ligand laminin-332 in squamous cell carcinomas, induced by oncogenic Ras and IκBα expression [281]. Similarly, the promotion of cell growth by α6β4, which was reported to be anchorage-independent [71,282], could still have been dependent on α6β4-mediated adhesion to autocrine-produced laminin. In support of this notion, in 3D culture of mammary spheroids, α6β4-mediated cell adhesion to autocrine produced laminin-332 conferred resistance to apoptosis by stimulating Rac1-Pak signaling and activation of NF-κB [283,284]. Moreover, Bertotti *et al*. [255] showed that the removal of the extracellular domain of β4 reduced anchorage-independent colony formation in soft agar.

Additionally, it has been suggested that suprabasal α6β4 contributes to cancer progression by enhancing proliferation of basal keratinocytes by relieving the growth inhibition of TGF-β [246]. TGF-β negatively regulates keratinocyte proliferation in the early stages of epidermal tumor promotion [285,286]. This growth inhibitory effect of TGF-β is dependent on cadherin-mediated cell-cell adhesion and PI3K, but not MAPK activity. Suprabasal α6β4 appears to perturb TGF-β signaling by blocking nuclear translocation of activated Smad2/3, resulting in increased cell proliferation and formation of skin papillomas and SCCs [246]. Surprisingly, tumorigenesis was further increased when mice expressed a mutant β4 subunit that lacked the cytoplasmic domain in the suprabasal layers of the epidermis, suggesting that α6, rather than β4 cytoplasmic domain might play a role in TGF-β signaling [246].

Role of α6β4 in angiogenesis

Within the vasculature, the expression of α6β4 in endothelial cells is dynamically regulated during angiogenesis and vessel maturation [287]. α6β4 is predominantly detected in small arterial vessels, where it may mediate strong endothelial cell adhesion, necessary to withstand the high shear rates in these vessels [19]. Contrary to β4, α6 is expressed in all vasculature, which suggests the presence of α6β1 in the absence of β4 [19,288]. Integrin α6β1 is known to promote angiogenesis; inhibition of α6 prevented endothelial cell migration and tube formation [289]. However, the role of β4 in angiogenesis is less clear. The exclusive presence of β4 in mature vessels suggests that it negatively regulates angiogenesis [287], and several studies report that α6β4 does not promote endothelial cell proliferation or growth of new vessels [19,287,290]. Furthermore, it has been recently suggested that downregulation of α6β4 is necessary for endothelial proliferation and tube formation during early stages of angiogenesis [288]. In line with this notion it was proposed that α6β4 can block angiogenesis by inducing endothelial cell death [291] (reviewed in [292]). On the other hand, experiments in mice in which the C-terminal domain of β4 was deleted, showed that α6β4 signaling is important for vascular remodeling and for a proficient angiogenic response to VEGF and basic fibroblast growth factor (bFGF) [290]. Furthermore, in mice lacking β4 in endothelial cells, hypoxia-induced arteriolar remodeling was defective, which was suggested to result from changed TGF-β signaling [19]. Alternatively, β4 can regulate angiogenesis via stimulation of translation and signaling of VEGF [266,293] (**Fig. 3**).

Although α6β4 seems to play a general regulatory role in angiogenesis, there is very little known about its role in tumor angiogenesis. In mice, carrying a deletion of the C-terminal domain of β4, vascularization in subcutaneously implanted tumors was impaired, suggesting that α6β4 promotes tumor angiogenesis [290]. However, the levels of tumor vascularization in a mammary gland tumor model were the same in mice carrying a similar β4 deletion as in β4 wild-type mice [252].

Role of α6β4 in invasion and metastasis

Unlike that of α3β1, the expression of α6β4 is positively correlated with tumor grade in most instances, indicating that this integrin promotes tumor progression and metastatic spread. The role of α6β4 in invasion and metastasis has been reviewed extensively over the last two decades, in articles primarily focusing on the mechanisms responsible for its tumor-promoting function [67,68,233,294,295]. However, there are certain cases in which α6β4 is negatively correlated with tumor invasion and formation of metastases. In order to elucidate the circumstances that determine the function of α6β4 in the final stages of cancer, it is important to understand under which conditions it contributes

to cellular migration and invasion, as well as to understand the differences between tumor settings, in which the role of α6β4 has been implicated.

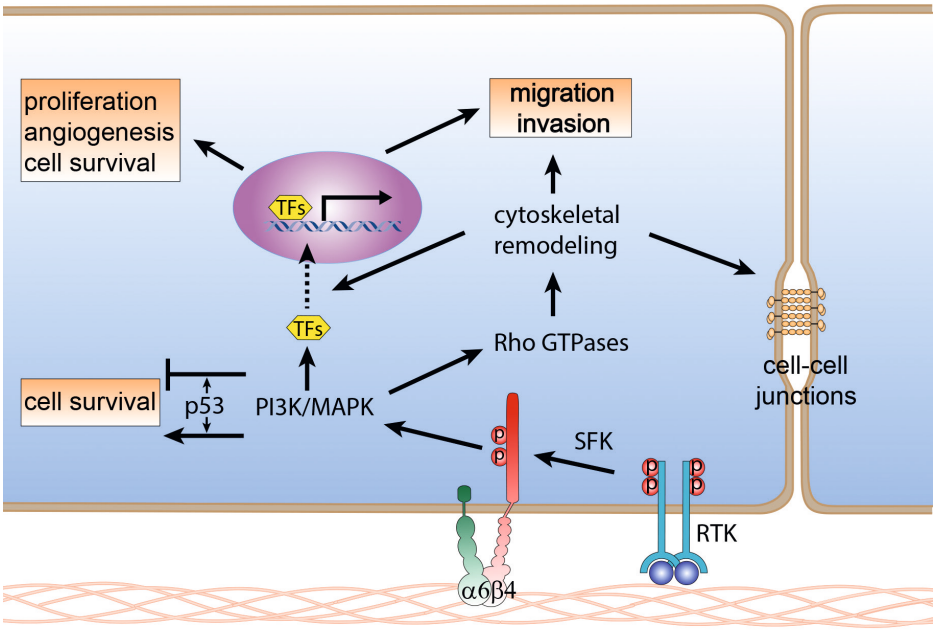


Figure 3: Integrin α6β4 regulates cell behavior by cooperating with RTKs. Integrin α6β4 amplifies intracellular signaling of RTKs. SFK that are activated by RTKs phosphorylate several tyrosine residues (red dots) in the signaling domain of the β4 cytoplasmic tail. These tyrosine residues act as docking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

Migration and invasion

It is generally accepted that HDs have to be disassembled before cells can migrate (**Fig. 4**). Indeed, studies using primary keratinocytes have revealed that classical HDs (type I HDs), which contain α6β4, CD151, plectin, BP180 and BP230, prevent cell migration [296,297]. However, α6β4 does not necessarily impede cell migration as part of type II HDs, a less complex type of HDs that only contains α6β4 and plectin [298]. Type II HDs are found in simple epithelia and many cultured epithelial cells, and in contrast to type I HDs, their components have a turnover rate that is fast enough not to limit the migration of cells [50,52,299].

In many transformed cells the number of HDs is reduced and they are completely and/or partially disassembled. In these cells, α6β4 facilitates growth-factor stimulated migration and invasion via its cytoplasmic domain (reviewed in [47]). Induction of carcinoma cell migration by EGF is associated with a redistribution of α6β4 from HDs to the leading edge of the cell (reviewed in [67]), where it colocalizes with actin in lamellipodia and filopodia [53,54]. How α6β4 associates with actin and how this contributes to migration is not clear. Like α3β1, α6β4 has been implicated in actin cytoskeletal dynamics during migration through the regulation of the Rho family of GTPases [300,301]. Although RhoA plays a crucial role in the retraction of the tail of the cells, both Rac1 and RhoA are required for stimulating cell migration on 2D substrates by inducing the formation of actin-based protrusive structures [302]. Rac1, which is activated by α6β4-mediated cell adhesion, promotes the formation of lamellipodia and, interestingly, the localization of α3β1 in these structures [303]. Integrin α6β4 supports PI3K-Rac1 signaling [268] downstream of several pro-migratory factors (*e.g.* EGF [304], HGF [305], PTHrP (parathyroid hormone-related protein) [306] and LPA (lysophosphatidic acid) [307]) (**Fig. 3**). EGF-induced activation of Rac1 requires both the extracellular and the cytoplasmic domain of the β4 subunit [304]. On the other hand, when it is part of HDs, ligation of α6β4 by laminin activates Rac1 independently of the signaling domain of β4 [308,309]. In addition to stimulating migration by increasing the activity of Rac1 in tumor cells, α6β4 has also been implicated in augmenting the activity of RhoA by a mechanism that involves suppression of the intracellular cAMP concentration by activating a cAMP specific phosphodiesterase [261,310].

Several studies have shown that α6β4 influences migration and invasion of tumor cells through the NFAT (nuclear factor of activated T-cells) transcription factors [311] (**Fig. 3**), which are activated downstream of Src and PI3K/Akt signaling [312](reviewed in [67]). α6β4-mediated activation of NFAT1 induces the transcription of autotaxin [313], which promotes LPA-induced cell motility and invasiveness [314]. In line with this, α6β4-mediated cell motility was decreased in breast carcinoma cells that were depleted of autotaxin [313]. NFAT5, a transcription factor responsible for the upregulation of the calcium-binding protein S100A4, can also be activated by α6β4-mediated cell adhesion [315]. S100A4 is a metastasis-promoting protein implicated in the invasion of a number of tumor types including colon and breast carcinomas (reviewed in [316,317]). Another protein that plays a role in the calcium-dependent regulation of migration is the transient receptor potential vanilloid channel (TRPV1). In the absence of TRPV1, both directional migration and β4 expression are reduced [318].

Several studies suggested that α6β4 can influence ovarian and breast cancer cell migration and invasion through the activation of FAK [259,319]. α6β4-mediated FAK activation was

observed upon ligation of α6β4 to laminin-332, but also to alternative ligands, such as CLCA1 and MUC5Ac [320,321]. Recent findings have shown that FAK can directly bind to β4 and suggest that this association is regulated by tyrosine phosphorylation of the β4 subunit [259].

During invasion, the ECM is often remodeled, enabling tumor cells to efficiently migrate and disseminate. α6β4 has been implicated in ECM remodeling through its ability to contribute to the activation of PI3K and RhoA, and subsequent induction of traction forces generated by the actomyosin cytoskeleton [322,323]. Additionally, α6β4 plays a role in remodeling of the ECM by supporting signals that lead to the production of MMP1 and MMP2 [325,326]. MMP levels can also be induced by several of its interactors, such as CD151 (reviewed in [327–329]).

Metastasis – intravasation, extravasation and niche preparation

Many studies have demonstrated a critical role of α6β4 in promoting the formation of metastases (reviewed in [78,330,331]). Moreover, α6β4 serves as a marker to detect distant metastases in the early stages of specific malignancies [101,332,333]. However, the mechanism underlying its pro-metastatic role has received little attention. Metastases occur when cancer cells invade into the blood or lymph vessels, travel through these systems and subsequently extravasate into the stroma of the target organ. α6β4 contributes to intravasation and extravasation of tumor cells by upregulating VEGF expression (**Fig. 3**), which enhances transendothelial permeability and migration of malignant cells [334–337]. The effect of α6β4 on VEGF expression appeared to be dependent on the signaling domain of β4 [337].

Following extravasation, tumor cells need to adhere, proliferate and grow in the new environment (*i.e.* the metastatic niche) in order to form a metastasis. Cells from specific tumors tend to form metastasis in certain organs. Recently, Hoshino *et al.* [338] suggested that exosomes can contribute to the formation of a metastatic niche in specific organs. They showed that expression of α6β4 and α6β1 on exosomes was associated with lung metastases in mice, and that blocking of exosomal α6β4 decreased the formation of such metastases.

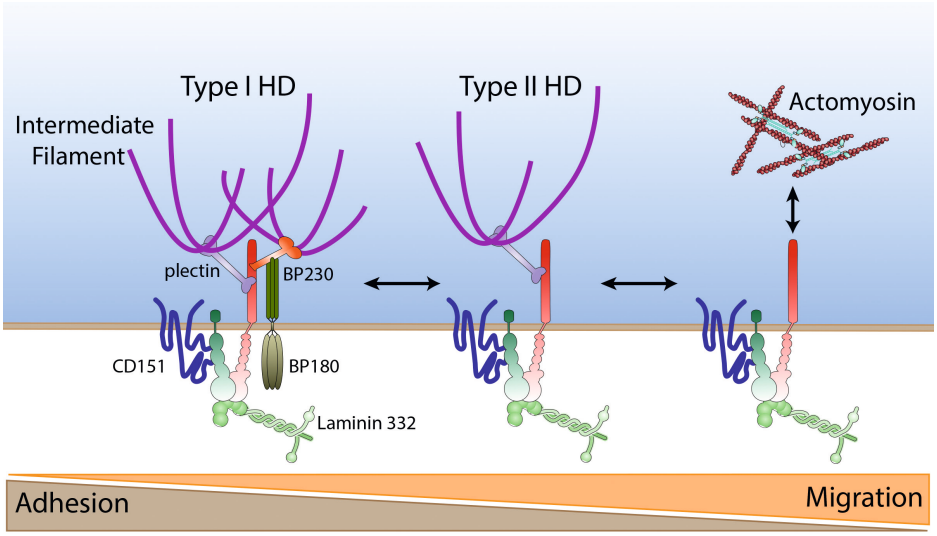


Figure 4: Integrin α6β4 regulates adhesion and migration by the formation of HDs. Type I HDs contribute to stable adhesion of basal epithelial cells to laminin-332 in the basement membrane and therefore inhibit cell migration. Although type II HDs contribute to adhesion as well, they are more dynamic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

Crosstalk with growth factors and hormones

The crosstalk between α6β4 and growth factor receptors not only plays a role in tumor growth (see previous chapters), but also strongly contributes to the α6β4-mediated tumor invasion, metastasis formation and cell migration. The synergy between α6β4 and several RTKs (*e.g.* c-Met [260,268], Ron [254,339], ErbB-2 [340], EGFR [258] and ErbB-3 [341]) primarily promotes tumor spreading and invasion. This tumor promoting function of α6β4, like its promotion of tumor growth, often involves the disassembly of HDs and subsequent β4-mediated signaling (reviewed in [47,68,80,342,343]). Additionally, α6β4 can have an impact on invasion and metastasis through regulating cell-cell junctions [252] (**Fig. 3**). In ErbB-2 transformed mammary epithelial cells, β4 promotes invasion and metastasis by disorganizing cell-cell junctions via SFK-dependent ErbB-2 activation of STAT3. Accordingly, in these cells the loss of β4 signaling restores cell-cell adhesion, and as a consequence cell invasion is compromised. However, this negative effect of β4 on cell-cell contacts is only seen in the presence of receptors of the EGFR family, and blocking these receptors promotes reassembly of cell-cell junctions [252]. Like α3β1, α6β4 was reported to promote the formation of cadherin dependent cell-cell junctions, resulting in restrained cell migration [344,345]. Furthermore, upon induction of EMT by TGF-β a decrease in E-cadherin expression has been correlated

with a reduction of the levels of β4 [346]. Such a reduction has also been shown in carcinoma cells in which EMT was induced by overexpression of Snail [347]. In contrast, suppression of the EMT regulator Slug resulted in a reduction of the α6β4 levels [348]. In addition to the reduced levels of β4 in tumor cells, which have undergone an EMT, there is evidence that β4 is less sialylated during EMT, which may further decrease the tumor promoting capacity of this integrin [349,350].

Besides a signaling cooperation between α6β4 and growth factor receptors, there is also crosstalk between α6β4 and hormones during tumor progression. Estrogen and PTHrP signaling was shown to enhance the pro-tumorigenic effects of α6β4 [351,352]. On the other hand, the expression of β4 in prostate cancer is reduced by the androgen receptor and the absence of β4 in the androgen-sensitive tumors reduces their invasiveness [353,354].

Targeted disruption of the crosstalk between α6β4 and growth factor receptors or between α6β4 and hormone receptors has been recognized as a potential form of therapy in the treatment of cancer [252,341,355]. However, because of its dual role in tumor development and promotion, targeting β4 can only be beneficial under well-defined and specific circumstances. In certain cancers, such as gastric carcinomas, the degree of β4 expression is inversely correlated with invasive potential and blocking β4 function would likely increase the tumor burden [276]. Furthermore, the analysis of transformed keratinocytes suggests that blocking the function of β4 in the early-stages of tumorigenesis might promote the disease [356].

Regulation of β4 expression

As previously discussed, the expression of β4 is frequently upregulated in cancer. For example, hypoxia increases the surface expression of α6β4 by promoting Rab11-dependent trafficking, resulting in increased breast tumor cell invasion [43]. Other factors and proteins implicated in the expression of β4 in tumors include Rac1 [357], TR3/Nur77 [358], PTHrP [352,359,360], IL24 [361] and H-Ras [362], ARRDC3 [363] and ZEB1 [364]. Expression of α6β4 is also regulated by palmitoylation of the α6 and β4 subunits and ablation of the protein acyl transferase, responsible for their palmitoylation results in accelerated degradation of α6β4 [365]. miRNAs have emerged in the past decade as key regulators of gene expression. They repress gene expression by blocking mRNA translation or promoting mRNA degradation [366]. Several miRNAs have been reported to regulate expression of β4. Overexpression of miR-221 and miR-222 resulted in reduced β4 expression, which was partially responsible for impaired invasion of breast cancer cells [367], while in the absence of miR-21 a higher expression level of β4 was correlated with a reduction in the rate of colorectal cancer cell migration

[366]. Furthermore, loss of miR-205 in prostate cancer resulted in reduced secretion of laminin-332 and β4 *in vitro* [368]. Therefore, the different expression patterns of β4 in cancer might be explained, at least to some extent, by specific miRNAs present in the different tissues.

Role of CD151 and α6 in α6β4-regulated tumorigenesis

Most studies have focused on the role of α6β4 in cancer without considering the fact that two isoforms of the α6 cytoplasmic domain (i.e. A and B) can be generated by alternative splicing [13,16]. In colorectal carcinomas, in which both a relative decrease and increase of β4 expression was observed during malignant transformation [369,370], the tumorigenic outcome of β4 positive cancers was dependent on which isoform of the α6 subunit was associated with β4 [371]. A relationship between the expression of specific α6 isoforms and tumor malignancy has also been observed in skin tumorigenesis [372]. As well as forming dimers with the β4 subunit, the α6 subunit can also dimerize with β1 to form the α6β1 heterodimer, which has a tumor promoting role and is correlated with a poor prognosis (discussed in introduction) [373]. Similar to α6β4, the role of α6β1 in cancer can be dependent on α6 isoform expression [374,375]. α6β1 and α6β4 are co-expressed in numerous cell lines. However, the ratio of these complexes varies considerably from one cell line to another [21]. Since α6 preferentially binds to the β4 subunit [231,376], the absence of β4 might lead to a switch from α6β4 to α6β1, and thus to promoting carcinogenesis in an α6β1-dependent manner. A switch from α6β4 to α6β1 has been observed during prostate cancer progression, in which expression of the androgen receptor suppresses α6β4 expression [241].

We have already discussed the impact of CD151-α3β1 on tumor progression. However, CD151 can also bind α6 and the presence of CD151-α6β4 in breast cancer was associated with tumor progression [204,377]. CD151 may affect the function of α6β4 through its association with PI4K or by regulating α6β4 trafficking, its incorporation into TEMs and/or its ligand binding activity [192,194,378]. Recently, it has been shown that CD151 is also involved in the crosstalk between α6β4 and RTKs [204,379] and that deletion of CD151 reduces β4 phosphorylation at specific serine residues [377,380]. These serine residues have been previously implicated in the regulation of the interaction between α6β4 and plectin [50,381]. While the interaction between α6β4 and CD151 is well-established, there is data supporting that α6β4 can also form complexes with two other tetraspanins (D6.1A and CD9) and thus might also influence tumorigenesis by interacting with these molecules [382,383].

CONCLUSIONS

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

Integrins influence multiple aspects of tumorigenesis through their impact on adhesion and their ability to enhance signaling pathways downstream of RTKs. One of the features of laminin-binding integrins that strongly influences tumorigenesis and tumor progression is their ability to mediate bi-directional signaling with small GTPases and to promote cytoskeletal remodeling. Several decades ago it was already known that tension-dependent changes in cell shape are necessary for progression of the cell cycle [384], and since then numerous studies have shown that integrin-mediated adhesion and spreading of cells is essential for nuclear translocation of transcription factors [309,385,386]. Therefore, the activation of GTPases by either α3β1- or α6β4-mediated cell adhesion not only influences tumor cell migration and invasion, but may also enable nuclear translocation of transcription factors to promote proliferation and survival of tumor cells that have not yet acquired the ability to grow anchorage independent. Furthermore, adhesion mediated by laminin-binding integrins, especially α6β4, might be essential for tumor development and progression simply by promoting pro-oncogenic signaling pathways.

The pro-migratory role of laminin-binding integrins is often connected to their ability to support PI3K-Rac1 signaling, which might be due to a lack of association of these integrins with the actomyosin cytoskeleton. Integrin α6β4 is connected with the intermediate filament system in type I and type II HDs, while α3β1, when associated with CD151, is incorporated into TEMs. Activation of Rac1 by either α3β1- or α6β4-mediated cell adhesion most likely occurs through synergy with aberrantly expressed and activated RTKs. Additionally, two tyrosine phosphorylation sites in the third fibronectin type III repeat of the β4 cytoplasmic domain (*i.e.* Y1494 and Y1526) have been reported to be sufficient for the activation of Rac [69,387]. Although these residues are located in N*XX*pY motifs, their structural environments are not compatible with binding to the PTB and SH2 binding domains of Shp2 and Shc, respectively [388]. The relevance of this mode of signaling therefore remains debatable.

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

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INTEGRIN α3β1 IN HAIR BULGE STEM CELLS MODULATES CCN2 EXPRESSION AND PROMOTES SKIN TUMORIGENESIS

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ABSTRACT

Epidermal-specific deletion of integrin α3β1 almost completely prevents the formation of papillomas during DMBA/TPA two-stage skin carcinogenesis. This dramatic decrease in tumorigenesis was thought to be due to an egress and premature differentiation of α3β1-depleted hair bulge stem cells (HB SCs), previously considered to be the cancer cells-of-origin in the DMBA/TPA model. Using a reporter mouse line with inducible deletion of α3β1 in HBs, we show that HB SCs remain confined to their niche regardless of the presence of α3β1 and are largely absent from skin tumors. However, tumor formation was significantly decreased in mice deficient for α3β1 in HB SCs. RNA sequencing of HB SCs isolated from short-term DMBA/TPA-treated skin showed α3β1-dependent expression of the matricellular protein connective tissue growth factor (CCN2), which was confirmed in vitro, where CCN2 promoted colony formation and 3D growth of transformed keratinocytes. Together, these findings show that HBs contribute to skin tumorigenesis in an α3β1-dependent manner and suggest a role of HB SCs in creating a permissive environment for tumor growth through the modulation of CCN2 secretion.

INTRODUCTION

Hair bulge (HB) contains one of the most studied and characterized stem cell (SC) compartments in mammalian skin. Located at the bottom of the hair follicles (HFs) in the resting stage of hair cycle (i.e. telogen), it is the main reservoir for cells that form elongated HFs during the growth phase of hair cycle called anagen [1–3]. The transient-amplifying progeny of HB SCs normally remains confined within HFs, however, under specific circumstances some of these cells can egress and contribute to the interfollicular epidermis (IFE). Such behavior has been observed during wound-healing, when HB keratinocytes migrate out of their niche into the newly formed epidermis [4,5].

It has been suggested that HB SCs also play a crucial role in cutaneous skin tumorigenesis. An increased expression of HB marker keratin 19 (K19) has been observed in human squamous cell carcinomas (SCCs) [6], while basal cell carcinomas and trichoblastomas were shown to upregulate the expression of the HB marker keratin 15 (K15) [7]. Furthermore, several studies have suggested that HB SCs represent the cells-of-origin of papillomas, benign tumors that can progress into invasive SCCs and are formed during the two-stage chemically induced mouse skin carcinogenesis protocol (DMBA/TPA treatment) [8–11]. The suggestion that HB SCs play a crucial role in DMBA/TPA-induced carcinogenesis was also made in our previously reported study in mice, lacking integrin α3β1 in epidermis (K14 Itga3 KO mice). Integrin α3β1 is a transmembrane receptor for laminins-332 and -511 in the epidermal basement membrane and functions as bi-directional signaling molecule [12,13]. Upon its epidermal deletion, mice exhibit an increased epidermal turnover, which coincides with the loss of label-retaining cells and, importantly, with the localization of K15-positive keratinocytes in IFE and upper parts of HFs (i.e. isthmus and infundibulum) [14]. As K14 Itga3 KO mice showed a near absence of DMBA/TPA-induced tumorigenesis, we hypothesized that this could be due to the egress of DMBA-primed K15-positive HB SCs and their loss through squamous differentiation in IFE [14]. However, the HB origin of DMBA/TPA-derived tumors is somewhat controversial. Recent studies reported a limited contribution of HB SCs to papillomas [15], and demonstrated the importance of SCs residing in isthmus instead [16]. Furthermore, it has been reported that K15 promoter, which is widely used to generate genetic deletions in HB SCs, also targets basal cells in IFE, isthmus and infundibulum [9] and, importantly, that K15 can be miss-expressed during tumorigenesis, reflecting the activity and responsiveness of basal epidermal cells to the loss of skin homeostasis [17]. Considering all this, we found it important to re-evaluate the mechanisms behind the absence of tumorigenesis in K14 Itga3 KO mice. Here, we exploit linage tracing and next-generation sequencing analysis of a mouse model with

inducible deletion of α3β1 in HBs to investigate the role of α3β1 in HB SCs and their contribution to skin tumorigenesis.

RESULTS

HB keratinocytes lacking integrin α3β1 stay confined within their niche and contribute normally to hair cycle

In order to target and visualize HB SCs and their progeny, we generated a mouse line, expressing an inducible Cre (CreER) under the HB-specific K19 promoter (K19-CreER) [18] in combination with mT/mG reporter transgene [19] (K19 Itga3 WT mice). To investigate the role of α3β1 in HB SCs, we have further introduced floxed Itga3 alleles to our mouse model (K19 Itga3 KO mice) (**Fig. 1a**), which resulted in an efficient deletion of α3β1 in Cre-induced GFP-positive cells (**Fig. 1b**). Tamoxifen administration induced GFP expression in the majority of HFs in both K19 Itga3 KO and WT mice (**Supplementary Fig. 1a**) and, as expected, the GFP-positive cells localized to HBs, with no obvious leakiness detected (**Fig. 1c-e**). Surprisingly, linage tracing of HB cells, induced in the first telogen phase of the hair cycle (P21), showed that HB SCs remain confined to their niche, regardless of the presence of α3β1, and do not egress into infundibulum and/or IFE (**Fig. 1c and e, Supplementary Fig. 1b**), as was expected based on previously reported observations of K15-positive cells in the IFE of mice with an epidermis-specific deletion of α3β1 (K14 Itga3 KO mice) [14]. Furthermore, linage tracing during the first hair cycle revealed that Itga3 KO and WT HB cells contribute to all layers of growing HFs, with no observed differences in hair cycle progression between K19 Itga3 KO and WT mice (**Fig 1e**). Taken together, these data demonstrate that α3β1 plays no major role in HBs of adult mice under homeostatic conditions.

Epidermal deletion of α3β1 causes *de novo* expression of K15 outside of HBs

The finding that HB cells do not egress from their niche into the IFE of K19 Itga3 KO mice casted doubt on whether the K15-positive cells, previously observed in the IFE of K14 Itga3 KO mice, truly originate from HBs [14]. To re-evaluate their origin, we turned to the K14 Itga3 KO and WT mouse models (**Fig. 2a**). In accordance with previous work on this mouse model, immunostaining of whole-mount tail epidermis from K14 Itga3 KO mice confirmed the presence of K15-positive and α3β1-depleted keratinocytes in isthmus, infundibulum and IFE [14] (**Fig. 2b**). Remarkably, we observed that α3β1-positive cells that had escaped Cre-recombinase in K14 Itga3 KO mice preferentially localized to HBs in both tail and back skin (**Fig. 2b, c**). We confirmed this non-stochastic localization with flow cytometry, which showed that the remaining α3-positive keratinocytes isolated from back epidermis of K14 Itga3 KO mice were twice more likely to originate from HBs compared to their counterpart isolated from WT mice (**Fig. 2d, Supplementary Fig. 2a**).

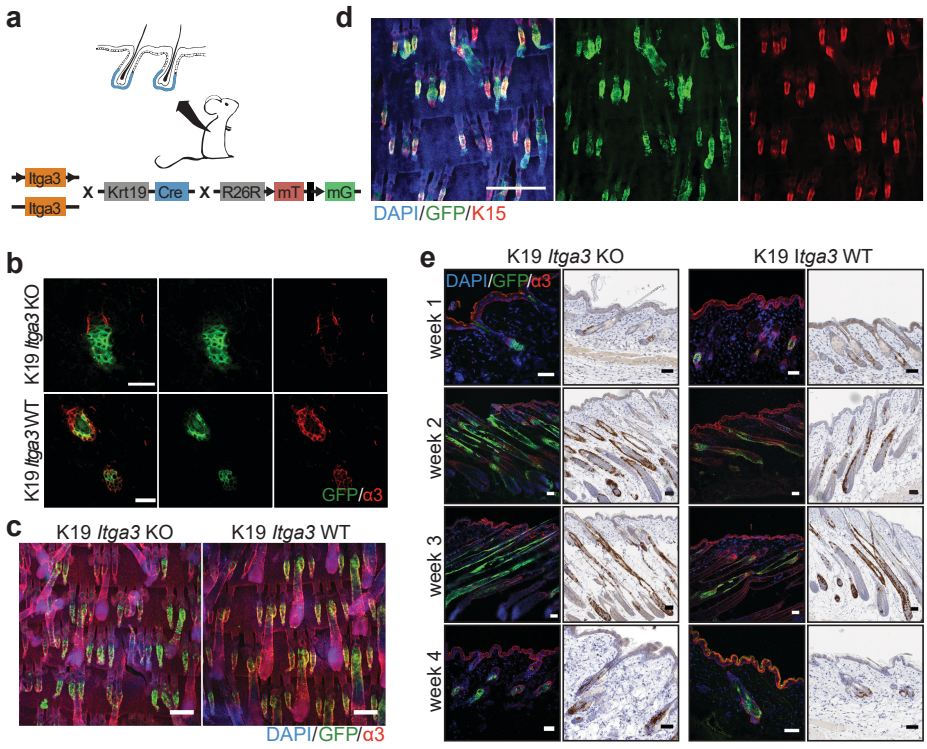


Figure 1: HB keratinocytes lacking integrin α3β1 stay confined within their niche and contribute normally to hair cycle. (**a**) Overview of the K19 Itga3 KO and WT mouse models. (**b**) Integrin α3β1 is expressed in Cre-induced GFP-positive keratinocytes of K19 Itga3 WT mice and efficiently deleted in GFP-positive keratinocytes of 7-week-old K19 Itga3 KO mice one week after tamoxifen treatment (back skin, scale bar: 30 μm). (**c**) Linage tracing of GFP-positive HB keratinocytes showing localization within their niche in K19 Itga3 KO and WT mice one week after tamoxifen treatment (whole mounts of tail epidermis, scale bar: 200 μm). Linage tracing of up to four weeks can be found in Supplementary figure S1b. (**d**) Whole mount of tail epidermis of K19 Itga3 WT mouse showing colocalization of Cre-induced GFP – positive cells and K15 marker in HBs (scale bar: 500 μm). (e) Linage tracing of GFP-positive HB SCs Cre-induced in telogen (P21) and followed for up to four weeks over whole hair cycle (until P49) in the back skin of K19 Itga3 KO and WT mice. Representative images of two to three mice per condition are shown (scale bar: 50 μm).

The relatively inefficient deletion of α3β1 in HBs in K14 Itga3 KO mice together with the absence of miss-localized HB keratinocytes in K19 Itga3 KO mice suggests that the loss of α3β1 in HB SCs does not lead to their egress, but rather that the miss-localized K15-positive keratinocytes originate from other epidermal compartments of K14 Itga3 KO mice. In order to determine whether K15 in these cells could be expressed de novo upon α3β1 deletion, we performed RT-qPCR of the RNA, isolated from tail and back epidermis of K14 Itga3 KO and WT mice. Indeed, an increased expression of K15 could be detected in the epidermis of K14 Itga3 KO, compared to WT mice (**Fig. 2e**), despite the absence of proliferating K15-positve cells residing in HBs (**Supplementary Fig.**

2b). We further confirmed that the miss-localized K15-positive keratinocytes were not derived from HB SCs by performing a quantitative flow cytometry analysis of the HB population size (CD34+, α6high), which was comparable between K14 Itga3 KO and WT mice (**Supplementary Fig. 2c, d**). This remained true even after mice were submitted to the short-term DMBA/TPA treatment, mimicking the initiation stage of tumorigenesis, during which an increased miss-localization of K15-positive keratinocytes had been previously reported for K14 Itga3 KO mice [14] (**Supplementary Fig. 2c, d**). All in all, this data strongly suggests that keratinocytes in IFE and upper parts of HFs express K15 de novo in the absence of α3β1 and provides new evidence that HB SCs remain confined within their niche regardless of whether they express α3β1.

The contribution of HB keratinocytes to newly formed IFE is increased in the absence of α3β1

The egress of HB SCs into IFE normally occurs during wound-healing, when HB keratinocytes contribute to the formation of neo-epidermis [4,5]. To test whether our model reflects these characteristics of HB SCs and to determine whether the presence of α3β1 affects induced egress of HB keratinocytes, we performed wounding experiments of K19 Itga3 KO and WT mice. The presence of α3β1 in HBs did not affect the closure of the wounds; no differences were observed in the length of neo-epidermis three days after the wounding and in the percentage of closed wounds five days after wounds were inflicted (**Fig. 3a, b**). As expected, Cre-induced, GFP-positive HB keratinocytes contributed to the formation of neo-epidermis upon wounding of both K19 Itga3 KO and WT mice (**Fig. 3c, d**). Whereas at the beginning of the wound re-epithelization no obvious differences in the contribution of HB-originating cells to the neo-epidermis could be observed between K19 Itga3 KO and WT mice (**Fig. 3c**), there was a small, but significant increase in the number of GFP-positive cells in the neo-epidermis of K19 Itga3 KO mice at the final stages of wound closure (**Fig. 3d**). This is consistent with previous observations that the absence of α3β1 promotes cell migration during wound-healing [20]. Together, these findings indicate that during wound healing, the egress of HB SCs into the neo-epidermis increases when α3β1 is absent from HB keratinocytes, which, however, does not alter the rate of re-epithelization.

The absence of α3β1 in HBs reduces susceptibility of mice to DMBA/TPA-mediated tumorigenesis

Next, we investigated the role of HB SC-residing α3β1 in skin carcinogenesis by submitting K19 Itga3 KO and WT mice to the complete DMBA/TPA carcinogenesis protocol. Tumors could be detected six weeks after the beginning of the treatment (P91-P97), regardless of the presence of α3β1 (**Fig. 4a**). Even though both, K19 Itga3 KO and WT mice developed numerous tumors by the end of the treatment (K19 Itga3

KO 33,8 and WT 49,1 tumors on average), there was a marked 30% decrease in tumor formation and a 15% decrease in the average tumor size upon the deletion of Itga3 in HB SCs (**Fig. 4a-b, Supplementary fig. 3a**). In line with this, K19 Itga3 KO mice exhibited significantly lower tumor burden compared to WT mice (K19 Itga3 KO 1313,5 and WT 2296,1 mm2 on average, **Fig. 4a**). Nearly all the tumors were benign papillomas and keratoacanthomas, with no notable difference in their prevalence between the two mouse lines (**Fig. 4c**).

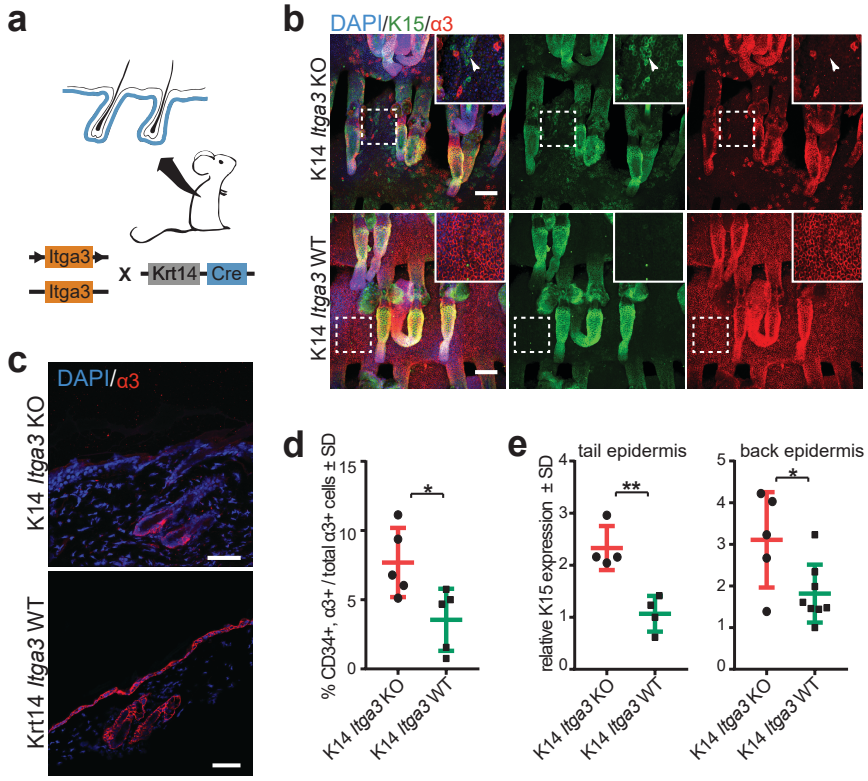


Figure 2: Epidermal deletion of α3β1 causes de novo expression of K15 outside of HBs. (**a**) Overview of the K14 Itga3 KO and WT mouse models. (**b**) Whole mounts of tail epidermis show the presence of α3β1-depleted K15-positive keratinocytes in upper parts of HFs and IFE of K14 Itga3 KO mice (white arrow heads). Remaining α3β1-positive keratinocytes in K14 Itga3 KO mice are preferentially localized to HBs (scale bar: 100 µm). (**c**) Staining for integrin α3 shows HB-localization of α3β1-positive keratinocytes in the back skin of 7-week-old K14 Itga3 KO mice. α3β1 is found in all basal keratinocytes of K14 Itga3 WT mice of similar age (scale bar: 50 µm). (**d**) FACS analysis of keratinocytes isolated from back skin epidermis. The chart shows the percentages of α3-positive HB cells (CD34-positive) in the total α3-positive population. Each dot represents a mouse. Gating strategy can be found in Supplementary figure S2a. (mean ± SD, unpaired t test, *P<0.05). (**e**) GAPDH-normalized relative mRNA expression of K15 is increased in the epidermis of back and tail skin of K14 Itga3 KO compared to WT mice. Each dot represents a mouse and is an average of technical duplicate or triplicate (mean ± SD, unpaired t test, *P<0.05, **P<0.005).

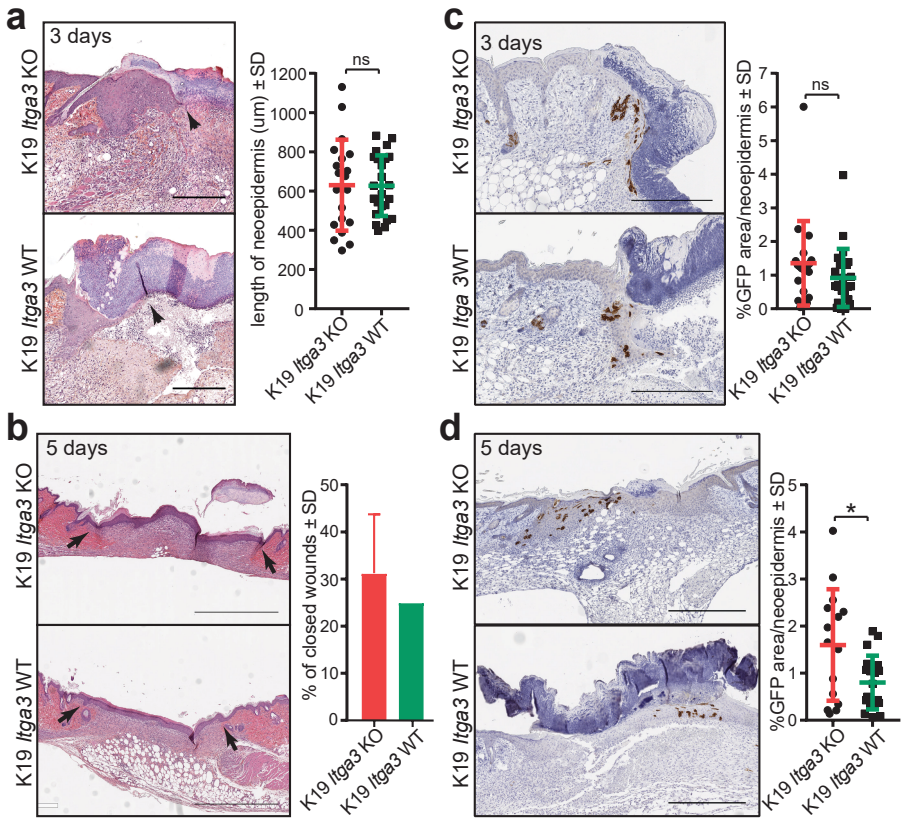


Figure 3: The contribution of HB keratinocytes to newly formed IFE is increased in the absence of α3β1. (**a-b**) H&E staining (left) and quantification (right) of wound-healing. Wound closure is comparable between K19 Itga3 KO and WT mice, 3 (**a**) and 5 days (**b**) after wounding. (**a**) Each dot represents the average length of the neo-epidermis (black arrows) per wound (mean ± SD, unpaired t test). Wounds of 6 K19 Itga3 WT and 5 K19 Itga3 KO mice were analyzed (scale bar: 300 μm). (**b**) Bars represent the percentage of closed wounds per mouse (mean ± SD). Wounds of 4 K19 Itga3 WT and 4 K19 Itga3 KO mice were analyzed (scale bar: 1 mm). (**c-d**) IHC staining for GFP (left) and quantification (right) of GFP-positive area per neo-epidermis of K19 Itga3 KO and WT mice. (**c**) HB-originating GFP-positive keratinocytes comparably contribute to neo-epidermis in K19 Itga3 KO and WT mice 3 days after wounding (scale bar: 300 μm). Each dot represents the percentage of GFP-positive area per wound (mean ± SD, unpaired t test). Wounds of 6 K19 Itga3 WT and 5 K19 Itga3 KO mice were analyzed. (**d**) Five days after the wounding, the contribution of the α3β1-deficient HB SCs to the newly formed epidermis is more extensive than that of the α3β1-proficient HB SCs. Each dot represents the percentage of GFP-positive area per wound (mean ± SD, unpaired t test, *P<0.05). Wounds of 5 K19 Itga3 WT and 4 K19 Itga3 KO mice were analyzed.

By the end of the treatment (P196), ulcerating tumors were observed in one K19 Itga3 KO and three WT mice, which were identified as SCCs (K19 Itga3 KO and WT) and keratoacanthomas with carcinomatous changes (K19 Itga3 WT) by histological analysis (**Supplementary fig. 3b**). As this incidence was too low to draw any conclusions about the role of α3β1 in the malignant progression of tumors, we selected seven K19 Itga3 KO and seven WT mice with low tumor burden and treated them with TPA for up to an additional 10 weeks, until they had to be sacrificed due to the tumor burden or ulceration of tumors. Tumor progression was more commonly observed in K19 Itga3 KO (5 out of 7) compared to K19 Itga3 WT mice (3 out of 7). Furthermore, K19 Itga3 KO mice developed also high malignancy grade tumors such as spindle cell sarcoma and mixed basal SCC in addition to the SCCs and keratoacanthomas with carcinomatous changes (**Supplementary fig. 3c**). Even though the yield of progressed tumors after this prolonged treatment was still too low to draw firm conclusions, the observed trend fits with the results of tumorigenesis experiments previously performed with K14 Itga3 KO and WT mice [14]. All in all, these data show that α3β1 in HB SCs promotes formation of benign tumors during DMBA/TPA treatment.

HB-derived keratinocytes are largely absent from skin tumors

The moderate reduction of tumorigenesis in the K19 Itga3 KO mice, compared to the near complete absence of tumor formation in mice carrying a targeted deletion of Itga3 in the whole epidermis, suggests that HB SCs might be the cells-of-origin for some, but not all DMBA/TPA-initiated tumors. To determine whether papillomas can arise from Cre-initiated HB keratinocytes in K19 Itga3 KO and WT mice, we have analyzed the cross-sectional areas of 321 and 365 tumors, respectively, for the presence of GFP. Remarkably, most tumors were negative for GFP and only one small papilloma isolated from K19 Itga3 WT mouse consisted almost entirely of GFP-positive cells (**Fig. 5a, b**). Consistent with their non-HB origin, all the analyzed tumors stained positive for α3β1 (**Fig. 5c**). 2,8% of tumors, isolated from the K19 Itga3 KO mice and 8,2% of the K19 Itga3 WT tumors contained patches of GFP-positive cells, accounting to up to 5% of the total tumor area (**Fig. 5b**). Such minor cell populations in otherwise monoclonal DMBA/TPA-derived papillomas have been recently described and shown to lack the activating mutation in Hras, the predominant proto-oncogene activated in DMBA/TPA-induced tumors [21]. As the assessment of the amount of HB-originating GFP-positive cells in the tumors was determined based on only one cross section, we reasoned that the actual numbers are likely higher. Indeed, an analysis of 10 cross sections (200 μm step size) in randomly selected tumors of four K19 Itga3 KO and four WT mice showed that respectively, 9,6% and 32,3% of the tumors contain regions of GFP-positive cells. These GFP-positive regions occupy less than 0,1% of the tumor area in the majority of tumors (**Fig. 5d, e**). The number of tumors, containing GFP-positive cells as well as the

GFP-positive regions were significantly reduced in tumors, originating from K19 Itga3 KO, compared to WT mice (**Fig. 5d**). Taken together, these data demonstrate that HB SCs are not the main tumor-initiating cells in the two-stage carcinogenesis model and that HB-derived keratinocytes constitute a minor cell population in some tumors.

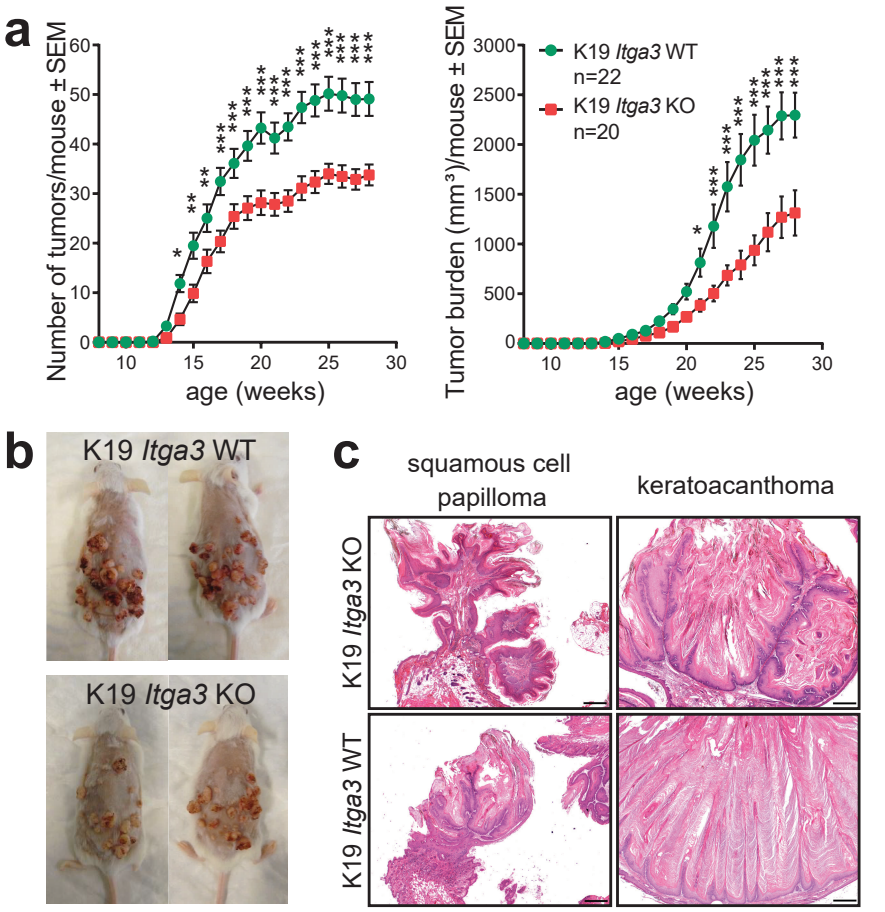


Figure 4: The absence of α3β1 in HBs reduces susceptibility of mice to DMBA/TPA-mediated tumorigenesis. (**a**) The number of tumors (left) and tumor burden (right) is decreased in K19 Itga3 KO compared to WT mice submitted to the DMBA/TPA-carcinogenesis protocol (mean ± SEM, unpaired t test, *P<0.05, **P<0.005, ***P<0.0005). (**b**) Representative macro images of K19 Itga3 KO and WT mice at the end of the treatment. (**c**) Histology of benign papillomas and keratoacanthomas, representing the majority of tumors isolated from K19 Itga3 KO and WT mice.

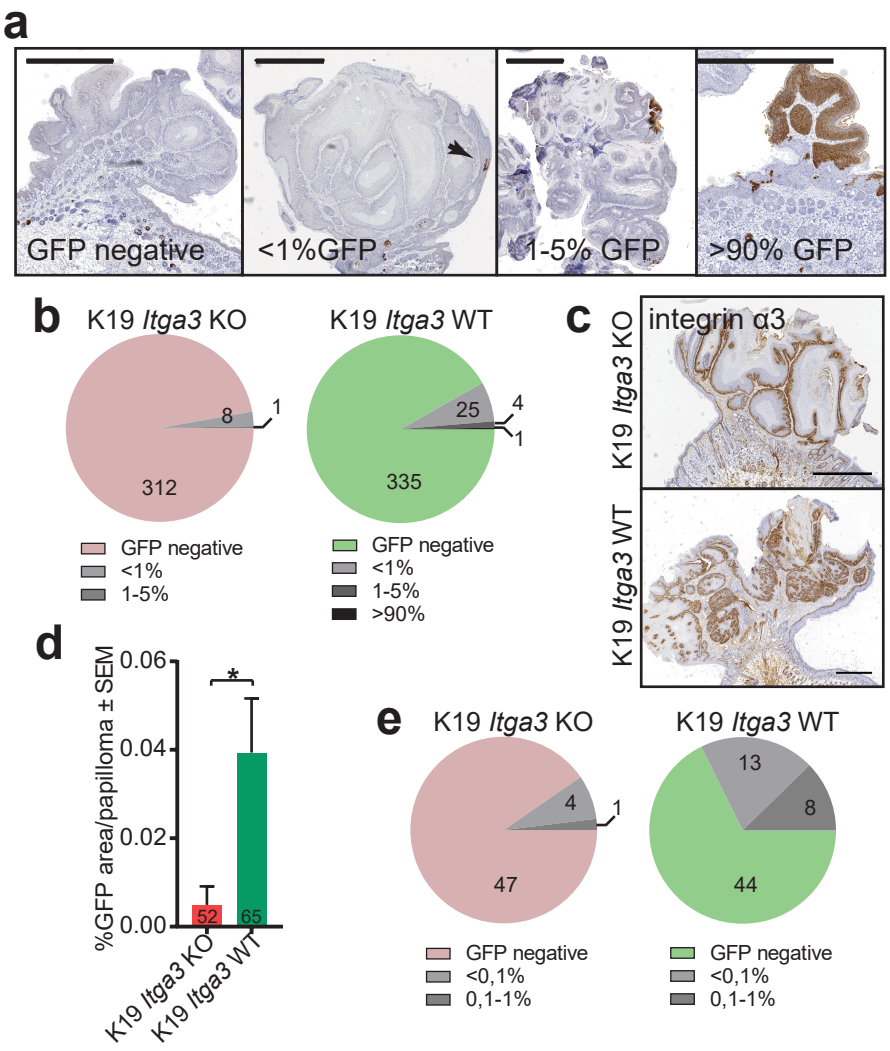


Figure 5: HB-derived keratinocytes are largely absent from skin tumors. (**a-b**) With rare exceptions, Cre-induced GFP-positive cells represent less than 1% of total tumor mass. (**a**) Representative IHC images stained for GFP and (**b**) quantification of GFP-positive area in cross-sections of tumors, isolated from 9 K19 Itga3 KO and 8 WT mice. The vast majority of tumors is GFP-negative (scale bar: 1mm). (**c**) Integrin α3β1 is strongly expressed in all tumors analyzed (scale bar: 1mm). (**d-e**) Analysis of GFP-positive area over 10 cross sections, cut every 200 µm of randomly selected tumors from 4 K19 Itga3 KO and 4 WT mice. (**d**) The contribution of GFP-positive HB-originating keratinocytes to tumors, isolated from K19 Itga3 KO is significantly reduced compared to WT mice (mean ± SEM, unpaired t test, *P<0.05). (**e**) The majority of tumors are GFP-negative in both, K19 Itga3 KO and WT mice. GFP was detected in 9,6% of K19 Itga3 KO and in 32,3% of K19 Itga3 WT tumors and did not exceed 1% of total tumor mass.

α3β1-depleted keratinocytes show an increased differentiation signature and decreased expression of CCN2 during the initiation stage of tumorigenesis

The non-HB origin of the majority of tumors together with the decreased tumorigenesis upon the deletion of HB-derived Itga3 indicate that α3β1 in HB SCs likely promotes tumorigenesis indirectly, i.e. through changes in the pro-tumorigenic environment or by directly affecting neighboring keratinocytes. Because the deletion of Itga3 in HB SCs had a larger effect on the initiation of tumorigenesis than on the rate of tumor growth (**Fig. 4a, Supplementary fig. 3a**), we investigated a potential role of α3β1 in establishing a tumor-supportive environment. To this end, we performed RNA sequencing of GFP-positive keratinocytes isolated from the skin of K19 Itga3 KO and WT mice during the initiation stage of tumorigenesis induced by short-term DMBA/TPA treatment, which has been shown to be sufficient for the outgrowth of papillomas [22,23]. This stage of two-stage chemical carcinogenesis, when pro-tumorigenic pathways are switched on, but tumors have not yet been formed, thus reflects the cell environment that can support tumor formation. At this time point, HB-originating GFP-positive cells can be found in both outer and inner layers of the growing HFs and, interestingly, in some cases in isthmus, infundibulum and IFE of K19 Itga3 KO and WT mice (**Fig. 6a**).

Gene expression profiling of the GFP-positive keratinocytes by RNA-sequencing confirmed their HB origin (e.g. high expression of CD34, Lgr5 and K15) (**Supplementary Fig. 4a**). A total of 15 protein-coding genes were significantly differentially expressed between the GFP-positive Itga3 KO and WT keratinocytes. Several of the hits that displayed an increased expression in Itga3 KO keratinocytes are known to be involved in squamous cell differentiation (**Fig. 6b**), which is in line with previously reported observations of the increased epidermal turnover upon the deletion of Itga3 [14]. Importantly, several of the hits belonged to the keratinocyte secretome, indicating that α3β1 could promote the formation of tumor-permissive environment through regulation of paracrine signaling of the HB SCs (**Fig. 6b**). The high expression of connective tissue growth factor CCN2 (also CTGF) was of particular interest, as this protein has a broad regulatory function in a variety of important biological and pathological processes, is a known integrin interactor and has been implemented in skin tumorigenesis before [24,25]. RNA sequencing data was validated using immunohistochemical and immunofluorescent (IF) staining, which confirmed that CCN2 localizes to HFs and HB SCs of short-term DMBA/TPA-treated back skin (**Fig. 6c, Supplementary Fig. 4b**), as has been previously reported [26,27]. Furthermore, IF staining confirmed the reduction of CCN2 expression in GFP-positive HFs lacking α3β1 (Fig 6c). In papillomas, CCN2 expression could be observed in isolated epithelial or stromal cells and occasionally in cell clusters, which did not correlate to the HB-originating GFP-positive areas in

consecutive sections of tumors, isolated from K19 Itga3 WT mice (**Supplementary fig. 4c, d**). Even though CCN2 could be detected in all K19 Itga3 WT tumors analyzed, a small number of CCN2-positive cells (the mean CCN2-positive surface is 0.27% of total tumor area) together with their non-HB origin indicates that CCN2 does not play a major role in the late stages of tumor growth (**Supplementary fig. 4c**).

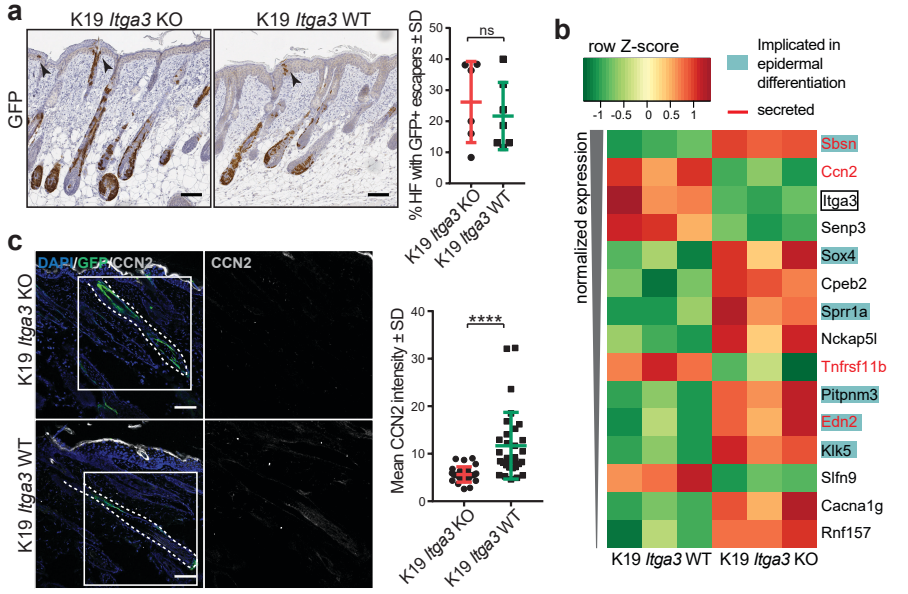


Figure 6: α3β1-depleted keratinocytes show an increased differentiation signature and decreased expression of CCN2 during the initiation stage of tumorigenesis. (**a**) GFP-positive Cre-induced HB SCs localize to growing HFs and, in some cases, to isthmus, infundibulum and IFE (black arrows) after short-term DMBA/TPA treatment in K19 Itga3 KO and WT mice. Left: IHC staining for GFP (scale bar: 100 μm). Right: quantification of the number of HFs, where GFP-positive cells were observed in upper parts of HFs and in adjacent IFE. Each dot represents a mouse (mean ± SD, unpaired t test). (**b**) Heat map of row-scaled significantly differentially expressed protein-coding genes of GFP-positive keratinocytes, isolated from 3 K19 Itga3 KO and 3 K10 Itga3 WT mice after short-term DMBA/TPA treatment. Protein-coding genes have an adjusted P<0.05 and an average normalized expression across all samples >4 (as calculated with Voom) and a logFC>0.6 between K19 Itga3 WT an KO mice. (**c**) IF staining (left) and quantification of mean intensity of the signal (right) for CCN2 in GFP-positive HFs after short-term DMBA/TPA treatment. Each dot represents a GFP-positive HF. HFs of 5 K19 Itga3 KO and 6 K19 Itga3 WT mice were quantified (mean ± SD, unpaired t test, P<0.0001).

Together, this data shows that during the initiation stage of tumorigenesis α3β1 in HB SCs suppresses HF differentiation and regulates the expression of CCN2 and several other proteins that are part of HB SC secretome. Thus, α3β1 might affect

the cell environment during early tumorigenesis through regulation of the paracrine signaling.

CCN2 expression is α3β1-dependent and promotes colony formation and 3D growth of *Hras* transformed keratinocytes *in vitro*

Next, we investigated whether α3β1 regulates the expression of CCN2 in Hras transformed keratinocytes, isolated from K14 Itga3 WT mice that underwent the full DMBA/TPA carcinogenesis protocol (MSCC WT and MSCC Itga3 KO keratinocytes) [14]. In agreement with our observations in mice, deletion of α3β1 in MSCC keratinocytes resulted in reduced levels of CCN2 mRNA (**Fig. 7a**). Furthermore, MSCC WT keratinocytes showed an increased expression of CCN2 when the conditions that occur during DMBA/TPA treatment were mimicked by application of either TPA or IL-6, the cytokine upregulated during DMBA/TPA tumorigenesis and crucial for tumor formation [28] (**Fig. 7a**). Integrin α3β1-dependent expression of CCN2 and its increase upon TPA and IL-6 treatment were confirmed at the protein level using IF and western blot analysis (**Fig. 7b, c, Supplementary fig. 5a**). Furthermore, IF analysis showed that MSCC WT keratinocytes secrete CCN2, which colocalized with deposited laminin-332 (**Fig. 7b, Supplementary fig. 5b**).

To assess whether CCN2 contributes to the tumorigenic properties of transformed keratinocytes in vitro, we generated two CCN2 KO clones using CRISPR/Cas9 with two distinct guide RNAs (MSCC CCN2 KO G1 and MSCC CCN2 KO G2) (**Fig. 8a**) and submitted them to colony formation assay. In line with observations in K14 Itga3 KO mice, the deletion of α3β1 resulted in a strong reduction of colony formation and in decreased colony size (**Fig. 8b and Supplementary fig. 6a**). Even though the deletion of CCN2 did not influence colony size, the colony-forming ability of the two CCN2 KO clones was significantly reduced compared to WT MSCC and control clones (**Fig. 8b and Supplementary fig. 6a**). Next, we tested whether secreted CCN2 can promote survival of transformed keratinocytes by treating Itga3 KO MSCCs and CCN2 KO clones with exogenous CCN2. CCN2 treatment significantly increased the colony formation of CCN2 KO MSCCs, albeit not to the level of the MSCC control clones (**Fig. 8c, Supplementary fig. 6b**). No differences in colony formation could be observed upon the treatment of Itga3 KO MSCCs with CCN2 (**Fig. 8d, Supplementary fig. 6b**), indicating that α3β1-mediated secretion of CCN2 may enhance the tumorigenic potential of keratinocytes, but is not sufficient for tumorigenesis. We further investigated whether α3β1 and CCN2 affect the three-dimensional (3D) growth of MSCC keratinocytes using Matrigel matrix. The results showed that at the beginning of spheroid formation, CCN2 expression was dependent on integrin α3β1 (**Fig. 8e**). Furthermore, spheroids needed α3β1 to successfully accumulate mass in the 3D matrix (Fig 8e, f). Importantly, even though

CCN2 KO clones still formed 3D spheroids, their size was significantly reduced compared to CCN2 control clones and MSCC WT keratinocytes (**Fig. 8f**). In 3D culture, seeding the CCN2 KO MSCC clones in the presence of two different concentrations of CCN2 slightly increased their growth potential (**Fig. 8g, Supplementary fig. 6c**), which was not observed when CCN2 MSCC cells were treated three days after seeding, when spheroids had already been formed **(Supplementary fig. 6d**).

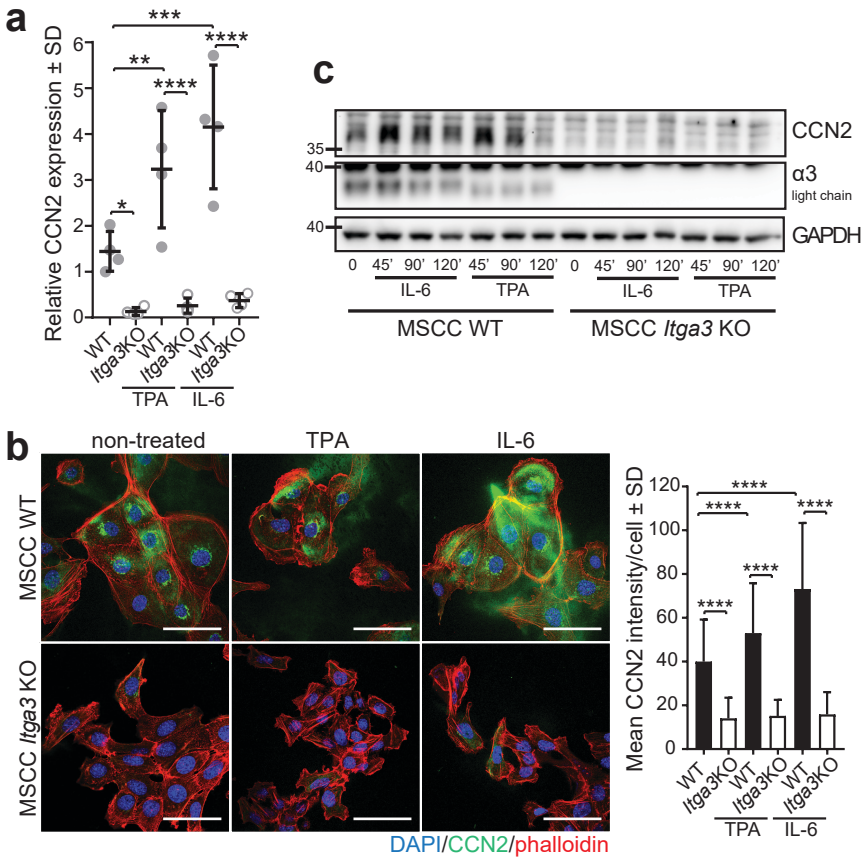


Figure 7: CCN2 expression in transformed keratinocytes is α3β1-dependent. (**a**) GAPDH-normalized relative mRNA expression of CCN2 is significantly decreased in non-stimulated as well as IL-6 and TPA-treated α3β1-depleted keratinocytes. The average of up to four independent measurements of technical duplicates of 4 independent samples is presented (mean ± SD, Fisher's LSD test, *P<0.05, **P<0.005, ***P<0.0005, ****P<0.0001). (**b**) IF (left) and quantification of the mean intensity (right) of CCN2 in non-stimulated, IL-6 and TPA-treated MSCC Itga3 KO and WT keratinocytes. Expression of CCN2 is α3β1-dependent and increases upon IL-6 and TPA treatment (scale bar: 50 μm). 90 cells imaged over 3 independent experiments were quantified (mean ± SD, Fisher's LSD test, P<0.0001). (**c**) Representative WB confirming α3β1-dependent and IL-6 and TPA-mediated CCN2 expression. Quantification can be found in Supplementary figure S5a.

This is in agreement with our previous observations of low CCN2 expression during late stages of tumor and spheroid growth (**Fig. 8e, Supplementary fig. 4c**) and could further indicate that exogenous CCN2 may enhance tumorigenic potential of transformed keratinocytes during early stages of tumorigenesis, however in later stages it likely does not play a major role. However, as CCN2 can associate with extracellular matrix components [25], which we observed also in this study (**Supplementary fig. 5b**), it is conceivable that the concentration of CCN2 that reached cells within the inner layers of spheroids was too low to promote the growth of already formed spheroids, despite the high concentration of CCN2 used (**Supplementary fig. 6d**). Finally, we confirmed that α3β1-expression is an essential prerequisite for CCN2-mediated promotion of tumorigenesis, as seeding Itga3 KO MSCC with CCN2 had no effect on their 3D growth (**Fig. 8h**).

Together, this data shows that CCN2 plays a role in supporting the tumorigenic potential of α3β1-expressing transformed keratinocytes in vitro, which makes it a likely player in our in vivo tumorigenesis mouse model.

DISCUSSION

The previous hypothesis on the mechanism behind the essential role of integrin α3β1 in DMBA/TPA-induced tumorigenesis heavily weighted on the idea that HB SCs represent the cancer cells-of-origin and that the deletion of α3β1 promotes their egress into IFE [14]. Here, we show that the deletion of α3β1 in HB keratinocytes causes a slight increase in their egress only during wound-healing. As the egress of HB SCs and α3β1-mediated suppression of keratinocyte migration during wound-healing is well established, such role of HB-residing α3β1 in wound re-epithelization is not unexpected [4,20,29]. More surprising is the observation that the deletion of α3β1 does not cause the egress of HB SCs under normal homeostatic conditions, even though α3β1-depleted, K15-positive keratinocytes are found in the upper parts of HF and in IFE of K14 Itga3 KO mice [14]. As the deletion of α3β1 causes an increased epidermal turnover in K14 Itga3 KO mice [14], our finding that these keratinocytes express K15 de novo ties well with the reported close relationship between K15 expression and the loss of homeostasis of the epidermal differentiation program in basal-like cells [17].

The DMBA/TPA-induced skin carcinogenesis model mimics the multi-stage nature of cancer, in which substantial time is needed for tumors to outgrow from cancer-initiating cells. In line with this, the importance of slow-cycling, label-retaining cells in DMBA/TPA-initiated tumorigenesis has long been established [30,31].

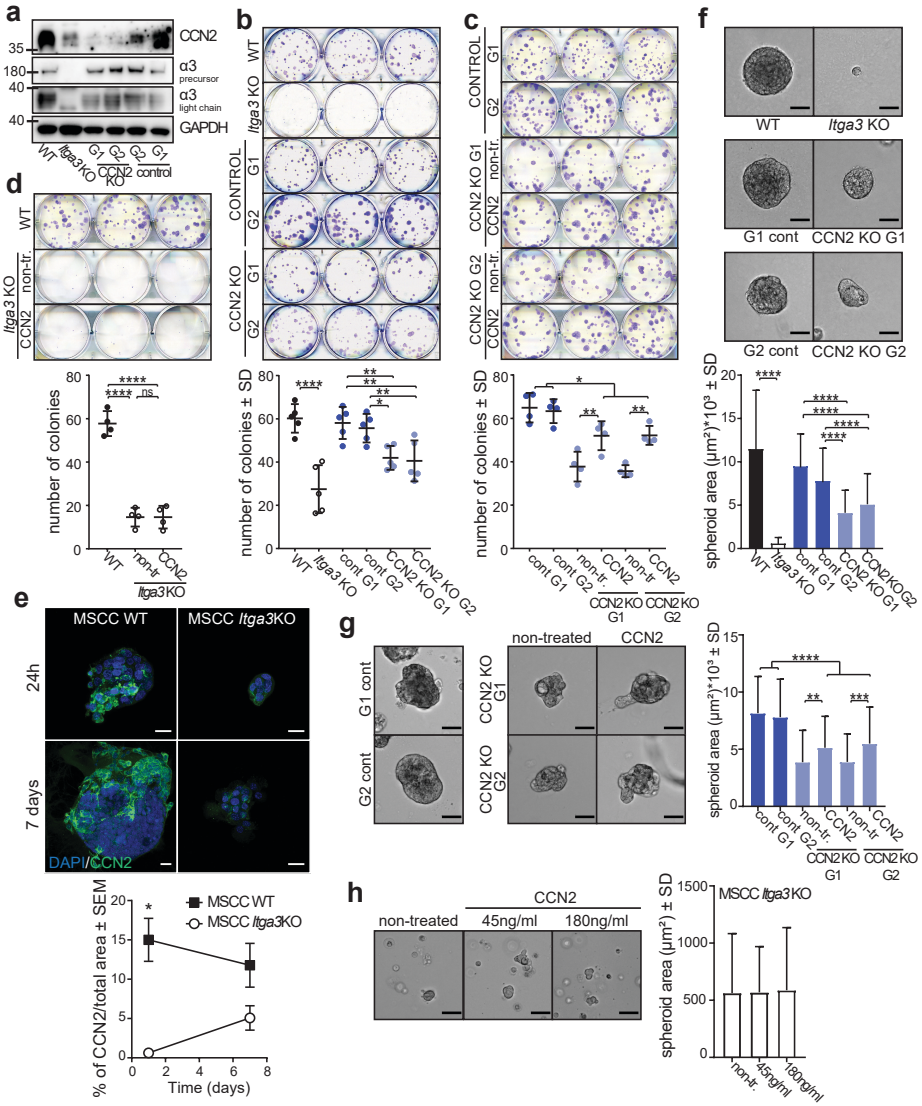


Figure 8: CCN2 promotes colony formation and 3D growth of transformed keratinocytes expressing α3β1. (**a**) WB of CCN2 and integrin α3-expression of selected CCN2 KO and control clones. (**b**) Representative image (top) and quantification (bottom) of colony formation assay of MSCC Itga3 WT and Itga3 KO cells and MSCC CCN2 KO G1, KO G2, control G1 and control G2 clones. Deletion of α3β1 results in a strong reduction of colony formation and colony size. Moderate reduction of colony formation can be seen upon CCN2 deletion. Quantification of colony size can be found in Supplementary figure S6a. Average values of technical triplicates of 5 independent experiments are presented (mean ± SD, Fisher's LSD test, *P<0.05, **P<0.005, ****P<0.0001). (**c**) Representative image (top) and quantification (bottom) of colony formation assay of MSCC CCN2 KO G1 and KO G2 clones, grown in control conditions or in the presence of 45 ng/ml CCN2, as well as CCN2 WT control G1 and

control G2 clones. Treatment with exogenous CCN2 significantly increases colony formation of CCN2 KO clones. Quantification of colony size can be found in Supplementary figure S6b. Average values of technical triplicates of 4 independent experiments are presented (mean ± SD, Fisher's LSD test, *P<0.05, **P<0.005). (**d**) No differences in the number of colonies can be observed upon CCN2 treatment of Itga3 KO transformed keratinocytes. Quantification of colony size can be found in Supplementary figure S6b. Average values of technical triplicates of 4 independent experiments are presented (mean ± SD, Fisher's LSD test, ****P<0.0001). (**e**) Quantification (left) and IF as maximum intensity projection (right) of CCN2 expression of MSCC Itga3 WT and KO spheroids, grown in 3D Matrigel matrix for 1 or 7 days (scale bar: 20 μm). The expression of CCN2 is α3β1-dependent, which is particularly prominent at the beginning of spheroid growth. The percentage of CCN2-positive area was quantified from 17 MSCC Itga3 KO and 30 MSCC Itga3 WT spheroids 1 day after seeding and from 15 MSCC Itga3 KO and 27 MSSC WT spheroids 7 days after seeding (mean ± SEM, unpaired t test, *P<0.05). (**f**) Spheroid growth in 3D Matrigel is α3β1-dependent and moderately reduced upon CCN2 deletion. Top: bright filed images of representative spheroids (scale bar: 50 μm). Bottom: size quantification of 60-80 spheroids measured over 3 to 4 independent experiments (mean ± SD, Fisher's LSD test, ****P<0.0001). (**g**) 3D growth of CCN2 KO MSCC spheroids shows small but significant increase when cells are seeded with 45 ng/ml of CCN2. Left: bright filed images of representative spheroids (scale bar: 50 μm). Right: size quantification of 85-90 spheroids measured over 3 independent experiments (mean ± SD, Fisher's LSD test, **P<0.005, ***P<0.0005 ****P<0.0001). (**h**) Seeding Itga3 KO MSCCs with 45 ng/ml or 180 ng/ml of CCN2 does not impact 3D growth pf spheroids. Left: bright filed images of representative spheroids (scale bar: 50 μm). Right: size quantification of 70 spheroids measured over 2 independent experiments (mean ± SD, One-way ANOVA P=0.9491).

Therefore, a loss of initiated label-retaining stem cells due to their premature terminal differentiation during increased epidermal turnover remains a plausible cause of the near complete absence of tumorigenesis in K14 Itga3 KO mice [14]. The finding that α3β1 affects epidermal turnover was confirmed in this study, where we an increased differentiation gene signature in GFP-positive cells, isolated from K19 Itga3 KO mice, although the differences in differentiation were not substantial enough to affect the hair cycle or hair growth in mice. This was expected, as hair cycle was not altered in K14 Itga3 KO mice of similar age [14], although the loss of integrin α3β1 has been shown to affect hair follicle maintenance and morphology in mice, bred onto C57Bl/6 background [14,32].

HB SCs have often been suggested to be the main cells-of-origin for DMBA/TPA-initiated tumors [33–36]. However, several studies have demonstrated that other epidermal cell populations can also act as cancer SCs in the DMBA/TPA model of skin carcinogenesis [9,15,16,30]. Considering also the fact that the K15 promoter, which is commonly used for HB-specific genetic modifications, does not target this compartment exclusively [9], there is an emerging consensus that several epidermal SC populations can serve as cells-of-origin for DMBA/TPA-initiated tumors, albeit that there are likely differences in their fate during the progression of the disease [11,30]. In this study, we showed that the contribution of HB SCs to skin tumors is minimal, and even further reduced in the K19 Itga3 KO mice. A similar observation was made by Goldstein and co-workers, when

they used the K19 promoter to target the deletion of the transcription factor Nfatc1 in HBs [37]. A recent, thorough study by Reeves et al. (2018) demonstrated that DMBA/TPA treatment induces papillomas that are mostly monoclonal but often contain additional minor populations at the edges of tumors. Interestingly, these minority populations, which resemble our HB-originating tumor patches, commonly do not possess driver Hras mutation and in carry a lower mutational load [21].

Despite their near absence in skin tumors, HB SCs affected tumor incidence and, to a much lesser degree, tumor size in an α3β1-dependent manner. The possibility that HB SCs can affect (rather than be) cancer-initiating cells has received undeservedly little attention. Only a very recent study by the research group of Valentina Greco demonstrated that HB SCs exhibit tolerance to Hras mutation and non-autonomously affect neighboring epithelial and stromal cells [38]. Our observations of HB-originating GFP-positive cells in IFE and upper parts of HFs during initiation of tumorigenesis indicate that α3β1 could affect neighboring cells and/or promote a permissive tumor environment in different epidermal niches. Furthermore, small HB-derived cell populations in tumors may also affect the survival and/or proliferation of neighboring tumor cells.

It is well established that the cellular environment, composed of fibroblasts, endothelial cells, and immune cells, plays a crucial role in development and progression of DMBA/TPA-initiated tumors [33,39–41]. Over the past few years, the role of integrins beyond adhesion and biomechanical transduction of signals has become more evident, especially in their ability to modulate the tumor microenvironment [42]. In particular, integrin α3β1 has been shown to regulate paracrine signaling and crosstalk between cells, among others by controlling the expression of secreted cellular proteins [43–45]. This is in line with our findings that the expression of CCN2 in HB keratinocytes in vivo and in mouse keratinocytes in vitro depends on α3β1. CCN2 has been implemented in skin tumorigenesis previously and is an interesting potential player in DMBA/TPA-induced skin cancer because its expression can be regulated by several proteins, essential for tumor formation in this model, such as FAK, YAP, TGFβ and Stat3 [8,24,25,46–48]. Moreover, it can directly interact with integrins, as well as with cytokines, such as TGFβ, and heparan sulfate proteoglycans, which are abundantly present in the basement membrane of the skin, and thus can modulate related signal transduction pathways and their crosstalk [25]. Furthermore, it was shown that the expression of CCN2 in mesenchymal cells in the skin and lungs affects a differentiation program of adjacent epithelial cells [49], which fits well with the role of α3β1 in the DMBA/TPA tumorigenesis model. As the expression of CCN2 in full-grown papillomas is very low and could not be correlated to HB-originating areas by histochemistry, its potential pro-tumorigenic

role likely occurs during the initiation stage of DMBA/TPA-driven tumorigenesis. This is supported by our in vitro studies, where CCN2 increased the clonogenic potential and initiation of 3D growth of transformed keratinocytes. However, it is clear that these in vitro studies cannot be used for drawing definitive conclusions concerning the role of CCN2 in vivo, and that further work is needed to explore this role. It would be particularly interesting to see if the deletion of CCN2 in HBs reduces the formation of papillomas in mice subjected to the two-stage carcinogenesis protocol.

It is important to note that the effect of CCN2 deletion on the *in vitro* tumorigenic properties of transformed keratinocytes is weak to moderate compared to the pronounced effect of the deletion of Itga3. Furthermore, the ability of CCN2 to promote the survival and outgrowth of transformed keratinocytes depends on the expression of α3β1. Thus, CCN2 may enhance tumorigenesis but is not essential for it. Considering also the fact that the deletion of α3β1 in HB SCs fails to completely recapitulate the effect of total epidermal α3β1 deletion on tumorigenesis, there must be additional α3β1-dependent functions that are relevant to tumor initiation and formation. Therefore, our study reopens the question of the mechanism behind the essential role of α3β1 in DMBA/TPA-driven tumorigenesis. Here, we disprove the original hypothesis that the dramatic effect of epidermal α3 deletion can be explained by the egress of HB SCs. Furthermore, we show that α3β1 in HB SCs contributes to tumorigenesis, however its moderate effect strongly indicates that there are additional mechanisms in play, which will have to be re-examined.

Even though K19 promoter targets the HB with high specificity, a limitation of the K19-CreER mouse model is the low efficiency of Cre-mediated recombination. We have observed Cre-induction in majority of HFs, however α3β1 has not been deleted in all the HBs, which could mean that the contribution of HB-originating cells to tumor mass has been understated. With this concern in mind we have analyzed a large number of tumors for HB-originating, GFP-positive cells. As we found only 1 tumor out of 365 to consist entirely of GFP-positive cells, we believe that the conclusion that HB SCs do not represent the main cancer cell-of-origin is credible. The remaining α3β1-positive cells in HBs of K19 Itga3 KO mice could also lead to skewed effects of the deletion of α3β1 in HBs in tumorigenesis assays. This is of lesser concern, as we observed non-efficient deletion of α3 also in the HBs of K14 Itga3 KO mice, i.e. the model in which DMBA/TPA-treatment resulted in near absence of tumor formation. Furthermore, it should be noted that differences between the two models may arise because of the different timing of Itga3 deletion in the K14 Itga3 KO and K19 Itga3 KO mice. However, the subtility of the phenotype of constitutive Itga3 epidermal deletion at the age when HB-specific Itga3 deletion was induced in K19-driven mouse model offers some reassurance that

our findings with the K19 Itga3 KO mice can be related to those with the K14 Itga3 KO mice [20].

In conclusion, we show that α3β1 in HB SC population indirectly contributes to skin tumorigenesis. Integrin α3β1-regulated expression of matricellular protein CCN2 during the initiation of tumorigenesis indicates that α3β1 might mediate paracrine signaling and thus promote the formation of a permissive tumor environment. The role of CCN2 as a potential player in α3β1-mediated tumorigenesis was demonstrated in transformed keratinocytes in vitro. Even though our findings elucidate only parts of the mechanism underlying the essential function of α3β1 in DMBA/TPA-driven tumorigenesis, they provide a new understanding of the role of HB SCs in skin tumorigenesis as well as offer an important insight into complex and diverse ways in which integrins can affect this disease.

MATERIALS AND METHODS

Generation of mice

K19-CreER mice [18] were intercrossed with mT/mG mice (Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo , Jackson Laboratory strain 007576) to obtain K19-CreER; mT/mG mice (K19 Itga3 WT mice). K19-CreER; mT/mG mice were further intercrossed with Itga3fl/fl (i.e. Itga3tm1Son/tm1Son according to Mouse Genome Informatics) to obtain K19-CreER; mT/mG; Itga3fl/fl (K19 Itga3 KO) mice. Epidermis-specific mice are named Krt14tm1(cre)Wbm (K14 Itga3 WT mice) and Krt14tm1(cre)Wbm; Itga3tm1Son/tm1Son (K14 Itga3 KO mice) according to the Mouse genome Informatics and have been described before [14]. All mice were bred onto an FVB/N background. 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.

Animal experiments

A DMBA/TPA-carcinogenesis protocol has been previously described [14]. Briefly, backs of 6-week old mice were shaved and after a week topically treated with 30μg (in 200μl acetone) of DMBA (Sigma, D3254), followed by bi-weekly topical applications of 12,34μg (in 200μl acetone) of TPA (Sigma, P1585) for 20 weeks. A similar procedure was used for short-term DMBA/TPA treatment; however, mice were only treated with four doses of TPA over two weeks following DMBA treatment. To induce Cre-recombinase, 5-week and 5-day old mice were injected intraperitoneally (IP) for four days with 2,5 mg/ml of tamoxifen (Sigma, T5648) dissolved in sunflower oil per day and (at 6 weeks) additionally topically treated with two doses of 200 μl of 20 mg/ml of tamoxifen dissolved in ethanol.

For linage tracing, 3-week old mice were given an intraperitoneal injection of 2,5 mg/ml of tamoxifen dissolved in sunflower oil per day for four consecutive days. After indicated time points, mice were killed, and the skin was isolated and processed for immunofluorescence, immunohistochemistry or flow cytometry analysis, and/or for RNA isolation. Wound-healing experiments were performed as described previously [50]. 5-week and 5-day old mice were given an intraperitoneal injection of 2,5 mg/ml of tamoxifen dissolved in sunflower oil per day for four consecutive days. 7-week old mice were anesthetized and shaved, and four full-thickness excision wounds of 4mm diameter were cut with small scissors (2 per either side of the dorsal midline). Complete wounds including surrounding tissue were excised 3 and 5 days after injury. Paraffin sections across the middle of the wounds were used for histological analysis of the wound closure. 10 consecutive sections cut every 100µm were used for quantification of the GFP-positive area per area of neo-epidermis.

Immunohistochemistry

Skin, tumors and tails were isolated, fixed in ethanolglacial acetic acid mixture (3:1), containing 2% of formaldehyde (EAF) and/or formaldehyde, embedded in paraffin, sectioned and stained for hematoxylin and eosin (H&E) and/or immunohistochemistry (see table 1). 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) or with the Aperio ScanScope (Aperio, Vista, CA, USA), using ImageScope software version 12.0.0 (Aperio). Tumor classification was performed blindly. Image analysis was performed using ImageJ [51,52].

Immunofluorescence and whole mounts

Skin was isolated and embedded in Tissue-Tek OCT (optimal cutting temperature) cryoprotectant. Cryosections of skin were prepared, fixed in ice-cold acetone, and blocked with 2% bovine serum albumin (BSA, Sigma) in PBS for 1h at room temperature. Whole mounts of tail epidermis were isolated as described previously [14], fixed in 4% paraformaldehyde in PBS and permeabilized and blocked in PB buffer (20 mM Hepes buffer, pH 7.2, containing 0.5% (vol/vol) TrotonX-100, 0.5% (wt/vol) skim milk powder, and 0.25% (vol/vol) fish skin gelatin). MSCC cells were fixed with 2% paraformaldehyde for 10 min, permeabilized with 0.2% Triton-X-100 for 5 min, and blocked with PBS containing 2% BSA for 1h at room temperature. Spheroids were retrieved from Matrigel by incubation with Cell recovery solution (CorningTM, 354253) for 1h at 4°C and subsequently resuspended in ice-cold PBS. Isolated spheroids were mounted on Poly-L-Lysine (Santa Cruz, 25988-63-0)-coated slides, fixed in 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.2% Triton-X-100 for 5 min, and blocked with PBS containing 2% BSA for 1 h at room temperature. Tissues, spheroids and cells

were incubated with the indicated primary antibodies (see Table 1) in 2% BSA in PBS (whole mounts: PB buffer) for 60 min (whole mounts and spheroids: overnight), followed by incubation with secondary antibodies diluted 1:200 for 60 min (whole mounts: overnight). All samples where counterstained with DAPI for 5 min at room temperature and, when indicated, filamentous actin was visualized using Alexa Fluor 488-conjugated phalloidin (Invitrogen). Cryosections were mounted in Vectashield (Vector Laboratories H-1000) and other samples in Mowiol. Samples were analyzed by Leica TCS SP5 confocal microscope with a 10 or 20x (NA 1.4) objective or 40 and 63x (NA 1.4) oil objective and processed using ImageJ [51,52].

Flow cytometry

Keratinocytes were isolated from mouse back skin as described before [53], washed in PBS containing 2% FCS, and incubated for 1 h at 4°C in primary antibody (see table 1) in PBS 2% FCS. In case of non-fluorophore conjugated antibodies, cells were subsequently incubated with donkey anti-goat Alexa Fluor 647 (Invitrogen; 1:200 dilution) antibody for 30 min at 4°C. Cells were analyzed on a Becton Dickinson FACS Calibur analyzer after the addition of indicated life/dead cell marker. For fluorescent activated cell sorting, GFP-positive cell population was obtained using a Becton Dickinson FACSAria IIu cell sorter.

RT-qPCR

Keratinocytes were isolated from mouse back skin as described before [50], pelleted by centrifugation and resuspended in Trizol reagent (Invitrogen, 15596018). Whole mounts of tail epidermis were isolated [14] and homogenized in Trizol reagent using Polytron, while keeping the temperature of the tissue at 4°C. 10cm dishes of semi-confluent cells were washed with cold PBS, scraped and collected in 2 ml of Trizol reagent. Total RNA was extracted according to the manufacturer's recommendations (Trizol reagent) and 3µg of purified RNA was used to synthesize the first-strand cDNA using First strand cDNA synthesis kit (Thermo scientific, K1612). Quantitative PCR analyses were performed using a Sybr Green gPCR Master mix (Thermo scientific, K0251) and ABI Prism 7500 Real Time PCR System (Applied Biosystems) real-time PCR system. Analysis of results was performed with 7500 Fast System SDS software v 1.4 (Applied Biosystems). Results were presented as relative quantification (RQ) of the ratio of K15 or CCN2 Ct over the GAPDH Ct. Following primers were used 5'-TGAGAAGGTGACCATGCAGA-3' and 5'-GGCAGCCAGAATCGGATCTC-3' for keratin 15, 5'-AGAACTGTGTACGGAGCGTG-3' and 5'-GTGCACCATCTTTGGCAGTG-3' for CCN2 and 5'-ACTCCACTCACGGCAAATTC-3' and 5'-TCTCCATGGTGGTGAAGA-3' for GAPDH.

Expression analysis of HB-originating keratinocytes

Keratinocytes from back skin of short-term DMBA/TPA-treated mice were isolated as described before and FACS-sorted for GFP-positive cells. The total RNA from was isolated using the RNeasy Mini Kit (74106, Qiagen), including an on-column DNase digestion (79254, Qiagen), according to the manufacturer's instructions. Quality and quantity of the total RNA was assessed by the 2100 Bioanalyzer using a Nano chip (Agilent, Santa Clara, CA). Total RNA samples having RIN>8 were subjected to library generation. Strand-specific libraries were generated using the TruSeq Stranded mRNA sample preparation kit (Illumina Inc., San Diego, RS-122-2101/2) according to the manufacturer's instructions (Illumina, Part # 15031047 Rev. E). Briefly, polyadenylated RNA from intact total RNA was purified using oligo-dT beads. Following purification, the RNA was fragmented, random primed and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen, part # 18064-014) with the addition of Actinomycin D. Second strand synthesis was performed using Polymerase I and RNaseH with replacement of dTTP for dUTP. The generated cDNA fragments were 3' end adenylated and ligated to Illumina Paired-end sequencing adapters and subsequently amplified by 12 cycles of PCR. The libraries were analyzed on a 2100 Bioanalyzer using a 7500 chip (Agilent, Santa Clara, CA), diluted and pooled equimolar into a multiplex sequencing pool. The libraries were sequenced with 65 base single reads on a HiSeq2500 using V4 chemistry (Illumina Inc., San Diego). Demultiplexing of the reads was performed with Illumina's bcl2fastq. Demultiplexed reads were aligned against the mouse reference genome (build 38) using TopHat (version 2.1.0, bowtie 1.1). TopHat was supplied with a known set of gene models (Gene Transfer Format file (GTF) Ensembl version 77) and was guided to use the first-strand as the library-type. As additional parameters --prefilter-multihits and --no coverage were used. In order to count the number of reads per gene, a custom script which is based on the same ideas as HTSeq-count has been used. For each sample, uniquely mapped sequencing reads were used in order to get the genecounts for all genes present in the GTF file. The strandedness of the mapped reads was taken into account. Differentially expressed analysis was performed using the R packages Limma/EdgeR. Reads that have zero counts across all samples were removed from the dataset. Samples were normalized to counts per million using the 'Voom' function from the Limma package. Gene expressions used for visualization purposes are normalized to 10 million reads and log2 transformed. Before log2 transformation, 1 pseudocount was added in order to avoid negative gene expressions.

Cell culture

MSCC WT and Itga3 KO cells were generated as described [14] and cultured in DMEM with 10% heat-inactivated FCS and antibiotics at 37°C in a humidified, 5% CO2 atmosphere. For CRISPR/Cas9-mediated CCN2 deletion, we cloned target sgRNAs against CCN2 (exon

2; guide1: 5'-ACTCCGATCTTGCGGTTGGC-3'; guide2: 5'-CTCCGATCTTGCGGTTGGCG-3') into pX330.pgkpur vector (a kind gift from the lab of Hein te Riele [54]). MSCC WT cells were transiently transfected with this vector using lipofectamine® 2000 (Invitrogen). Cells were selected with 5 µg/ml of puromycin and puromycin-resistant bulk population was single-cell cloned. Clones were analyzed for the expression of CCN2 using WB analysis. For IL-6 and TPA stimulation of MSCC we added 10 ng/ml of recombinant human IL-6 (R&D Systems, 206-IL) or 100 ng/ml of TPA to DMEM 10% FCS and incubated cells with the mixture for 45 min (for IF and RT-qPCR analysis) or for 45, 90 and 120 min (for WB analysis). For 3D cell culture, 70 µl of growth factor reduced Matrigel Basement Membrane Matrix (Corning, 354230) was pipetted per well of chilled 96-well plate and incubated for 30 min at 37°C. 1000 cells in cold DMEM containing 10% FCS and 2% Matrigel were seeded on top of Matrigel layer and grown for up to 7 days. For CCN2-treatment experiments, cells were seeded in the presence of 45 ng/ml or 180 ng/ml of mouse recombinant CCN2 (Biovendor, RD272589025) and cultured for up to 9 days. Where indicated, 180 ng/ml of CCN2 was added to growth medium of non-treated spheroids on day 3. Medium and CCN2 was refreshed every 3 days of 3D cell culture.

Colony formation assay

Hundred cells in DMEM 10 % FCS were seeded per well of a 6-well plate and incubated at 37°C in a humidified, 5% CO2 atmosphere for 6 days. Where indicated, 45 ng/ml of CCN2 was added to cells during seeding. Colonies were fixed in chilled methanol for 20 min, washed with PBS and stained with crystal violet. Stained plates were scanned and colony number and size was quantified using ImageJ [51,52].

Western Blot

Protein lysates were obtained from sub-confluent cell cultures by lysis in RIPA buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 4 mM EDTA (pH 7.5), 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with 1.5 mM Na_3VO_4, 15 mM NaF (Cell Signaling) and protease inhibitor cocktail (Sigma). Lysates were cleared by centrifugation at 14.000 x g for 20 min at 4°C and eluted in sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 12.5 mM EDTA, 0.02% bromophenol blue) with final concentration of 2% β-mercaptoethanol and denatured at 95°C for 10 min. Proteins were separated by electrophoresis using Bolt Novex 4–12% gradient Bis-Tris gels (Invitrogen), transferred to Immobilon-P transfer membranes (Millipore Corp) and blocked for 1h in 2% BSA in TBST buffer (10 mM Tris (pH 7.5), 150 mM NaCl, and 0.3% Tween-20). The blocked membranes were incubated overnight at 4°C with primary antibodies (see table 1) in TBST containing 2% BSA, following by 1 h hour incubation at room temperature with horseradish peroxidase–conjugated goat anti-mouse IgG or goat anti-rabbit IgG (diluted 1:5000 in 2% BSA in TBST buffer). After washing, the bound antibodies were detected

by enhanced chemiluminescence using or Clarity™ Western ECL Substrate (Bio-Rad) and signal intensities were quantified using ImageJ [48,49].

Antibodies

Primary antibodies used are listed in Table 1. Secondary antibodies were: donkey anti-rabbit Alexa 594, donkey anti-goat Alexa 594, donkey anti-goat Alexa 647, goat-anti mouse Alexa 647 (Invitrogen), stabilized goat anti-mouse HRP-conjugated and stabilized goat anti-rabbit HRP-conjugated (BioRad).

Antigen	Name	Type	Application	Dilution	Source
Integrin α3		Rabbit pAb	WB	1:2000	Home made
Integrin α3	AF2787	Goat pAb	IF	1:100	R&D
Integrin α3	AF2787	Goat pAb	FACS	1:100	R&D
Integrin α3	sc-374242	Mouse mAb	IHC	1:500	Santa Cruz
Integrin α6-PE	eBioGoH3	Rat mAb	FACS	1:200	eBioscience
CCN2	E-5	Mouse mAb	WB	1:800	Santa Cruz
CCN2	L-20	Goat pAb	IF	1:100	Santa Cruz
CCN2	L-20	Goat pAb	IHC		Santa Cruz
CD34-FITC	RAM34	Rat mAb	FACS	1:100	eBioscience
GAPDH	CB1001	Mouse mAb	WB	1:1000	Calbiochem
GFP	ab6556	Rabbit pAb	IHC	1:2000	Abcam
Keratin 15	MA1-90929	Mouse mAb	IF	1:200	Thermo scientific
Keratin 15	MA1-90929	Mouse mAb	IHC	1:200	Thermo scientific
Ki67	PSX1028	Rabbit pAb	IHC	1:750	Monosan
Laminin-332	R14	Rabbit pAb	IF	1:400	Kind gift of M. Aumailey

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

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (version 7.0c). Graphs represent the mean and error bars standard deviation (SD) or standard error of mean (SEM), as indicated per graph. Unpaired two-tailed t test was used for comparisons of experimental groups with a control group. One-way ANOVA was used to analyze experiments with more than two groups and two-way ANOVA was performed on experimental data where we investigated the effect of different treatments on two distinct cell types. Type I errors were reduced by testing only planned comparisons among a relatively small number of means. Planned comparisons were conducted using Fisher's Least Significant Difference test after a global ANOVA was determined to be

significant. The significant values shown are described in appropriate figure legends. Results with P value lower than 0.05 were considered significantly different from the null hypothesis.

DATA AVAILABILITY

The RNA sequencing data from this publication have been deposited to the GEO database and assigned the identifier GSE135983.

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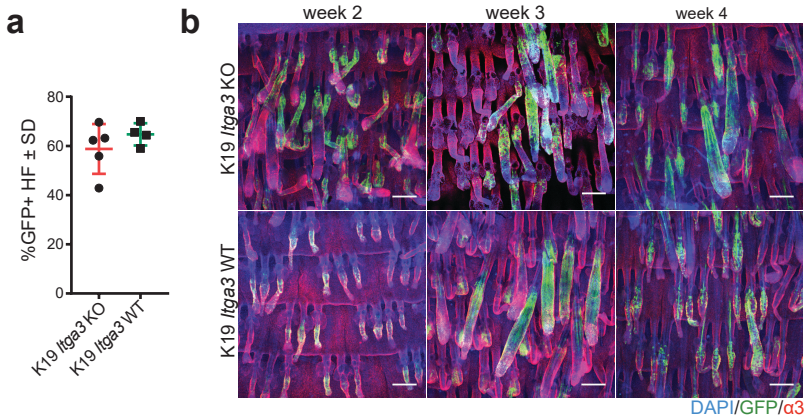
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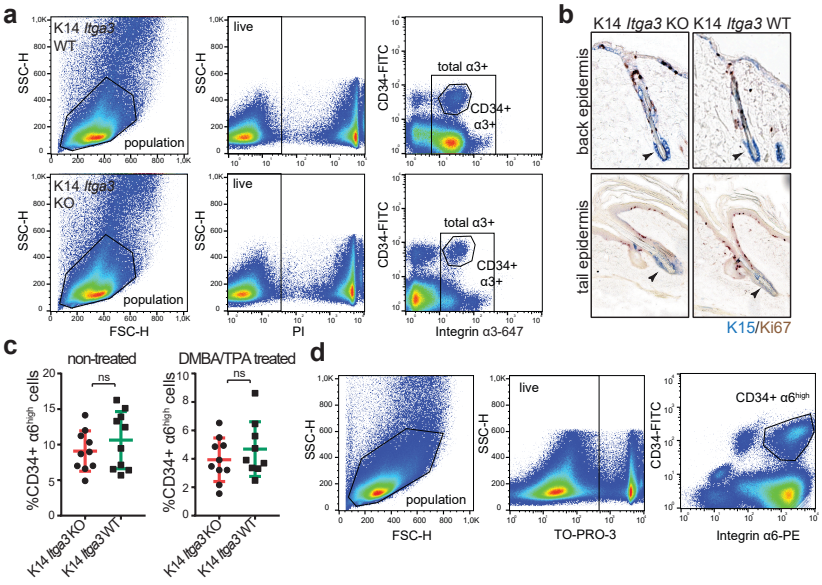
SUPPLEMENTARY FIGURES

Supplementary figure 1



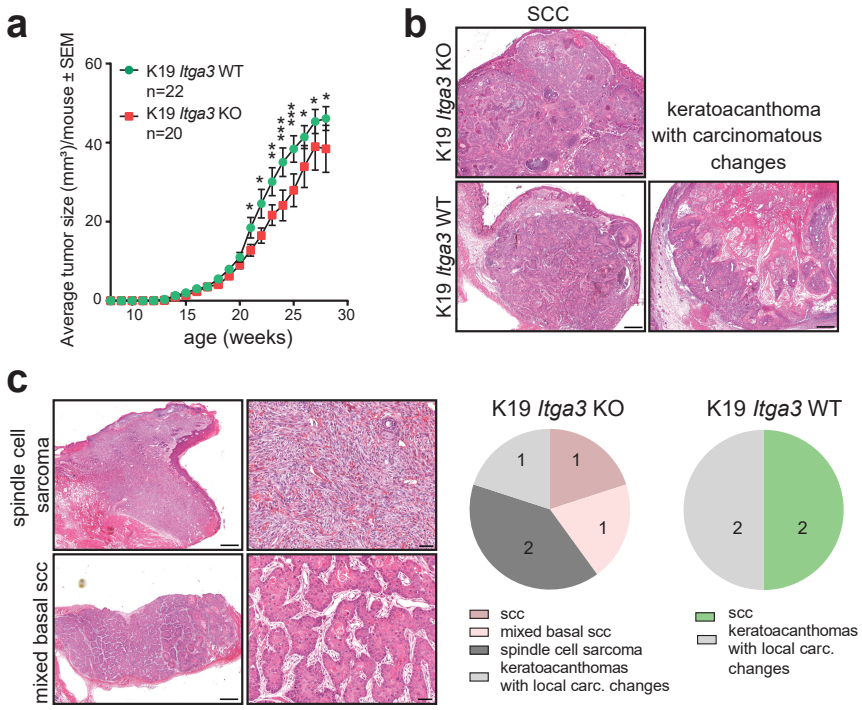
(**a**) Quantification of the percentage of GFP-positive HFs in the back skin of 7-week-old K19 Itga3 KO and WT mice one week after tamoxifen treatment. Each dot represents a mouse (mean ± SD, unpaired t test). (**b**) Linage tracing of GFP-positive HB keratinocytes in K19 Itga3 KO and WT mice at up to four weeks after tamoxifen treatment (whole mounts of tail epidermis, scale bar: 200 μm).

Supplementary figure 2



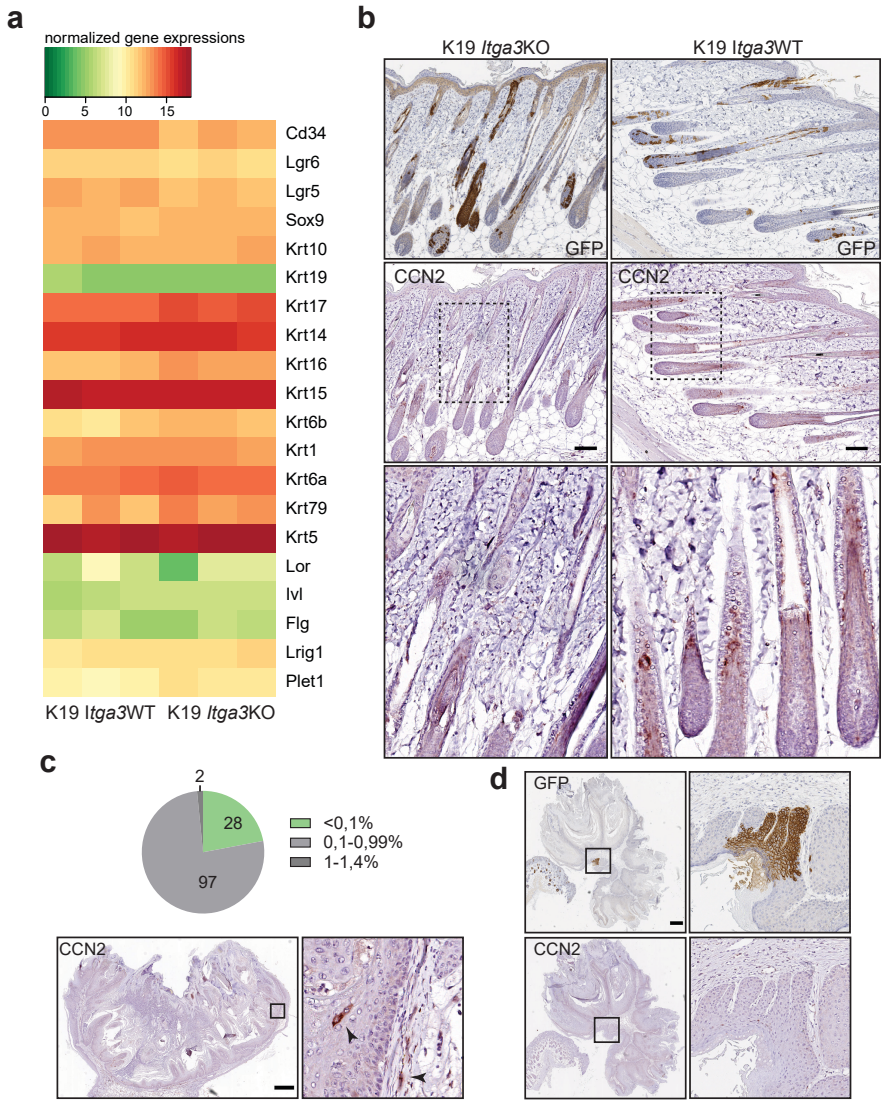
(**a**) Gating strategy of figure 2d. (**b**) Double IHC staining for proliferation marker Ki67 and HB marker K15 shows the absence of proliferation of K15-positive HBs in back and tail epidermis of K14 Itga3 KO and WT mice. (**c**) FACS quantification of the HB population (CD34+, α6high) in the back skin of K14 Itga3 KO and WT mice in homeostatic conditions and after short-term DMBA/TPA treatment (mean ± SD, unpaired t test). (**d**) Gating strategy of Supplementary figure S2c.

Supplementary figure 3



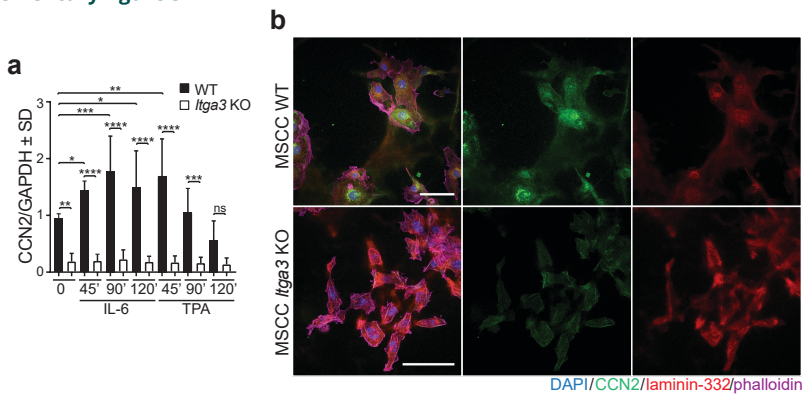
(**a**) The average tumor size is slightly, but significantly decreased in K19 Itga3 KO compared to WT mice submitted to DMBA/TPA-carcinogenesis protocol (mean ± SEM, unpaired t test, *P<0.05, **P<0.005, ***P<0.0005). (**b**) Histology of progressed tumors at the end of DMBA/TPA treatment: SCC, isolated from K19 Itga3 KO and WT mice and keratoacanthomas with carcinomatous changes, isolated from K19 Itga3WT mouse. Progressed tumors were observed in only one K19 Itga3 KO and three WT mice at the end of the treatment. (**c**) Histology (left) and quantification (right) of the progressed tumors of 7 K19 Itga3 KO and 7 WT mice, kept on prolonged TPA treatment for up to additional 10 weeks. 5 K19 Itga3 KO (1 tumor/mouse) and 3 K19 Itga3 WT (1 or 2 tumors/mouse) mice showed tumor progression. In addition to the SCCs and keratoacanthomas with carcinomatous changes, K19 Itga3 KO mice also developed spindle cell sarcomas and mixed basal SCC.

Supplementary figure 4



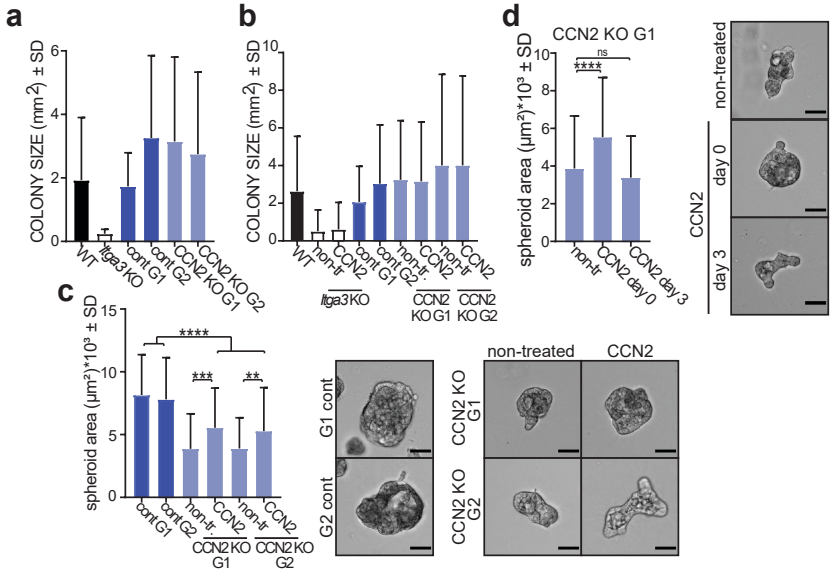
(**a**) A heat map showing expression of selected epidermal markers, selected from gene-list, in GFP-positive HB-originating keratinocytes, isolated from the skin of short-term DMBA/TPA-treated K19 Itga3 KO and WT mice. (**b**) IHC staining for GFP and CCN2 in the skin of short-term DMBA/TPA-treated K19 Itga3 KO and WT mice (scale bar: 100 µm). (**c**) Sparse CCN2-positive cells can be observed in epithelia and stoma of all tumors of K19 Itga3 WT mice (arrow heads). Top: Quantification of the CCN2-positive surface in cross-section of 127 tumors, isolated from 6 K19 Itga3 WT mice. CCN2 represents less than 1% of total tumor surface in the majority of tumors. Bottom: representative IHC staining for CCN2 (scale bar: 500 µm). (**d**) Consecutive section of papilloma, isolated from K19 Itga3 WT mouse, stained for GFP and CCN2. No overlap of the two markers can be observed (scale bar: 500 µm).

Supplementary figure 5



(**a**) Quantification of WB from figure 7c. Bars represent the mean of 5 independent experiments (mean ± SD Fisher's LSD test, *P<0.05, **P<0.005, ***P<0.0005, ****P<0.0001). (**b**) IF images showing co-localization of excreted CCN2 and laminin-332 in the culture of non-treated MSCC Itga3 KO and WT keratinocytes.

Supplementary figure 6



(**a**) Quantification of the colony size from colony formation from figure 8b. Total colonies from 3 independent experiments were quantified (n=246-563, mean ±SD). (**b**) Quantification of the colony size from colony formation from figure c and d. Total colonies from 3 independent experiments were quantified (n=166-779, mean ± SD). (**c**) 3D growth of CCN2 KO MSCC spheroids shows small but significant increase when cells are seeded with 180 ng/ml of CCN2. Left: bright filed images of representative spheroids (scale bar: 50 μm). Right: size quantification of 90 spheroids measured over 3 independent experiments (mean ± SD, Fisher's LSD test, **P<0.005, ***P<0.0005, ****P<0.0001). (**d**) Whereas seeding MSCC CCN2 G1 clone with CCN2 increases its 3D growth, such effect is not observed when exogenous CCN2 (180 ng/ml) is added when spheroids have already formed 3 days after seeding. Left: size quantification of 90 spheroids measured over 2-3 independent experiments (mean ± SD, Fisher's LSD test, ****P<0.0001). Right: bright filed images of representative spheroids (scale bar: 50 μm).



INTEGRIN α3β1 IS A KEY REGULATOR OF SEVERAL PRO-TUMORIGENIC PATHWAYS DURING SKIN CARCINOGENESIS

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ABSTRACT

Integrin α3β1 plays a crucial role in tumor formation in the two-stage chemical carcinogenesis model (DMBA/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 activation of several pro-tumorigenic signaling pathways during the initiation of DMBA/TPA-driven tumorigenesis. In transformed keratinocytes, α3β1-mediated FAK/Src-activation leads to in vitro growth of spheroids and to strong Akt and Stat3 activation, when the α3β1-binding partner tetraspanin CD151 is present to stabilize cell-cell adhesion and to promote Smad2 phosphorylation. Remarkably, α3β1 and CD151 can support Akt and Stat3 activity independently of α3β1 ligation by laminin-332 and as such control essential survival signals required for suprabasal keratin-10 expression during keratinocyte differentiation. These data demonstrate that α3β1 together with CD151 regulate signaling pathways that control the survival of differentiating keratinocytes and provide mechanistic understanding of the essential role of α3β1 in early stages of skin cancer development.

INTRODUCTION

Over the last 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 bi-weekly applications of the phorbol ester TPA, stimulating an increased production of growth factors and inflammatory cytokines. This leads to sustained epidermal hyperplasia and development of benign tumors papillomas, which can progress to invasive carcinomas upon further TPA treatment [1]. The most prominent factors leading to papilloma development are stromal and inflammatory responses, and activation of several growth factor signaling pathways [2,3]. In addition to the Raf/MEK/ERK cascade [4,5], resulting from the activating *Hras1* mutation and TPA treatment, FAK/Src [6–8], PI3K/Akt [9,10], JAK/Stat3 [11–13] and TGFβ/Smad [14,15] 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 [16]. 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 mechano- and biochemical signals. They play diverse roles in tumorigenesis and tumor progression [17]. Integrin α3β1 often exerts opposing functions in cancer, switching from a tumor promoting to suppressing mechanism, depending on the cancer type/driving mechanism, cell environment and stage of the disease [18, 19]. Together with integrin α6β4, α3β1 mediates cell-matrix adhesion of basal keratinocytes by binding the extracellular matrix (ECM) proteins laminin-332 and -511. Furthermore, α3β1 can stabilize E-cadherin-based cell-cell junctions, especially when forming a complex with the tetraspanin CD151 [18]. Here, we investigate the impact of α3β1 on activation of several key signaling pathways during the initiation phase of mouse skin tumorigenesis. Our study uncovers the crucial role of α3β1 in activation of FAK, Akt and Stat3 in transformed keratinocytes and thus provides mechanistic understanding of the tumor-promoting function of α3β1 during skin carcinogenesis.

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*

In order to assess the impact of α3β1 on DMBA/TPA-mediated pro-tumorigenic signaling, we subjected mice with an epidermis-specific deletion of α3β1 (*Itgα3*-KO mice) and wild-type mice (*Itgα3*-WT mice) to topical short-term DMBA/TPA treatment, when pro-tumorigenic pathways are switched on, but tumors have not yet been formed (**Fig. 1a**).

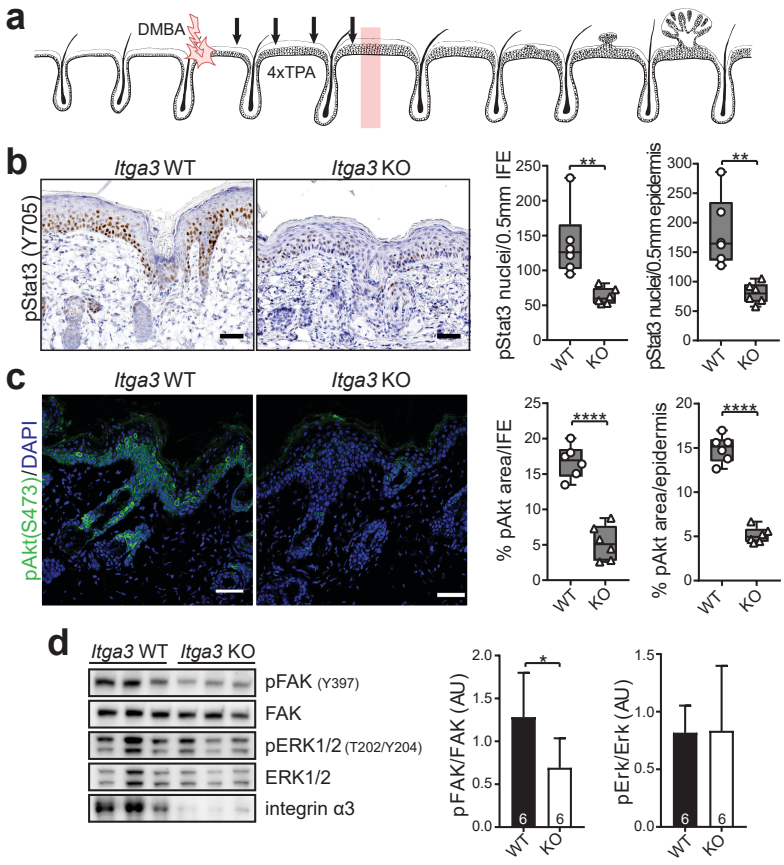


Figure 1: Integrin α3β1 is required for full activation of Stat3, Akt and FAK signaling during the initiation of DMBA/TPA tumorigenesis *in vivo*. (**a**) Overview of the short-term DMBA/TPA treatment (Box: time of analysis). (**b-d**) Analysis of the epidermis, isolated from short-term DMBA/TPA-treated *Itgα3*-WT and -KO mice. (**b**) Representative IHC images and quantification of the number of pStat3 (Y705)-positive nuclei in the 500 µm of interfollicular or total epidermis. Box and whisker plot, dot = mouse (average of 8 quantified images). (**c**) IF images of pAkt staining and quantifications of pAkt-positive area in interfollicular or total epidermis. Box and whisker plot, dot = mouse (average of 10 quantified images). (**d**) WB and quantifications of the protein levels of pFAK Y397 and pERK1/2 (T202/Y204) of 6 *Itgα3*-WT and -KO mice (mean±SD). IFE, interfollicular epidermis. Scale: 50 µm. Statistics: unpaired t test, *P<0.05, **P<0.005, ****P<0.0001.

Signaling through the IL-6/JAK/STAT3 and PI3K/Akt/mTOR pathways are critical for the initiation and progression of papillomas [9–12,20]. Epidermal depletion of α3β1 compromises both pathways, as judged by the strong reduction of the activity of Stat3 and Akt in the epidermis of *Itgα3*-KO compared to -WT mice (**Fig. 1b-c**). We also observed a reduction of phosphorylated FAK (pFAK) in short-term DMBA/TPA treated skin of *Itgα3*-KO mice (**Fig. 1d**). However, the loss of α3β1 did not affect pERK1/2 (**Fig. 1d**), which is consistent with unperturbed phosphorylation of ERK1/2 in *Stat3*-KO mice [12]. The reduction of active FAK upon the deletion of α3β1 is in line with the role of integrin-mediated adhesion in FAK/Src signaling [17], and the requirement of FAK for development of papillomas in mice [7].

Together, this data demonstrates that α3β1 is required for full activation of the Stat3, Akt and FAK pro-tumorigenic signaling pathways during the first stage of skin tumorigenesis.

α3β1-mediated activation of Stat3, Akt and FAK/Src is crucial for 3D growth of transformed keratinocytes *in vitro*

To further investigate the role of α3β1 in pro-tumorigenic signaling, we made use of *Hras1* transformed keratinocytes, isolated from *Itgα3*-WT mice that underwent full DMBA/TPA treatment (MSCC *Itgα3*-KO and -WT keratinocytes) [16]. Consistent with the lack of tumorigenesis in two-stage carcinogenesis [16] and the reduced pro-tumorigenic signaling in *Itgα3*-KO mice, the growth of MSCC keratinocytes in 3D Matrigel depended on the presence of α3β1 and its ability to support Stat3 and Akt signaling (**Fig. 2a-c**). The ability of α3β1 to support growth of transformed keratinocytes is in line with the high-level expression of α3β1 (**Supplementary fig. 1a**) and the activity of Stat3 [12] and Akt [21] in papillomas, as well as with Akt- and Stat3-dependent progression of the cell cycle via activation of cyclin D during DMBA/TPA-treatment [9,12]. Interestingly, MSCC spheroids exhibited co-dependent activation of Stat3 and Akt: while treatment with Akt (MK2206) or mTOR (AZD8055) inhibitors ablated Stat3 activity (**Fig. 2a**), treatment with Stat3 inhibitors (niclosamide, stattic) strongly reduced phosphorylation of Akt (**Fig. 2b, Supplementary fig. 1b**). In line with this, the expression of constitutively activated Stat3 (CA-Stat3) in *Itgα3*-KO keratinocytes restored Akt activation and vice versa, the expression of myristoylated Akt (myrAkt), which renders Akt constitutively active, activated Stat3 (**Fig. 2a-b**). Such crosstalk between the PI3K/Akt and Stat3 pathways has been demonstrated before in different types of tumors [22–24]. Furthermore, several functional links have been established between Stat3 and Akt signaling pathways, such as TEC kinases, which activate Stat3 downstream of PI3K [25] 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 [26].

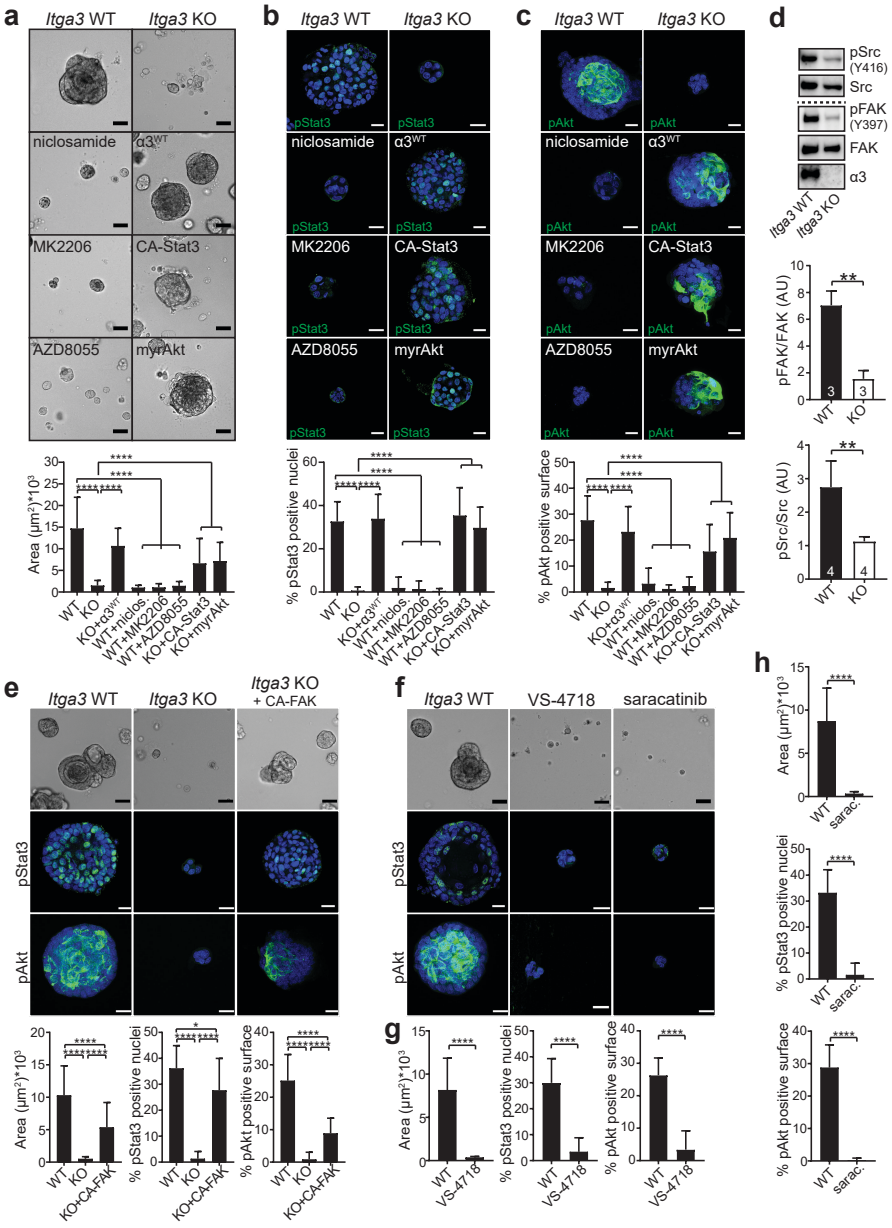


Figure 2: α3β1-mediated activation of Stat3, Akt and FAK/Src is crucial for 3D growth of transformed keratinocytes *in vitro*. (**a-c**) Analysis of *Itga3*-WT spheroids, treated with 2µM niclosamide, 10µM MK2206, or 500nM AZD8055 and *Itga3*-KO spheroids, transfected with wild-type α3 (α3^{WT}), constitutively active Stat3 (CA-Stat3), or myristoylated Akt (MyrAkt). (**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 show reduced pFAK (Y397) and pSrc

(Y416) protein levels in *Itgα3*-KO compared to WT spheroids. (**e**) Representative bright field/IF images and quantifications of the area (n=80), pStat3 (Y705)-positive nuclei (n=20) and pAkt (S473)-positive area (n=20) of *Itgα3*-KO, WT, and *Itgα3*-KO spheroids transfected with constitutively active FAK (CA-FAK) construct. (**f-h**) Representative bright field/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 non-treated, 10μM saracatinib-treated and 5μM VS-4718-treated *Itgα3*-WT spheroids. Scale: 20μm (IF), 50μm (bright field). Statistics: mean ± SD, d, f, h: unpaired t test, a-c, e: Sidak's multiple comparisons test, *P<0.05, **P<0.005, ****P<0.0001.

Consistent with our *in vivo* data (**Fig. 1d**), the absence of α3β1 in spheroids resulted in reduced activation of FAK, as well as Src (**Fig. 2d**). The FAK/Src complex presents a likely link between α3β1 and the PI3K/Akt and Stat3 pathways [27–29]. This hypothesis is supported by our finding that *Itgα3*-KO spheroids expressing constitutively active FAK (CA-FAK) (**Supplementary Fig. 1b**) showed an increased Stat3 activation and, albeit moderately, increased Akt activation and 3D growth (**Fig. 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 keratinocytes (**Fig. 2f-h**).

Our results strongly suggest that α3β1 promotes growth of papillomas via FAK/Src signaling, which supports co-dependent 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, pStat3 and pAkt-positive keratinocytes can be observed in both basal and suprabasal cell layers of the epidermis (**Fig. 1b-c, 3a**). In fact, suprabasal keratinocytes often exhibit elevated levels of pAkt compared to basal cells (**Fig. 3a**), which is consistent with previous observations [30]. Such high suprabasal activity of Akt and Stat3 is surprising considering the fact that laminin-332 and -511, the ligands of α3β1, are only found in the basement membrane, underlying the basal keratinocytes (**Fig. 3b**). Because in hyperplastic mouse skin, α3β1 is also expressed by suprabasal, differentiating keratinocytes (**Fig. 3b**), unconventionally, α3β1 may support pro-tumorigenic Stat3 and Akt signaling independently of its ligation by laminin. To test this hypothesis, we reconstituted *Itgα3*-KO MSCC keratinocytes with either wild-type human α3 ($\alpha3^{WT}$) or an α3 mutant ($\alpha3^{G163A}$), which is unable to bind to laminin-332 [31] (**Supplementary fig 2a-b**). The MSCC-$\alpha3^{WT}$ and -$\alpha3^{G163A}$ keratinocytes grown in spheroids exhibit tight cell-cell adhesion with laminin-332 deposited only by cells forming the outermost basal cell layer (**Fig. 3c**). In these cells, α3β1 localizes at the cell-ECM interface. Additionally, α3β1 is present in the inner cell layers at cell-cell contacts (**Fig. 3c**). Consistent with our *in vivo* observations, ligation of α3β1 to laminin-332 was not needed for strong activation of Stat3 and Akt (**Fig. 3d-e**). The

expression of α3^{G163A} also almost completely restored growth of spheroids (**Fig. 3d-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 keratinocyte growth (**Supplementary fig. 2c**). Integrin signaling has been conventionally linked to ligation of integrins by ECM proteins, leading to their association with actomyosin cytoskeleton and activation of FAK and Src family kinases [32]. Even though the expression of α3^{G163A} leads to reduced FAK activation and a small decrease in Src phosphorylation (**Fig. 3f**), Itga3-KO spheroids exhibit a much more prominent reduction of FAK/Src signaling (**Fig. 2d**). Thus, α3β1 can support FAK/Src activation at the level, sufficient to drive Stat3 and Akt signaling, independently of its ligation by laminin-332. Non-ligated α3β1 may mediate phosphorylation of FAK directly if clustered by other integrins or integrin-associated proteins. Alternatively, α3β1 may support the activation of other integrins, which promote FAK phosphorylation.

We conclude that α3β1 is present in cell-cell contacts also in suprabasal keratinocytes, 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 α3β1 is known to stabilize cell-cell contacts [33] and to associate with E-cadherin [34]. The ability of α3β1 to control cell-cell junction stability is dependent on its binding to tetraspanin CD151 [35–38], a tumor-promoter in DMBA/TPA carcinogenesis model [39,40]. Furthermore, CD151 has been shown to promote TPA-induced Stat3 phosphorylation in mouse and human keratinocytes [39]. We thus wondered whether α3β1-CD151 complexes play a central role in pro-tumorigenic signaling, especially in suprabasal cells. Interestingly, we observed reduced expression of CD151 in the epidermis of *Itga3*-KO compared to -WT mice (**Fig. 4a, Supplementary fig. 3a-b**), which is in line with observations in leukemic K562 cells [41-42]. Since it was shown that suppression of α3β1 expression by RNAi affects clustering of CD151 and promotes its homodimerization [43-44], our data may suggest that the stability of CD151 in mouse epidermis depends on its ability to form a complex with α3β1. As expected, deletion of CD151 in MSCC keratinocytes 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 keratinocytes in the inner cell layers of the spheroids (**Fig. 4b**). Furthermore, *CD151*-KO MSCCs exhibited decreased Smad2 phosphorylation, which is induced by TGFβ and promoted by association of α3β1 and E-cadherin [34] (**Fig. 4c**). We observed reduced pSmad2 also in the epidermis of *Itga3*-KO mice (**Fig. 4d, Supplementary fig. 3c**), demonstrating a role of α3β1-CD151 in

sustaining the pro-tumorigenic TGFβ/Smad2/3 pathway [14,15]. The deletion of *Cd151* in MSCCs resulted in strongly reduced pAkt and in impaired nuclear translocation of pStat3 (**Fig. 4e**). Remarkably, the reduced activity of Akt and Stat3 did not affect the 3D growth of *Cd151*-KO keratinocytes (**Fig. 4e**). A similar trend was observed when spheroids were grown in the presence of the E-cadherin function-blocking antibody DECMA-1 (**Supplementary fig. 3d**), further supporting a role of CD151 and cell-cell junctions in promoting Stat3 and Akt activity.

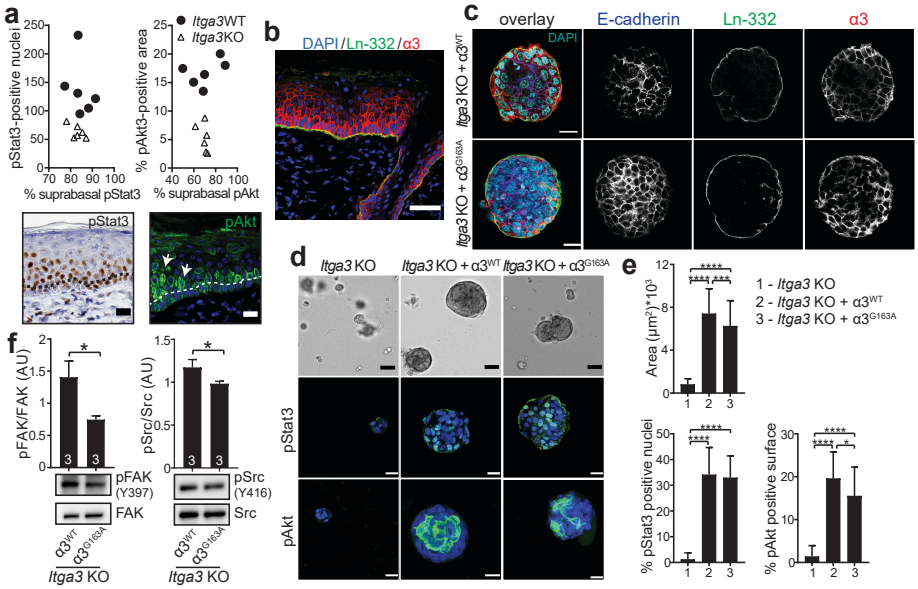


Figure 3: α3β1 supports Stat3 and Akt signaling independently of its ligation by laminin matrix. (**a**) Quantifications (dot=mouse, averaged 8 images) and representative images of the percentage of pStat3-positive nuclei and pAkt-positive area located suprabasally in the short-term DMBA/TPA-treated interfollicular epidermis of *Itga3*-KO and WT mice. Dotted line delineates suprabasal keratinocytes. High pAkt expression is commonly observed in suprabasal keratinocytes (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 (Scale: 50 μm). (**c**) IF images of *Itga3*-KO spheroids, transfected with wild-type α3 ($α3^{WT}$) or laminin-binding mutant G163A ($α3^{G163A}$). α3β1 co-localizes with laminin-332 at the outer layer of spheroids and with E-cadherin in cell-cell contacts (scale: 20μm). (**d-e**) Representative bright field/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 $α3^{WT}$ or $α3^{G163A}$ (Scale: 20 μm (IF), 50 μm (bright field)). (**f**) WB and quantification showing levels of pFAK (Y397) and pSrc (Y416) in MSCC-$α3^{WT}$ and -$α3^{G163A}$ spheroids. Statistics: mean ± SD, e: Sidak's multiple comparisons test, f: unpaired t test, *P<0.05, ***P<0.0005, ****P<0.0001.

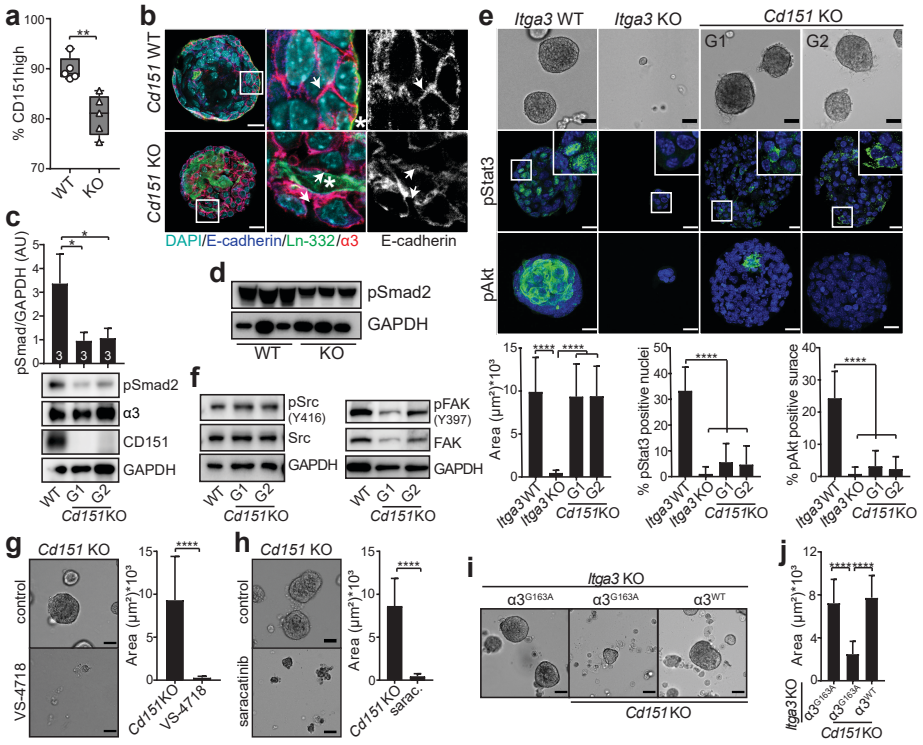


Figure 4: Deletion of CD151 reduces cell-cell contact integrity, resulting in reduced activation of Stat3 and Akt but not reduced FAK/Src signaling. (**a**) Flow cytometry quantification showing reduced expression of CD151 upon the deletion of α3β1 in epidermis. Box and whisker plot, dot = mouse (gating strategy: 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 keratinocyte layers (asterisk) in the absence of CD151 (Scale: 20µm). (**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 to WT mice (quantification: Figure S3c). (**e**) Representative bright field/IF images and quantifications of the area (n=80), pStat3 (Y705)-positive nuclei (n=22-25) and pAkt (S473)-positive area (n=28) of of *Itga3*-KO and WT spheroids and *Itga3*-WT spheroids with deletion of *Cd151*, obtained with two distinct CRISPR/Cas9 gRNAs (G1 and G2) (Scale: 20 µm (IF), 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=80) of the size of *Cd151*-KO spheroids treated with (g) 10 µM saracatinib and (**h**) 5 µM VS-4718 (Scale: 50µm). (**i**) Bright-field images and (j) quantifications (n=85) of the size of MSCC-α3^{G163A} spheroids and MSCC-α3^{WT} and α3^{G163A} spheroids with deletion of *Cd151* (Scale: 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.

Because *Cd151*-KO and -WT spheroids showed similar levels of active Src and FAK (**Fig. 4f, Supplementary fig. 3d**), 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 (**Fig. 4g-h**), thus demonstrating that FAK/Src signaling supports

proliferation of these cells, despite their low Akt and Stat3 activity. Since α3β1-mediated adhesion to laminin-332 increases FAK activation (**Fig. 3f**), the presence of laminin-332 and α3β1 in the inner layers of Cd151-KO spheroids (**Fig. 4b**) could promote ECM-adhesion-supported pro-survival signaling and thus explain their sole dependency on FAK/Src signaling for growth. Indeed, the deletion of CD151 in MSCC-α3^{G163A} cells and treatment of and MSCC-α3^{WT} *Cd151*-KO spheroids with function-blocking J143 antibody resulted in strongly reduced spheroid size (**Fig. 4i-j, Supplementary fig. 3f-g**). However, it should be noted that the levels of pFAK and pSrc were comparable in MSCC-α3^{G163A} and MSCC-α3^{G163A} *Cd151*-KO spheroids (**Fig. S3h**) but higher than those observed in *Itga3*-KO spheroids (**Fig. 2d**, **3f**). Therefore, α3β1 can still support some FAK/Src activity independently of laminin or CD151-association.

Together, our data shows that even though FAK/Src activation is required, it is not sufficient to support Akt and Stat3 signaling in our model. In transformed keratinocytes, efficient Akt and Stat3 activation also depends on the cell-cell contact-stabilizing function of CD151, a binding partner of α3β1.

α3β1 and CD151 are needed for 3D growth of differentiating keratinocytes

Previous studies showed that Akt and Stat3 play important roles in the differentiation of keratinocytes [9,45]. While Stat3 signaling prevents terminal differentiation of keratinocytes through regulation of the transcription factor AP-1 [45], activation of Akt promotes the survival of differentiating keratinocytes [30,46]. The activation of PI3K/Akt in primary mouse keratinocytes occurs concomitantly with the expression of keratinocyte differentiation markers and, as in *Itga3*-WT MSCC spheroids, depends on the E-cadherin-mediated adhesion and the activity of the Src family kinases [30]. We therefore wondered whether α3β1 and CD151 play a role in the differentiation of MSCCs through the regulation of Akt and Stat3 signaling. In *Itga3*-WT MSCC spheroids we could observe a single outer layer of keratin-14 positive basal keratinocytes that adhere to laminin-332 and express integrin α6β4, and a variable number of inner, post-mitotic suprabasal cells that express keratin-10 (**Fig. 5a and Supplementary fig. 4a**). The *Itga3*-KO spheroids were small, containing only a few α6β4-expressing keratinocytes and no keratin-10-positive cells. Interestingly, several of the keratinocytes in the outer cell layer of MSCC-α3^{G163A} spheroids exhibited expression of keratin-10 together with laminin-332. As this was not found in WT spheroids, it is likely that α3β1-laminin-332 ligation prevents aberrant differentiation (**Fig. 5a**). Strikingly, none of the *CD151*-KO keratinocytes expressed keratin-10, while laminin-332 colocalized with α6β4 in addition to α3β1 in outer and inner layers of the spheroids (**Fig. 4a and 5a**). Furthermore, consistent with the roles of Akt and Stat3 in promoting survival of differentiating

keratinocytes, activated Akt and Stat3 could be observed in the keratin-10-positive differentiating *Itgα3*-WT and $\alpha3^{G163A}$ transformed keratinocytes (**Fig. 5b**).

Thus, while sustained 3D growth of basal-like *Cd151*-KO keratinocytes primarily depends on FAK/Src signaling and less on strong activity of Akt and Stat3, the growth of spheroids that similarly to papillomas contain differentiating keratinocyte layers requires FAK/Src, Stat3 and Akt activation (**Fig. 2a,b and g**).

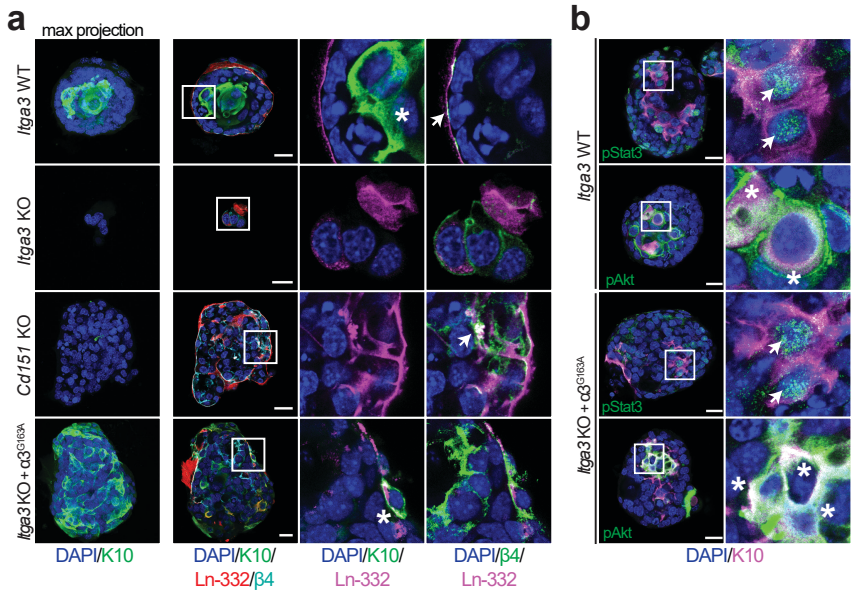


Figure 5: α3β1 and CD151 are needed for 3D growth of differentiating keratinocytes. (**a**) IF staining shows the absence of differentiation marker keratin-10 (asterisk) and co-localization of integrin α6β4 and laminin-332 (arrow) in the inner layers of spheroids in the absence of Cd151. Such basal-like phenotype is largely absent in *Itgα3*-KO spheroids, transfected with laminin-binding mutant G163A ($\alpha3^{G163A}$), which show high expression of keratin-10 and its localization with laminin-332 in outer spheroid layers (asterisk). (**b**) IF staining shows the presence of pAkt (asterisk) and nuclear localization of pStat3 (arrows) in keratin-10-positive *Itgα3*-WT and *Itgα3*-KO spheroids expressing $\alpha3^{G163A}$. Scale: 20 μm

Final remarks

We previously showed that *Itgα3*-KO and *Cd151*-KO mice displayed increased epidermal turnover and loss of slow-cycling stem cells [16,40,47]. However, skin thickness and number of proliferative keratinocytes were similar between *Itgα3*-KO and WT mice during DMBA/TPA treatment (**Supplementary fig. 4b**) [16], which suggests that the increased rate of proliferation is coupled with enhanced differentiation to maintain the faster turnover of keratinocytes in the *Itgα3*-KO mice. The question, therefore, arises how to relate these observations to our present findings. In *Itgα3*-KO mice, decreased Stat3

and Akt activity may accelerate terminal differentiation of the suprabasal keratinocytes, leading to increased rate of proliferation of basal keratinocytes and the loss of slow-cycling cells. As it is generally accepted that DMBA-initiated basal keratinocytes must persist long enough in hyperplastic skin to acquire additional mutations leading to outgrowth of clonal papillomas, eventual tumor outgrowth could depend on the Akt and Stat3-regulated survival of suprabasal keratinocytes. However, the increased epidermal turnover can be detected also in non-treated skin of Itga3-KO mice, where α3β1 is mostly restricted to the basal keratinocytes in much thinner epidermis. Thus, the loss of stem cells is DMBA/TPA-primed skin of *Itga3*-KO mice is likely caused by a combination of a reduced adhesion strength of *Itga3*-KO basal keratinocytes, reflected in reduced FAK/Src activation, and an increased rate of terminal differentiation, promoted by decreased Akt and Stat3 activation in α3β1-depleted suprabasal keratinocytes.

In conclusion, α3β1 is a key regulator of several pro-tumorigenic pathways during initiation of DMBA/TPA-mediated tumorigenesis. In transformed keratinocytes, α3β1-mediated FAK/Src-activation leads to strong Akt and Stat3 signaling when CD151 is present, promoting cell-cell stability and Smad2 phosphorylation. Remarkably, α3β1-CD151 can support Akt and Stat3 activity independently of α3β1 ligation by laminin-332 and as such promote pro-survival signaling in suprabasal differentiating keratinocytes, which likely delays epidermal turnover and enables eventual papilloma formation. For tumor outgrowth transformed keratinocytes depend on CD151-independent α3β1-mediated FAK/Src activation, which promotes proliferation of basal keratinocytes, and on α3β1-CD151 mediated Stat3 and Akt activation in suprabasal layers, which is required for 3D growth of differentiating transformed keratinocytes (**Fig. 6**).

MATERIALS AND METHODS

Animal experiments

Epidermis-specific Krt14$^{tm1(cre)Wbm}$; *Itga3*$^{tm1Son/tm1Son}$ (*Itga3*-KO mice) and Krt14$^{tm1(cre)Wbm}$ (*Itga3*-WT mice) on FVB/N background have been previously described [16]. DMBA/TPA tumorigenesis has been done as before [16]. Briefly, backs of 6-week old mice were shaved and after a week topically treated with 30μg (in 200μl acetone) of DMBA (Sigma, D3254), followed by bi-weekly topical applications of 12,34μg (in 200μl acetone) of TPA (Sigma, P1585) for 20 weeks. For short-term DMBA/TPA treatment mice were treated with four doses of TPA over two weeks following DMBA treatment. Thereafter, mice were killed, skin was isolated and processed for immunofluorescence, immunohistochemistry analysis and/or protein lysate preparation. 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.

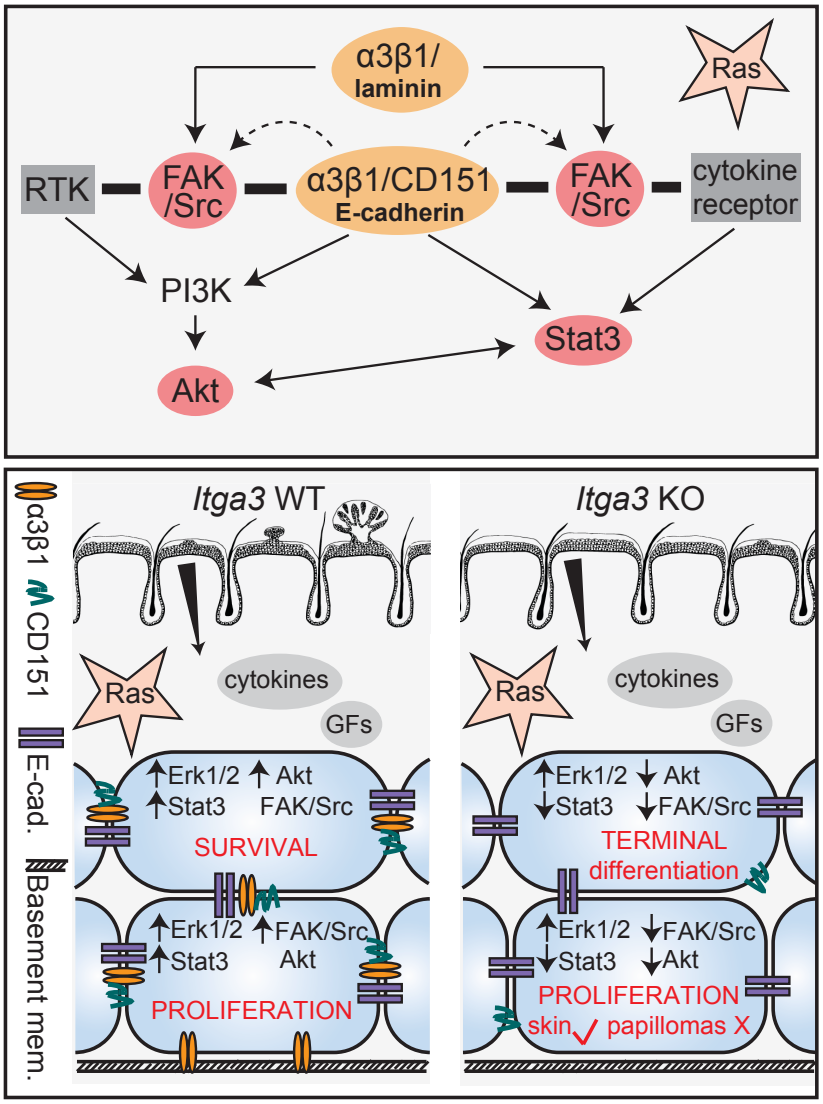


Figure 6: The model of α3β1-mediated pro-tumorigenic signaling in transformed keratinocytes. In *Hras1*-transformed keratinocytes, integrin-mediated FAK/Src-activation leads to strong Akt and Stat3 activation when CD151 is present, promoting cell-cell adhesion stability (top). Basal keratinocytes in hyperplastic skin during the initiation of tumorigenesis proliferate regardless of the presence of α3β1, which supports moderate induction of pAkt and strong activation of Stat3 and FAK/Src in this layer. In suprabasal keratinocytes, α3β1 is not ligated by laminin-332 but localizes to cell-cell contacts with its binding partner CD151. Here, CD151 and α3β1 induce pro-survival Stat3 and Akt signaling, which delays terminal differentiation of suprabasal keratinocytes. This allows outgrowth of well-differentiated papillomas, which need α3β1-mediated FAK/Src activation in basal keratinocytes for proliferation and Stat3 and Akt activation in basal/suprabasal layers for eventual outgrowth (bottom). RTK: receptor tyrosine kinase; GF: growth factor.

Cell culture and generation of stable cell lines

To obtain *Itgα3*-KO MSCC cells, stably expressing wild-type α3 (MSCC *Itgα3*-KO $\alpha3^{WT}$), full-length human α3A cDNA was ligated into pUC18-a3. After digestion with SphI, α3A cDNA was ligated into LZRS-IRES-zeo, a modified LZRS retroviral vector conferring resistance to zeocin [48]. Retroviral vector was introduced into the Phoenix packaging cells by the calcium phosphate precipitation method, and virus-containing supernatant was collected [48]. MSCC *Itgα3*-KO cells were infected with the recombinant virus by the 1,2-dioleoyl-3-trimethylammonio-propane (DOTAP) method (Boehringer). After infection overnight at 37°C, infected cells were selected with 0.2 mg ml^{-1} zeocin (Invitrogen). Cells expressing α3β1 were obtained by fluorescence activated cell sorting. To obtain *Itgα3*-KO MSCC cells, stably expressing laminin-binding mutant $\alpha3^{G163A}$ [31] (MSCC-$\alpha3^{G163A}$), $\alpha3^{G163A}$ in pBJ-1 expression vector (a kind gift of Yoshikazu Takada, University of California, Davis, USA) was transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions and bulk populations were sorted for α3-expressing cells using fluorescence activated cell sorting. Retroviral expression vector pBABE-puro with construct encoding constitutively active Stat3 [49] (a kind gift of D. Peeper, NKI, the Netherlands) or active myristoylated Akt [50] (a kind gift of R. Bernards, NKI, the Netherlands) was used to generate MSCC *Itgα3*-KO CAStat3 and MSCC *Itgα3*-KO myrAkt cells, using retroviral transduction as described above. Cells expressing CAStat3 or myrAkt were selected with 5μg/ml of puromycin (Invitrogen). MSCC *Itgα3* KO cells expressing constitutively active FAK (MSCC *Itgα3*-KO CAFAK) were obtained by lentiviral transduction of the pLV-neo-CD2-FAK vector (#37013 Addgene; deposited by Bob Weinberg [51]) followed by 600 μg ml^{-1} of G-418 solution (Roche).

To generate CD151-deficient MSCC keratinocytes, target sgRNAs against *Cd151* (Guide1: 5'-GTTCCGTCGCTCCTTGAAAGTGG -3' and Guide2: 5'- CACGGCCTACATCTTAGTGGTGG-3') were cloned into pX330-U6-Chimeric_BB-CBh-hSpCas9 (#42230 Addgene; deposited by Feng Zhang [52]). Cells were transfected with plasmids containing sgRNA using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. CD151-negative cell lines were obtained by fluorescent activated cell sorting. MSCC-$\alpha3^{WT}$ *Cd151*-KO and MSCC-$\alpha3^{G163A}$ *Cd151*-KO keratinocytes were generated as described above using sgRNA guide 1.

All cell lines were cultured in DMEM with 10% heat-inactivated FCS and antibiotics at 37°C in a humidified, 5% CO_2 atmosphere.

Matrigel cell culture

For 3D cell culture, 70μl of growth factor-reduced Matrigel Basement Membrane Matrix (Corning, 354230) was pipetted per well of 96-well plate and incubated for 30min at

37°C. 1000 cells in cold DMEM containing 10%FCS and 2% Matrigel were seeded on top of the Matrigel layer and grown for 7 days with or without inhibitors/function-blocking antibodies.

For the size measurements, spheroids were imaged with Zeiss AxioObserver Z1 inverted microscope, utilizing 5x and 10x objectives and a Hamamatsu ORCA AG Black and White CCD camera and using Zeiss ZEN software. Area of the spheroids was measured manually using ImageJ [53,54].

For inhibition studies, MSCC cells were seeded with 10 μM saracatinib, 10 μM MK2206, 500 nM AZD8055, 5 μM VS-4718 or 2 μM niclosamide (Selleckchem) or 2 μM stattic (Selleckchem) added to the DMEM with 10% heat-inactivated FCS and antibiotics. For function-blocking experiments, MSCC were grown in Matrigel in the presence of 10 μg ml^{-1} of anti-α3 J143 [55] or anti E-cadherin DECMA-1 antibody (Life technologies, 16-3249-82). Spheroids were retrieved from Matrigel by incubation with Cell recovery solution (Corning, 354253) for 1h at 4°C and subsequent resuspension in ice-cold PBS. For BrdU treatment, spheroids were incubated for 4h before harvesting with 50μM of BrdU (DakoCytomation).

Antibodies

For IHC, primary antibodies were used: rabbit- anti pStat3 Y705 (Cell Signaling, #9145, 1:100), rabbit anti-Ki67 (Abcam, ab15580, 1:3000) and mouse anti-integrin α3 (Santa Cruz, sc-374242, 1:500). For IF, we used primary antibodies: goat anti-mouse integrin α3 (R&D, AF2787, 1:50), mouse anti-human integrin α3 J143 ([49], 1:100), rabbit anti-pStat3 Y705 (Cell Signaling, 9131S, 1:100), rabbit anti-pAkt S473 (Cell Signaling, 9271, 1:100), rat anti-integrin β4 (BD Pharm., 346.11A, 1:50), rabbit anti-laminin-332 R14 (1:400, kind gift of M. Aumailey), rabbit anti-keratin 14 (Covance, PRB-155P, 1:400), rat anti-E-cadherin DECMA-1 (Life technologies, 16-3249-82, 1:100), mouse anti-BrdU (DakoCytomation, 1:50), mouse anti-E-cadherin (BD bioscience, #610182, 1:100) and mouse anti-keratin 10 DE-K10 (non-diluted supernatant, kind gift of D. Ivanyi [56]). 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, goat anti-rat Alexa Fluor 647, donkey anti-mouse Alexa Fluor 647 and goat anti-mouse Alexa Fluor 568 (Invitrogen). For flow cytometry we used primary rat anti-CD151 (R&D, MAB4609, 1:50) and mouse anti-human integrin α3 J143 ([55], 1:100) antibodies. For secondary antibodies donkey anti-rat PE (Biolegend, #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-pFAK Y397 (Invitrogen, 44-624G, 1:1000), mouse anti-FAK (BD transduction lab., #610087, 1:1000), rabbit anti-pERK1/2

T202/Y204 (Cell Signaling, #4376s, 1:1000), rabbit anti-ERK1/2 (Cell signaling, #9102, 1:1000), rabbit anti-integrin α3 (homemade, 1:2000), rabbit anti-pSrc Y416 (Cell signaling, #2101, 1:1000), rabbit anti-Src (Cell signaling, #2123, 1:1000), rabbit anti-CD151 140190 ([57], 1:500), rabbit anti-pSmad2 S465/467 (Cell signaling, #3108S, 1:1000), mouse anti-GAPDH (Calbiochem, CB1001, 1:1000) and mouse anti-tubulin (Sigma, B-5-1-2, 1:5000). As secondary antibodies we used stabilized goat anti-mouse HRP-conjugated and stabilized goat anti-rabbit HRP-conjugated (BioRad).

Immunohistochemistry

Tumors and skin were isolated, fixed in EAF and/or formaldehyde, embedded in paraffin, sectioned and stained. Images were taken with the Aperio ScanScope (Aperio, Vista, CA, USA), using ImageScope software version 12.0.0 (Aperio). Count of phospho-Stat3 positive nuclei was performed blindly. Image analysis was performed using ImageJ [53,54].

Immunofluorescence

Skin was isolated, embedded in Tissue-Tek OCT (optimal cutting temperature) cryoprotectant and frozen. Frozen skin was cryosectioned, sections were fixed in ice-cold acetone and blocked with 2% bovine serum albumin (BSA, Sigma) in PBS for 1h at room temperature. Isolated spheroids were mounted on Poly-L-Lysine (Santa Cruz, 25988-63-0)-coated slides, fixed in 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.2% Triton-X-100 for 5 min, and blocked with PBS containing 2% BSA for 1h at room temperature. Tissues or spheroids were incubated with primary antibodies in 2% BSA in PBS overnight, followed by incubation with secondary antibodies diluted 1:200 for 60-90 min. All samples where counterstained with DAPI for 5 min at room temperature. Cryosections were mounted in Vectashield (Vector Laboratories H-1000) and skin sections in mowiol. Samples were analyzed by Leica TCS SP5 confocal microscope with 40 and 63x (NA 1.4) oil objectives. When spheroids were analyzed, the thickness of spheroids was determined and z-stacks with step size every 1.2-1.3µm were acquired. All images were processed using ImageJ [53,54]. The number of pStat3 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 pStat3-positive nuclei was calculated by dividing the number of pStat3-positive nuclei in image with total number of nuceli based on DAPI staining. Percentage of pAkt-positive area in mouse epidermis was calculated using the Analyze Particle function, with delineated total or interfollicular epidermis as a region of interest (ROI). Percentage of pAkt-positive area in spheroids was calculated on maximum intensity projected z-stacks, with delineated surface of spheroids based on DAPI staining as a ROI. pAkt-positive area was divided by the total ROI area to define the percentage of pAkt-positive area.

Flow cytometry

Keratinocytes were isolated from mouse back skin as described before [58] or MSSC keratinocytes were trypsinized. Cells were further washed in PBS containing 2% FCS, incubated for 1h at 4°C with primary antibody in PBS 2% FCS, washed and incubated with secondary antibody for 30 min at 4°C. Cells were analyzed on a Becton Dickinson FACS Calibur analyzer after addition of the indicated life/dead cell marker. For fluorescence activated cell sorting, α3-positive or CD151-negative cell populations were obtained using a Becton Dickinson FACSAria IIu cell sorter.

Western Blotting

Protein lysates were obtained from keratinocytes, isolated from mouse back skin as described before [52], from isolated spheroids, or from sub-confluent cell cultures by lysis in RIPA buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 4 mM EDTA (pH 7.5), 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with 1.5 mM Na_3VO_4, 15 mM NaF (Cell Signaling) and protease inhibitor cocktail (Sigma). Lysates were cleared by centrifugation at 14.000 x g for 20 min at 4°C and eluted in sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 12.5 mM EDTA, 0.02% bromophenol blue) with final concentration of 2% β-mercaptoethanol and denatured at 95°C for 10 min. Proteins were separated by electrophoresis using Bolt Novex 4–12% gradient Bis-Tris gels (Invitrogen), transferred to Immobilon-P transfer membranes (Millipore Corp) and blocked for 1h in 2% BSA in TBST buffer (10 mM Tris (pH 7.5), 150 mM NaCl, and 0.3% Tween-20). The blocked membranes were incubated overnight at 4°C with primary antibodies in TBST containing 2% BSA. After washing, membranes were incubated for 1 h with horseradish peroxidase–conjugated goat anti-mouse IgG or goat anti-rabbit IgG (diluted 1:5000 in 2% BSA in TBST buffer). After washing, the bound antibodies were detected by enhanced chemiluminescence using or Clarity™ Western ECL Substrate (Bio-Rad) or Amersham ECL Western Blotting Detection Reagent (GE Healthcare) as described by the manufacturer. Signal intensities were quantified using ImageJ [53,54].

Adhesion assay

For adhesion assays, 6-well plates were coated with laminin-332-rich matrix, obtained by growing RAC-11P cells [59] 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, $1*10^5$ MSCC cells 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 5x and 10x objectives, a Hamamatsu ORCA AG Black and White CCD camera, and 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 P-value lower than 0.05 were considered significantly different from the null hypothesis.

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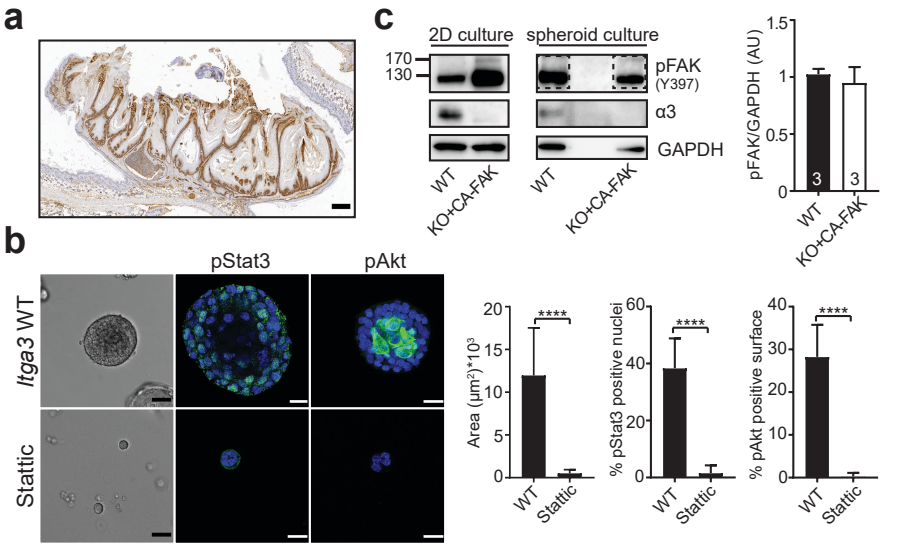
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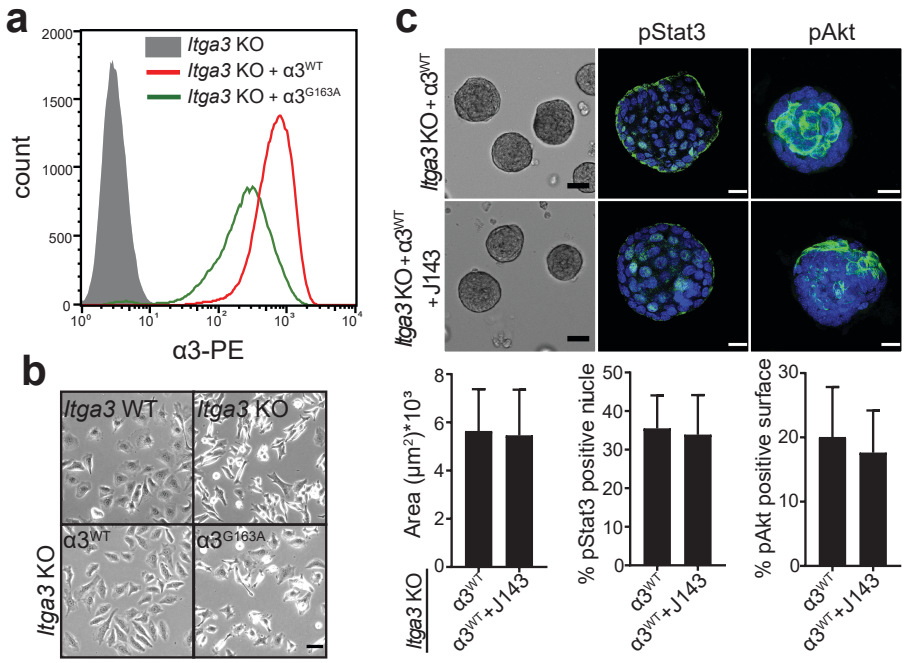
SUPPLEMENTARY FIGURES

Supplementary figure 1



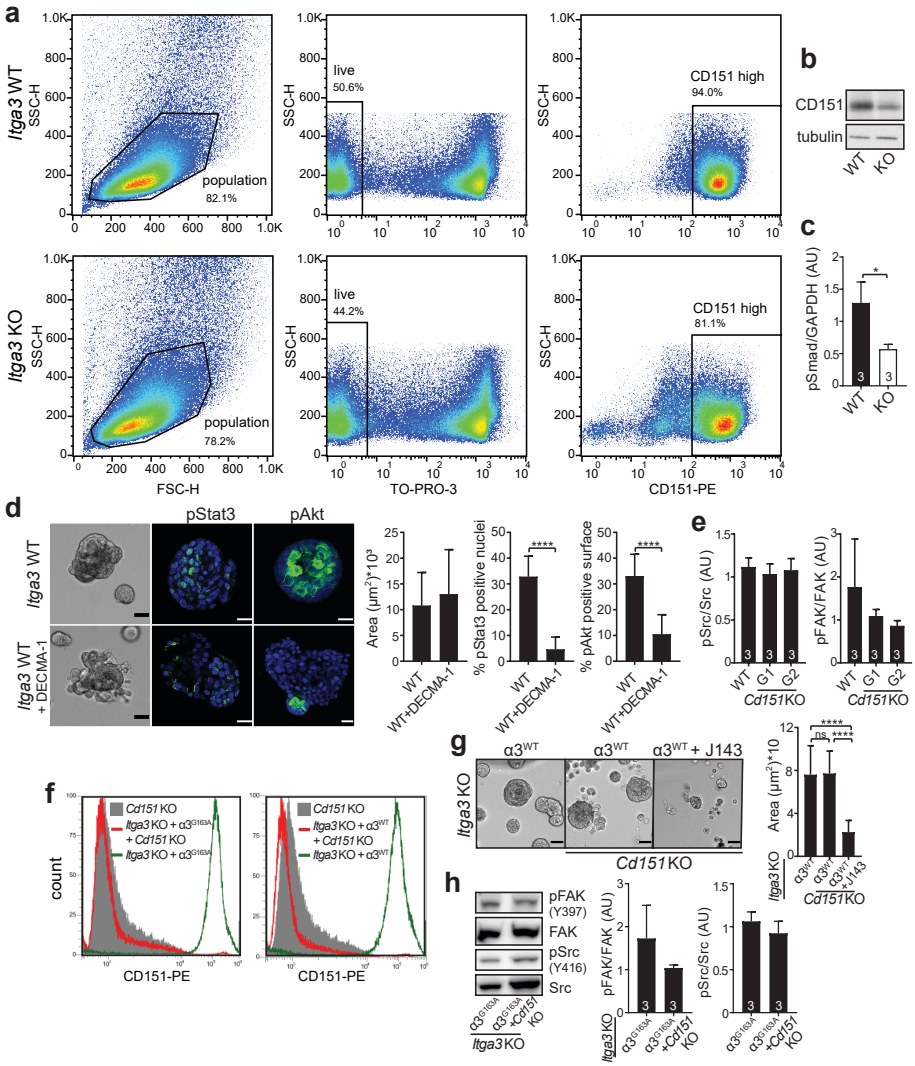
(**a**) IHC staining for integrin α3β1 in papilloma, formed after long-term DMBA/TPA-treatment of *Itgα3*-WT mice (Scale: 500 µm). (**b**) Representative bright field/IF images and quantifications of the area (n=80), pStat3 (Y705)-positive nuclei (n=20) and pAkt (S473)-positive area (n=20) of non-treated and 2 µM stattic-treated *Itgα3*-WT spheroids. Scale: 20 µm (IF), 50 µm (bright field). Statistics: mean ± SD, unpaired t test, ****P<0.0001. (**c**) Left: WB showing pFAK (Y397) expression in *Itgα3*-WT and *Itgα3*-KO MSCC keratinocytes transfected with a construct encoding constitutively active FAK (CA-FAK), grown in 2D or as spheroids. Even though 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 activation of endogenous FAK (lower band). Right: quantification of total pFAK expression in spheroids (as indicated by the dash-line box) Statistics: mean ± SD

Supplementary figure 2



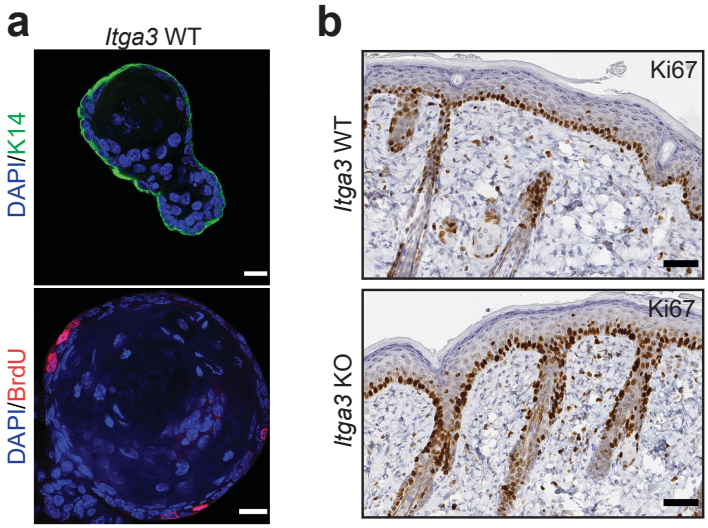
(**a**) Histogram of surface levels of α3β1 in MSCC *Itga3*-KO spheroids and MSCC *Itga3*-KO spheroids with stable expression of $\alpha3^{WT}$ or α3 laminin-binding mutant G163A ($\alpha3^{G163A}$). (**b**) MSCC *Itga3*-KO and MSCC-$\alpha3^{G163A}$ show reduced adhesion to laminin-rich matrix compared to MSCC *Itga3*-WT and MSCC-$\alpha3^{WT}$ keratinocytes. (**c**) Representative bright field/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 $\alpha3^{WT}$ construct and treated with function-blocking J143 antibody (1µg/ml) (Scale: 20µm (IF), 50µm (bright field)). Statistics: mean ± SD, unpaired t test.

Supplementary figure 3



(**a**) Representative gating strategy of flow cytometry quantifications from Fig. 4a. (**b**) WB showing reduced expression of CD151 upon the deletion of α3β1 in mouse epidermis. (**c**) Quantification of the WB from the figure 4d. (**d**) Representative bright field/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 (10 µg ml−1) (Scale: 20 µm (IF), 50 µm (bright field)). (**e**) Quantification of the WB from the figure 4e. (**f**) Histogram of surface levels of CD151 in MSCC-*Cd151* KO, -α3^{G163A}, -α3^{WT} spheroids and MSCC-α3^{G163A} and -α3^{WT} spheroids with CD151 deletion. (g) Bright-field images and quantifications (n=85) of the size of MSCC-α3^{WT} spheroids, -α3^{WT} *Cd151* KO and -α3^{WT} *Cd151* KO spheroids treated with α3-blocking J143 antibody (10 µg ml−1). Scale: 50 µm. (h) WB and quantification of the pFAK and pSrc levels in MSCC-α3^{G163A} and MSCC-α3^{G163A} 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.

Supplementary figure 4



(**a**) IF staining of *Itga3*-WT MSCC spheroids showing keratin-14 expression and BrdU-positive proliferating keratinocytes in outer cell layer. (**b**) Ki67 staining of short-term DMBA/TPA-treated *Itga3*-WT and KO skin shows that majority of suprabasal keratinocytes are non-mitotic regardless of the presence of α3.



ABSENCE OF INTEGRIN α3β1 PROMOTES THE PROGRESSION OF HER2-DRIVEN BREAST CANCER IN VIVO

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ABSTRACT

Background

HER2-driven breast cancer is correlated with poor prognosis, especially during its later stages. Numerous studies have shown the importance of the integrin α3β1 during the initiation and progression of breast cancer, however its role in this disease is complex and often opposite during different stages and in different types of tumors. In this study we aim to elucidate the role of integrin α3β1 in a genetically engineered mouse model of HER2-driven mammary tumorigenesis.

Methods

To investigate the role of α3β1 in HER2-driven tumorigenesis *in vivo* we generated a HER2-driven MMTV-cNeu mouse model of mammary tumorigenesis with targeted deletion of Itga3 (Itga3 KO mice). We have further used several established triple-negative and HER2-overexpressing human mammary carcinoma cell lines and generated ITGA3-knockout cells to investigate the role of α3β1 *in vitro*. Invasion of cells was assessed using Matrigel and Matrigel/collagen I coated transwell assays under static or interstitial fluid flow conditions. The role of α3β1 in initial adhesion to laminin and collagen was assessed using adhesion assays and immunofluorescence.

Results

Tumor onset in mice was independent of the presence of α3β1. In contrast, the depletion of α3β1 reduced the survival of mice and increased tumor growth and vascularization. Furthermore, Itga3 KO mice were significantly more likely to develop lung metastases and had an increased metastatic burden compared to WT mice. *In vitro*, the deletion of ITGA3 caused a significant increase in cellular invasion of HER2-overexpressing SKBR3, AU565 and BT474 cells, but not of triple-negative MDA-MB-231. This invasion suppressing function of α3β1 in HER2-driven cells depended on the composition of the extracellular matrix and the interstitial fluid flow.

Conclusion

Downregulation of α3β1 in a HER2-driven mouse model and in HER2-overexpressing human mammary carcinoma cells promotes progression and invasiveness of tumors. The invasion suppressive role of α3β1 was not observed in triple-negative mammary carcinoma cells, illustrating the tumor type specific and complex function of α3β1 in breast cancer.

INTRODUCTION

Gene amplification and/or overexpression of a member of the epidermal growth factor receptor family, epidermal growth factor receptor 2 (HER2), is observed in around 20% of invasive breast cancers [1]. Initial studies of transgenic mice expressing an activated Neu (i.e. the rat homolog of HER2) under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter provided direct evidence that HER2 acts as a mammary oncogene [2,3]. The phosphorylation of the intracellular tyrosine kinase domain of HER2 results in activation of PI3K/AKT and MAPK/ERK pathways, leading to increased cell proliferation and survival [4,5]. Despite numerous studies and medical advances, HER2-overexpression and activation remain linked with poor prognosis due to their correlation with shorter disease-free intervals and an increased risk of metastasis [6,7]. Therefore, there is a need to better understand HER2-driven breast cancer, especially at its late stages.

Integrins, a family of transmembrane glycoproteins consisting of 18 α- and 8 β-subunits that form 24 distinct heterodimeric receptors, play an important role in cancer progression. Integrins are primarily involved in cell-matrix adhesion and serve as mechanochemical transducers that generate biochemical signals [8,9]. Over recent years it has become clear that the function of integrins within both, tumor cells and tumor environment, is highly complex, which is reflected by the fact that they often play opposing roles in initiation and progression of different tumor types [10]. This is especially prominent for integrin α3β1, a laminin-332 and -511-binding integrin that is expressed mostly in epithelia of kidneys, lungs, intestine, skin, bladder and stomach. Among others, α3β1 can be found in cell-cell contacts and focal adhesions (FAs), dynamic protein adhesion complexes that form mechanical links between the extracellular matrix and the actomyosin cytoskeleton [11]. The regulation of FAs and consequent reorganization of the associated actin cytoskeleton are important determinants for cell migration. The presence of α3β1 has been associated with promotion and suppression of different stages and diverse types of tumors through its interactions with integrin associated proteins (such as tetraspanin CD151), changes in cell adhesion and/or migration, or via the induction of α3β1-mediated signaling [12]. Independent studies looking for correlations between α3β1 and breast cancer in selections of human tumor samples reported all possible outcomes – lack of correlation [13], positive correlation with tumor progression and angiogenesis [14] and correlation between the downregulation of α3β1 and increased invasiveness, resulting in reduced survival [15–17]. This illustrates that there is a need to investigate its clinical significance in relation to the phenotypical and histological variants of specific types of tumors.

Integrin α3β1 was shown to be essential for initiation, proliferation and invasiveness of basal tumors that are adherent to the pre-existing or newly deposited laminin matrix [18,19]. Furthermore, its role in promoting HER2-negative breast cancer *in vivo* and *in vitro* in human breast cancer cell line MDA-MB-231 has been demonstrated in several studies [19–21]. However, the role of α3β1 in HER2-driven mammary tumorigenesis has not been properly addressed yet. In this study, we investigated the impact of the α3β1 deletion in a mouse model of HER2-driven tumorigenesis *in vivo* and in human mammary carcinoma cell lines *in vitro*. With this, we aim to add to the understanding of the complex function of α3β1 as a breast cancer marker and therefore to its clinical potential.

RESULTS

Integrin α3β1 is not needed for the onset of HER2-driven mammary tumorigenesis

To investigate the role of integrin α3β1 in HER2-mediated mammary tumorigenesis and tumor progression *in vivo*, we employed the widely used breast cancer mouse model, MMTV-cNeu, designed to promote development of mammary tumors as a result of overexpression of HER2/Neu oncogene under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter [2]. Additionally, mice harbored the MMTV-Cre transgene in the presence (MMTV-Cre; MMTV-cNeu; *Itga3*$^{fl/fl}$ mice, i.e. Itga3 KO mice) or absence of floxed *Itga3* alleles (MMTV-Cre; MMTV-cNeu mice, i.e. Itga3 WT mice).

HER2-mediated tumor onset and tumor number were not affected by the absence of integrin α3. First palpable tumors could be detected at day 126 in Itga3 WT and at day 133 in Itga3 KO group (**Fig. 1a**). On average, Itga3 WT and KO mice had developed 5.3 and 4.6 palpable tumors, respectively, when they were sacrificed at the defined humane endpoint (**Fig. 1b**). Histological analysis of the tumors revealed that mice developed two types of mammary adenocarcinomas, i.e. solid and cystic/hemorrhagic tumors (**Supplementary fig. 1a**), which were equally represented in both groups (**Supplementary fig. 1b**). The absence of α3 protein expression in the tumors of Itga3 KO group was confirmed by western blot analysis (**Fig. 1c**). Furthermore, immunohistochemistry showed high levels of expression of HER2/*Neu* (**Fig. 1d**), confirming their origin from cells driven by the expression of this oncogene. Tumors also showed high levels of E-cadherin, β-catenin, Plet1 and keratin 18, which in combination with the absence of basal markers integrin β4, Laminin-332 and keratin 5 indicates their luminal origin [22–24]. Abundant collagen I was observed in tumor stroma. No differences in the distribution and/or expression of these markers could be observed between tumors isolated from Itga3 WT or Itga3 KO mice (**Fig. 1e**). Together, these data

show that α3 does not play an obvious role in initial development and characteristics of HER2-dependent tumors *in vivo*.

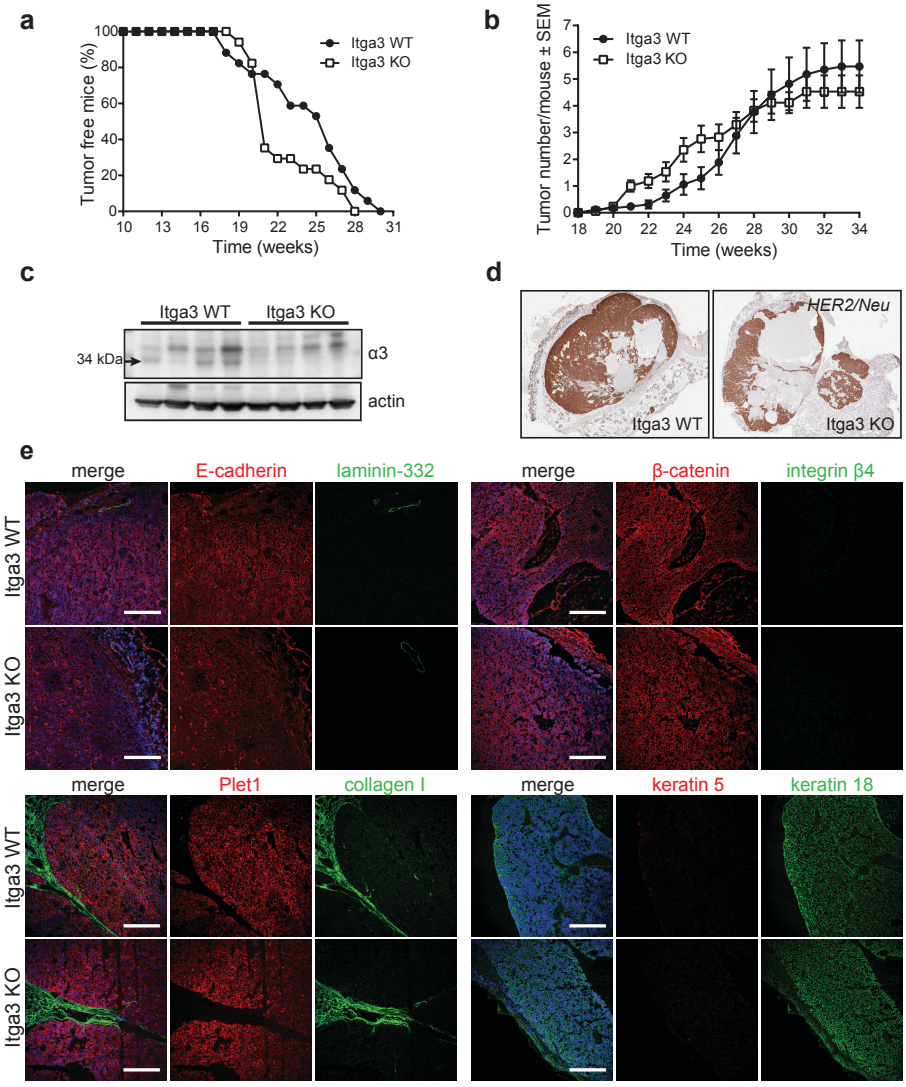


Figure 1: Integrin α3β1 is not needed for the onset of HER2-driven mammary tumorigenesis. (**a**) Kaplan-Meier plots of tumor-free survival in Itga3 KO and WT mice show that first palpable tumors could be detected at comparable age in both genotypes. (**b**) The number of tumors, measured macroscopically over time, was similar between Itga3 KO and WT mice (n=17). (**c**) The western blot of four randomly selected, representative tumors isolated from Itga3 KO and WT mice shows clear deletion of α3 in Itga3 KO mice. (**d**) Representative images of immunohistochemical staining for HER2/Neu in Itga3 KO and WT tumors show its strong expression, which was observed in all the analyzed tumors. (**e**) Representative images of immunofluorescent staining show the absence of basal markers β4, keratin 5 and laminin-332, which is also a main ligand for integrin α3β1. E-cadherin, β-catenin, Plet1, collagen I and keratin 18 were strongly expressed in all tumors. No difference was observed between Itga3 KO and WT mice. Scale bar: 200 μm.

The absence of integrin α3 promotes HER2-dependent tumor growth and metastases formation

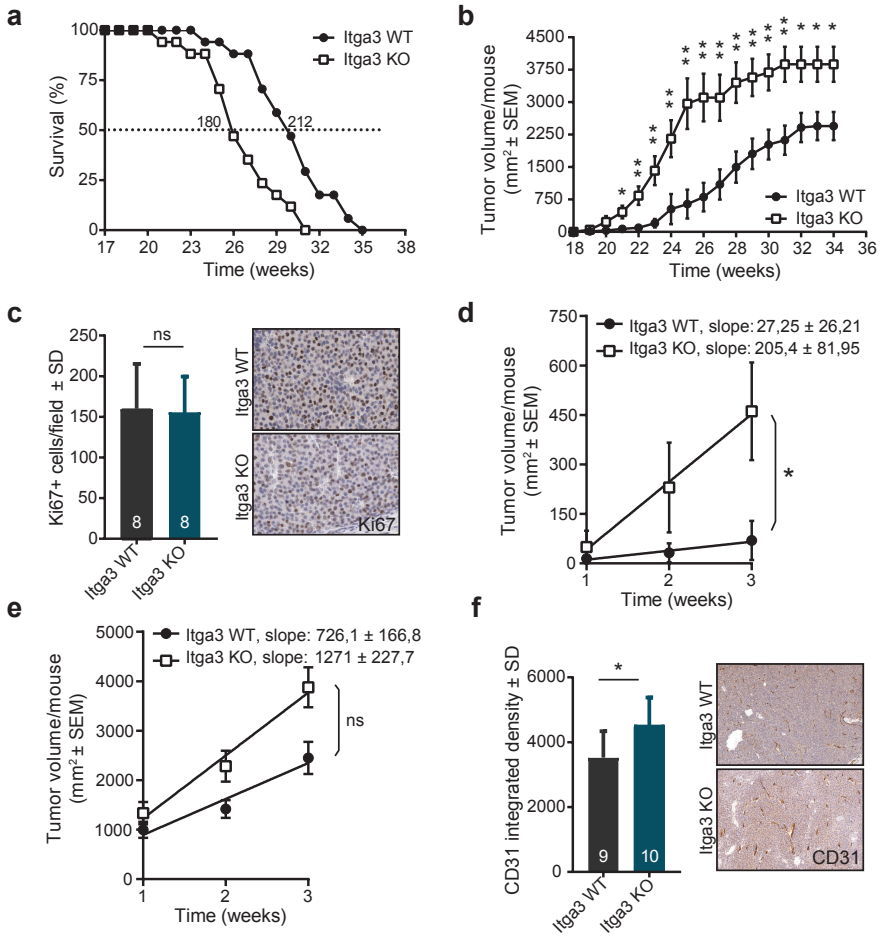


Figure 2: The absence of integrin α3 promotes HER2-dependent tumor growth and vascularization. (**a**) Survival Kaplan-Meier plots comparing Itga3 KO and WT mice. The median age when half of the mice per group needed to be sacrificed was 180 days for Itga3 KO mice and a month later (212 days) for Itga3 WT mice (n=17). (**b**) Itga3 KO mice developed significantly bigger tumors from the age of 21 weeks on (n=17; unpaired t test, *P<0.05, **P<0.005). (**c**) No difference in the number of proliferating Ki67-positive tumor cells was observed between Itga3 KO and WT mice. Left: Ki67-positive cells were counted in five fields of randomly selected tumors from 8 mice per genotype (unpaired t test, P=0.7168). Right: representative tumor images of immunohistochemical staining for Ki67. (**d-e**) Plots showing the fitted linear regression of average tumor sizes during (**d**) first and (**e**) last three weeks of tumor growth per mouse. The slopes of trend lines are significantly different during first weeks of tumorigenesis, showing an increased growth rate in Itga3 KO mice. Such difference is not observed during the last three weeks of tumor growth before mice were sacrificed (n=17, unpaired t test, *P<0.05). (**f**) Vascularity of tumors, quantified as integrated density of CD31 stained samples (left), was increased in Itga3 KO mice. Five fields of randomly selected tumors from 9 WT and 10 KO mice were analyzed (unpaired t test, P=0.0176). Right: representative tumor images of immunohistochemical staining for CD31.

Despite the similar tumor onset in both groups, the survival of Itga3 KO mice was reduced compared to the WT group (**Fig. 2a**). In line with this, tumor volume was significantly increased in the absence of α3 (**Fig. 2b**). Interestingly, analysis of Ki67-positive cells in solid areas of Itga3 KO and WT tumors, isolated at the time the mice had to be sacrificed, showed no significant differences in the number of proliferating cells (**Fig. 2c**). Similarly, there were no differences in the number of apoptotic cells between Itga3 WT and Itga3 KO mice, as detected by cleaved Caspase-3 staining in solid and cystic adenocarcinomas (**Supplementary fig. 1c**). To investigate whether the differences in tumor volume between Itga3 KO and WT mice originated from different growth rates during the first weeks of tumor formation (i.e. in the early stages of oncogenesis), we determined the slopes of trend lines of average tumor size per mouse for both, Itga3 KO and WT groups, during the first 3 weeks after tumors were detected (Figure 1d) and the last 3 weeks before individual mouse had to be sacrificed (**Fig. 1e**). Comparison of the slopes of the growth trend lines showed that Itga3 KO compared to WT tumors exhibited faster volumetric growth at the onset of tumorigenesis, whereas their growth rate during the last 3 weeks before the final time point was comparable. This could explain the differences in tumor volume, yet similar cell proliferation and apoptosis of the analyzed Itga3 KO and WT tumors at the time of sacrifice. Furthermore, histological analysis of blood vessel density in solid tumors, determined by measuring CD31 positive areas, showed an increased vascularization of Itga3 KO compared to WT tumors (**Fig. 2f**), which could offer a further explanation for the larger tumors in the Itga3 KO mice.

The analysis of the organs of mice at the final time point showed the presence of metastases only in lungs, which is common in MMTV-cNeu mice [25]. Pulmonary metastasis occurred in both, Itga3 KO and WT mice, but while metastases were detected only in 40% of the Itga3 WT mice, nearly all the Itga3 KO mice had pulmonary lesions (**Fig. 3a**). Furthermore, the Itga3 KO mice had a significantly higher number of metastases (**Fig. 3b**), which, despite the comparable average size of metastases between both groups (**Fig. 3c**), resulted in significantly increased metastatic burden in Itga3 KO, compared to WT mice (**Fig. 3d**). HER2/*Neu* positive lung metastases could be observed within blood vessels (bloodborne metastases) or escaping the vasculature and invading the surrounding lung parenchyma (invasive metastatic lesions). Both types of metastases could be detected in Itga3 WT and KO mice (**Fig. 3e**). A moderate increase in the percentage of invasive lesions out of their total number was observed in the Itga3 KO mice (**Fig. 3f**). Importantly, invasive metastatic lesions were detected in 37,5% of metastasis-bearing Itga3 WT mice, whereas in the Itga3 KO mice this number raised to 70% (**Fig. 3g**). In order to investigate whether the absence of α3 promotes tumor progression and invasiveness through changes in the downstream signaling of HER2, we have analyzed primary tumors and metastases for the activation of protein kinase B (pAkt), the mitogen activated protein

kinase 1 and 2 (pErk1/2) and eukaryotic translation initiation factor 4E-binding protein 1 (p4E-BP1), a downstream target of mTOR signaling pathway [5]. No differences were observed between Itga3 WT and KO mice (**Supplementary fig. 2**), suggesting that major alterations in MAPK and Phosphoinositide 3-kinase (PI3K) signaling do not lie at the basis of the metastasis-promoting effect of the absence of α3β1. Together, these findings demonstrate that the absence of α3 promotes tumor growth and vascularization and strongly increases the invasive and metastatic potential of HER2-driven breast cancer, resulting in reduced overall survival of Itga3 KO mice.

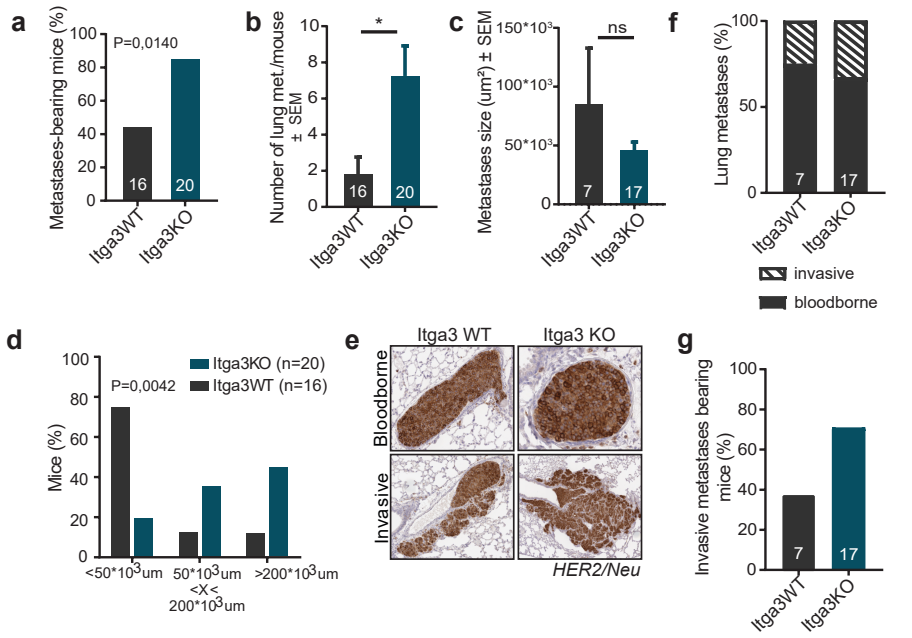


Figure 3: The absence of integrin α3 increases invasiveness of HER2-driven tumors. (**a**) Significantly more Itga3 KO compared to WT mice developed lung metastasis (chi-square (Fisher's exact) test). (**b**) The number of metastasis, counted in H&E stained sections of lungs, was significantly increased in Itga3 KO mice (unpaired t test, P=0.0119). (**c**) Metastases were of similar sizes in Itga3 KO and WT mice (unpaired t test, P=0.2222). (**d**) Itga3 KO compared to WT mice had significantly larger total metastatic burden, i.e. total metastatic area in lungs (chi-square test). (**e**) Representative images of invasive and bloodborne metastasis in lungs of Itga3 KO and WT mice, stained for HER2/Neu. (**f**) The percentage of bloodborne and invasive out of total pulmonary metastases shows small increase in the number of invasive metastases in Itga3 KO mice. (**g**) The percentage of invasive metastasis-bearing mice per genotype out of the total number of metastases-bearing mice. Almost twice as many Itga3 KO as WT mice displayed invasive metastases.

Reduced expression of α3 is associated with increased invasiveness of HER2+, but not of triple-negative human mammary carcinoma cells

Next, we investigated whether reduced expression of α3 increases the invasiveness of human mammary carcinoma cells. The surface expression of α3 was analyzed in three established human HER2+ (SKBR3, AU565 and BT474) and three triple-negative (MDA-MB-231, BT-20 and Hs 578T) mammary carcinoma cell lines using flow cytometry. The surface levels of α3 were reproducibly lower in all three HER2+ cell lines (**Fig. 4a**), which was confirmed by western blot analysis of total cell lysates (**Supplementary fig. 3a**). This is in line with previous observations of the α3 protein levels in MDA-MB-231 and SKBR3 cells [20] and suggests that downregulation of α3β1 integrin is associated with HER2-driven mammary tumorigenesis and tumor progression. Available RNA sequencing data from a panel of HER2+ and triple-negative-enriched breast cancer cell lines [26] also showed a negative correlation between ERBB2 and ITGA3 expression (**Fig. 4b**), although no such clear correlation could be observed in larger and more diverse breast cancer cell line panel [27,28], indicating that the regulation of α3β1 expression could be breast cancer cell type-dependent (**Supplementary fig. 3b**). In line with this, luminal-like breast cancer cell lines form a clear cluster of low ITGA3 expression in both datasets (**Supplementary fig. 3c and 3d**). Interestingly, both datasets showed relatively low ITGA3 expression in several HER2+ cell lines despite ITGA3 gene amplification (**Fig. 4c, Supplementary fig. 3e**). Together, these data indicate that HER2-driven, luminal-like breast cancer cells exhibit lower ITGA3 expression than triple-negative breast cancer cells.

To assess whether α3β1 influences the invasive potential of HER-driven human mammary carcinoma cells, we generated SKBR3, AU565 and BT474 ITGA3 KO cell lines by CRISPR/Cas9 technology (**Fig. 5a**). No difference in HER2-levels or downstream Akt signaling was observed between ITGA3 WT and KO cells (**Supplementary fig. 4a**). For comparison, we have also deleted α3 in triple-negative MDA-MB-231 cells (**Fig. 5a**). Cell invasion was assessed in Transwell chambers with membranes coated with a mixture of Matrigel and Collagen I, which was abundantly present around primary tumors in our *in vivo* model (**Fig. 1e**), and using 10% FCS as chemoattractant. To mimic high interstitial fluid flow in tumors, caused by angiogenesis and increased vascular permeability, pressure was applied to the tumor cells by an overlying column of serum-free medium in the upper chamber [29]. To control for the flow rates in the different experiments we measured the volume of the medium that has passed through the gel and membrane (**Supplementary fig. 4b**). As shown in **figure 5b**, there were no significant differences in the number of invading cells for triple-negative MDA-MB-231 ITGA3 KO and WT cells. In contrast and consistent with *in vivo* observations, all HER2+ cell lines exhibited a significantly higher invasion in the absence of α3 (**Fig. 5b**). Together,

these data suggest that the absence of α3 promotes migration of HER2+, but not triple-negative carcinoma cells in an environment with interstitial fluid pressure.

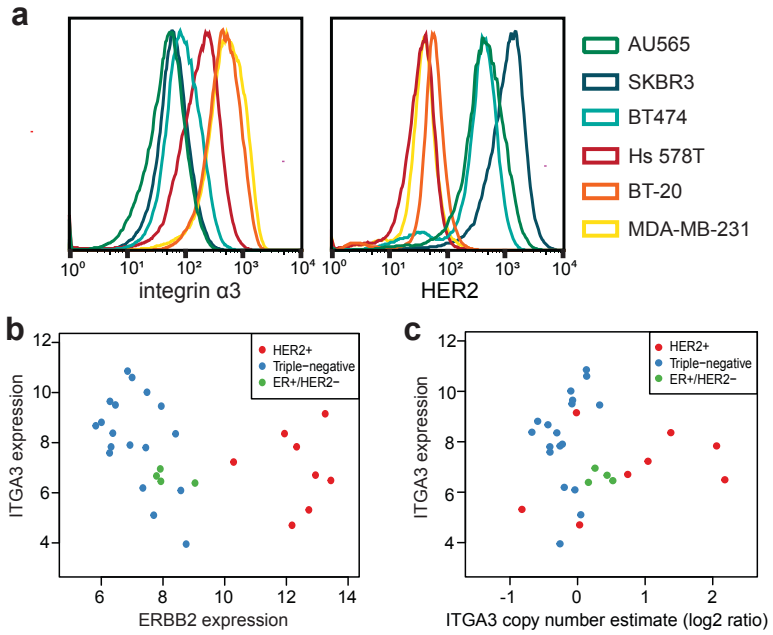


Figure 4: Decreased ITGA3 expression in HER2+, compared to triple-negative human carcinoma cells. (**a**) Flow cytometry histograms of signal intensity for α3 (left) and HER2 (right) staining of six human mammary carcinoma cell lines shows lower surface expression of α3 in HER2+ cells. 50*10^3 cells were analyzed per experiment; representative graphs of 3 independent experiments. (**b**) Scatter plot of ITGA3 and ERBB2 expression for HER2+ and triple-negative-enriched breast cancer panel [26] shows negative correlation between ITGA3 and ERBB2 expression (Spearman's rho -0.46, P=0.01, n=30). (**c**) Scatter plot of ITGA3 gene copy number estimates against ITGA3 expression for HER2+ and triple-negative-enriched breast cancer panel [26]. Despite ITGA3 amplification in several HER2+ cell lines, their expression of ITGA3 remains relatively low.

The invasion suppressing function of α3 in HER2+ cells depends on the extracellular matrix composition and the interstitial fluid flow

We further investigated whether the microenvironment, such as interstitial fluid flow and extracellular matrix composition, plays a role in the increased invasive potential of HER2+ carcinoma cells lacking α3. First, we allowed cells to invade through the membrane, coated with a mixture of Matrigel and collagen I in the absence of fluid flow. Under these conditions the deletion of α3 in MDA-MB-231 cells significantly impaired their invasive potential, which is in line with previous reports [20,21]. In contrast, all three HER2-driven carcinoma cell lines exhibited very low invasive potential, with no obvious differences between the ITGA3 KO and WT cells (**Fig. 5c**). Next, we assessed the role of collagen I in extracellular matrix by performing the invasion assays under

interstitial fluid flow, but with membranes, coated only with Matrigel. As before, the flow rates through the coated membranes were similar for the different cell lines (**Supplementary fig. 4c**). However, different from that observed with membrane coated with a collagen-Matrigel mixture, no significant differences in cell invasion were observed between the ITGA3 KO and WT HER+ cells (**Fig. 5d**). Therefore, the increased invasive potential of ITGA3 KO HER2+ carcinoma cells strongly depends on the presence of the interstitial fluid flow and a collagen I-rich extracellular matrix.

Interestingly, the number of ITGA3 WT HER2+ cells invading the Matrigel was increased to a level comparable to that of the invading ITGA3 KO cells, which is similar in both collagen-Matrigel and Matrigel gels under fluid flow conditions (**Fig. 5b and 5d**). This finding made us wonder whether α3β1 contributes to adhesion of cells to collagen, leading to faster 'passive' migration of ITGA3 KO cells through the collagen I-rich extracellular matrix when fluid pressure is applied. To investigate the effect of α3 deletion on the adhesion of cells to collagen, we performed short-term adhesion assays. HER2+ SKBR3, AU565 and BT474 and triple-negative MDA-MB-231 cells were seeded on matrices rich in laminin-332 or collagen I and allowed to adhere for 30 min, after which the number of adherent cells was quantified. As expected, the adhesion of all ITGA3 KO cell lines to laminin-332-rich matrices was strongly reduced. Deletion of ITGA3 also resulted in a moderate, but significant decrease in adhesion of triple-negative MDA-MB-231 and HER2+ SKBR3 and AU565 (but not BT474) cells to collagen I (**Fig. 6a**). Furthermore, the spread area and size of FAs of ITGA3 KO SKBR3 cells was significantly reduced compared to WT cells (**Fig. 6b-e**). No changes in the surface expression of the collagen receptors α1β1 and α2β1 integrin were observed in ITGA3 KO cells (**Supplementary fig. 5a**). However, IF staining of SKBR3 ITGA3 KO cells showed reduced clustering of integrin α2 and its binding partner β1 to FAs during the first 30 min of adhesion to collagen I (**Fig. 6f**). This suggests that α3β1 may promote adhesion and spreading of cells on collagen by promoting the clustering of collagen-binding integrins and thus the formation of FAs. In line with this, the invasion-suppressing effect of α3β1, observed during the invasion of HER2+ cells through a Matrigel/collagen I mixture under fluid flow conditions was not dependent on the adhesive activity of this integrin. No clear differences in invasion were observed between SKBR3 and AU565 cells, treated with α3 function-blocking antibody J143, which prevents the ligation of α3β1 by laminin, and non-blocking control antibody A3-X8 [30]. As observed before, the depletion of α3 significantly increased migration of both cell lines (**Fig. 6g, Supplementary fig. 5c**).

Together, our data suggest that the reduction of α3 allows HER2-drived carcinoma cells to migrate and invade faster through highly vascularized, collagen I-rich tumor stroma, which could be partially explained through the decreased adhesion to collagen I.

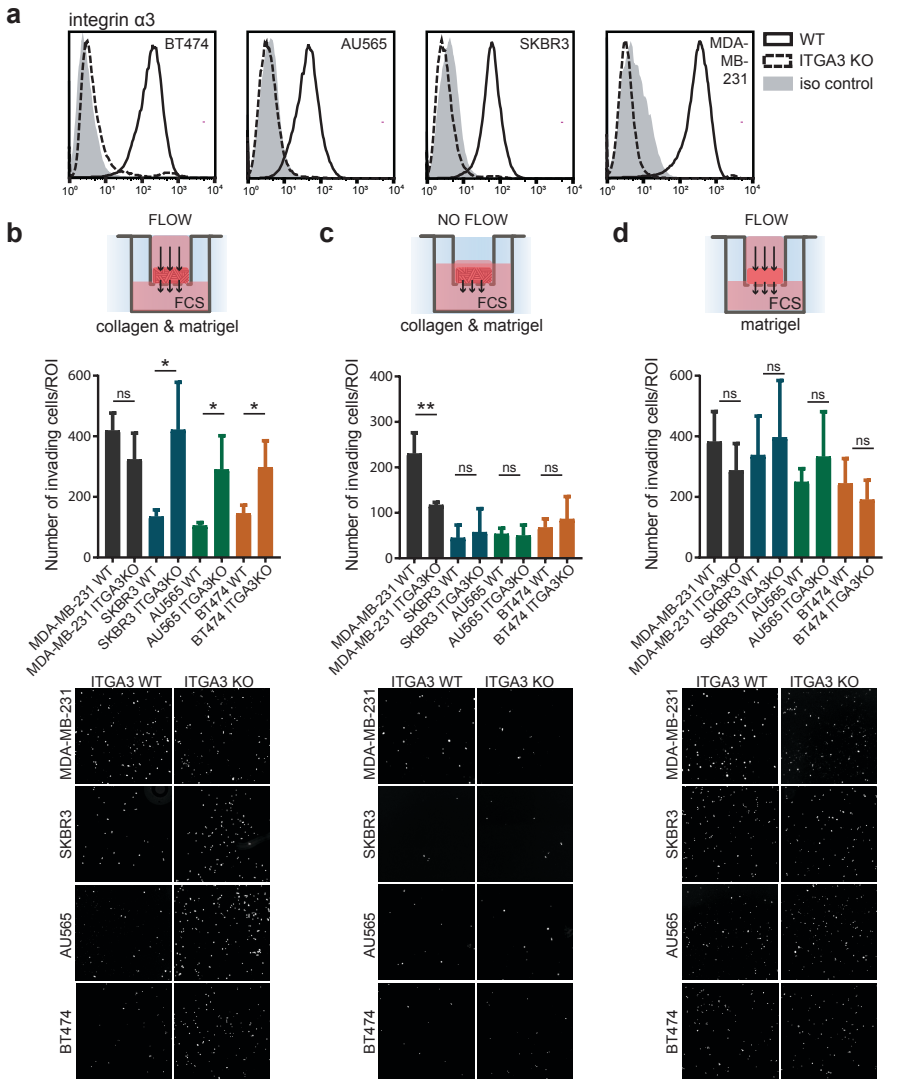


Figure 5: α3 reduction causes increased invasiveness of HER2+, but not triple-negative human carcinoma cells. (**a**) Flow cytometry histograms of signal intensity of α3 shows successful α3 deletion in AU565, SKBR3, BT474 and MDA-MB-231cells. (**b-d**) Invasion assays through gel (Matrigel-collagen I or Matrigel only)-coated membrane. Bottom: representative images of the part of the membrane, showing DAPI-stained nuclei of invading cells. Top: analysis of experiments, performed in duplicate and repeated three times. (mean ±SD, unpaired t test, *P<0.05, **P<0.005) (**b**) The reduction of α3 increases invasiveness of HER2+ carcinoma cells through the mixture of collagen I and Matrigel under interstitial fluid flow conditions. (**c**) Under static conditions HER2+ cells show strongly reduced and α3-independent invasion. Contrary, the invasion of triple-negative MDA-MB-231 is α3-dependent. (**d**) The absence of collagen I causes an increased invasion of α3WT HER2+ carcinoma cells, resulting in similar levels of invasion between α3KO and WT cells.

DISCUSSION

In this study we show that loss of the α3β1 integrin promotes HER2-driven luminal-type of breast cancer in vivo. In this tumor model, the initial steps of the tumorigenesis developed independently of the presence of α3β1, however, α3β1-depleted tumors grew bigger, were highly vascularized and, importantly, displayed strongly increased metastatic potential.

The studies, describing the essential role of α3β1 in tumor initiation, usually investigated epithelial tumors of basal nature, i.e. originating from epithelial cells anchored to a laminin-rich basement membrane, such as non-melanoma skin tumors and ovarian cancers [31,32]. Similarly, α3β1 supports tumor initiation and progression in basal-type of breast cancer [18,19,33]. Such pivotal role of α3β1 in basal-type tumors is often linked to its ability to support oncogenic MAPK signaling upon its ligation by laminin-332 [34], which may be crucial for proliferation and survival of tumor cells in early stages, when cells still depend on adhesion for their proliferation. This mechanism has indeed been observed in an *in vivo* mouse model of the basal type of breast cancer, in which α3β1 promotes proliferation and survival of tumor cells through activation of the FAK-PAK1-ERK1/2 signaling pathway [18]. Furthermore, the ability of α3β1 to support and sustain activation of signaling pathways upon its ligation to laminin might be necessary also during the later stages of breast cancer when cells acquire additional mutations, i.e. during epithelial-mesenchymal transition and invasion [19,33].

In contrast with this evident pro-tumorigenic function of α3β1 when bound to laminin, its laminin-independent role, such as described in our model, remains more elusive. We observed no clear differences in activation of pro-survival and proliferation-promoting pathways between Itga3 KO and WT mice. The downstream signaling of HER2 that drives formation and progression of tumors therefore appears to be independent of α3β1, enabling normal tumor onset and initial tumor development in Itga3 KO mice. This finding is seemingly in contradiction with a previously reported study by Novitskaya *et al.* [35], showing that α3β1 (in complex with CD151) supports the growth of SKBR3 and BT474 cells in Matrigel by promoting the homodimerization and activation of HER2 via inhibition of RhoA. However, the downregulation of α3 only affected the phosphorylation and homodimerization of HER2, whereas Akt signaling (as we confirm in this study) and phosphorylation of HER3, another member of epidermal growth factor receptor family, was unperturbed. Therefore, it seems likely that in their model the majority of pro-survival and proliferative signaling came from the dimerization of HER2 with HER3, i.e. the most potent mitogenic signaling dimer in the family [36]. Furthermore, it has been shown that HER2 activates pro-metastatic RhoA and RhoC in vivo and in vitro [37].

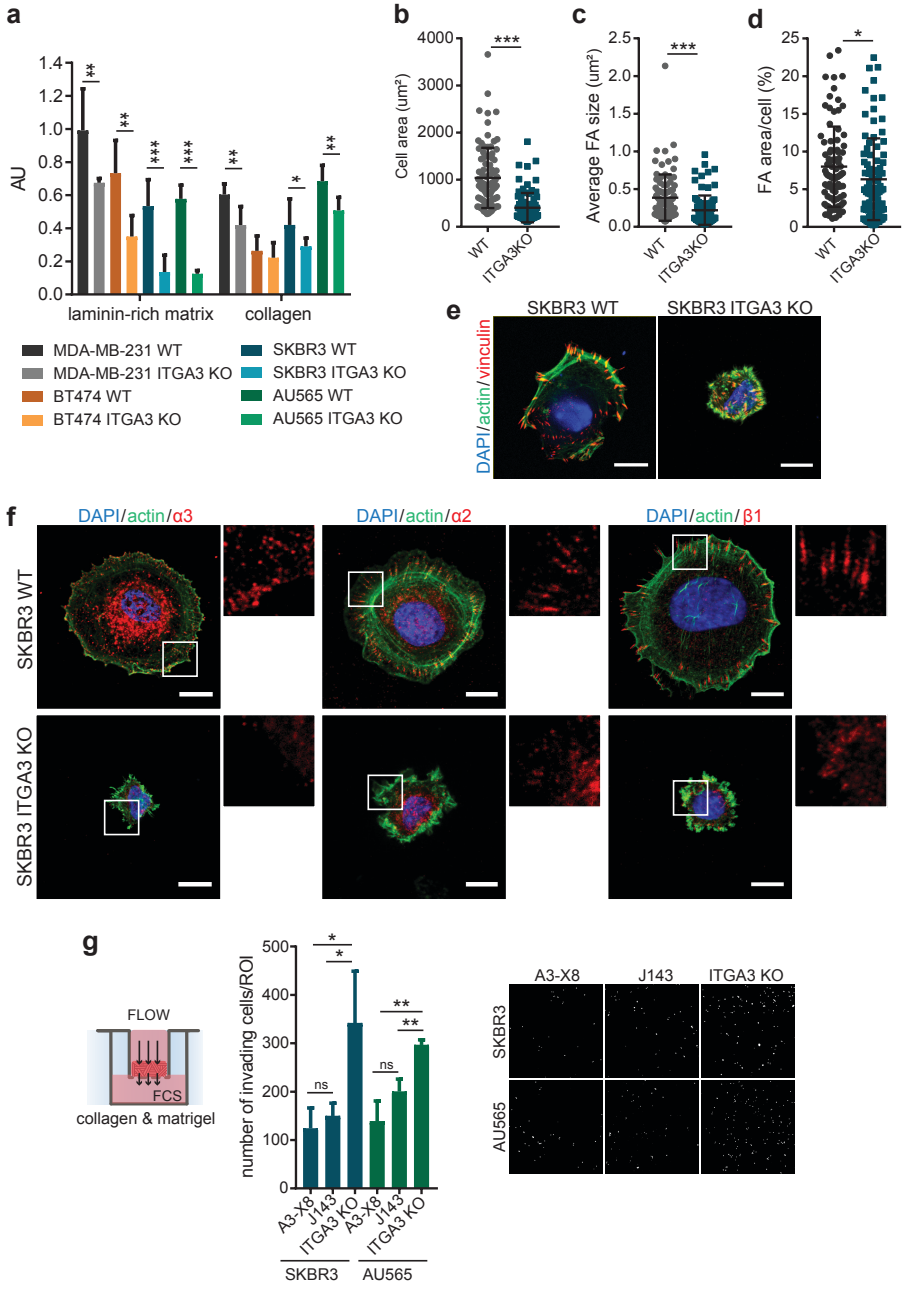


Figure 6: α3-mediated changes in collagen adhesion and FA formation. (**a**) Short-term adhesion assays of HER- MDA-MB-231 and HER2+ BT474, AU565 and SKBR3 on laminin-rich matrix and collagen I. Experiments were performed in triplicate and repeated three times (mean ±SD, unpaired t test, *P<0.05, **P<0.005, ***P<0.0001). (**b**) α3KO SKBR3 show significantly decreased cell area, (**c**) reduced size of focal adhesions and (**d**) reduced total adhesion area per cell after 30 min of adhesion to collagen I.

Experiments were performed three times with 30 cells analyzed per experiment (total n=90, unpaired t test, *P<0.05, **P<0.005, ***P<0.0005). (**e**) Representative image of SKBR3 ITGA3 KO and WT cell, used for quantifications (**b-d**), stained for actin and vinculin (scale bar: 10 μm). (**f**) Representative images of SKBR3 ITGA3 KO and WT cells, allowed to adhere to collagen I-coated coverslips for 30 min and stained for actin and integrins α3, α2 or β1. ITGA3 KO cells show reduced clustering of integrins in adhesion complexes. Note that α3 and α2 signal was enhanced by biotin-conjugated secondary antibody, resulting in unspecific biotin staining in the center of the cell (supplemental figure 4b) (scale bar: 10 μm). (**g**) Invasion assays through the mixture of collagen I and Matrigel-coated membrane. The reduction of α3 increases invasiveness of HER2+ carcinoma cells under interstitial fluid flow conditions, which cannot be recapitulated with blocking adhesion of α3 to laminin by addition of function-blocking J143 antibody (A3-X8: control non-blocking antibody). Left: analysis of experiments, performed in duplicate and repeated three times. (mean ±SD, unpaired t test, *P<0.05, **P<0.005) Right: representative images of the part of the membrane, showing DAPI-stained nuclei of invading cells.

Despite the similar proliferation rate and the fact that we observed no differences in HER2-driven signaling events between Itga3 KO and WT mice at the time when mice were sacrificed, the absence of α3β1 promoted tumor growth during the early stage of tumorigenesis. One of the obstacles that tumor cells must overcome during this stage of tumor mass accumulation is the absence of vascularity, and consequent hypoxic environment and lack of nutrients. It is possible that the increased angiogenesis that we observed in Itga3 KO mice in late stage of tumorigenesis could have contributed to differences in tumor growth rate during the first weeks of fast tumor mass accumulation, when earlier and/or increased vessel formation would likely result in strong growth advantage. In line with this it has been observed that the reduction of α3β1 in prostate carcinoma cells promoted their proliferation via changes in the interaction between tumor and stromal cells [38].

High vascularization and vascular permeability of tumors leads to interstitial fluid flow that is increased compared to normal tissues, promoting dissemination of cells and metastases formation [39]. Therefore, an increased angiogenesis of Itga3 KO tumors might already (partially) explain their faster progression and increased invasion. Furthermore, our data show that fluid flow and collagen I-rich extracellular matrices play a crucial role in an increased invasive potential of ITGA3 KO HER2+ SKBR3, AU565 and BT474 cells. Such increased invasiveness can be partially explained by their reduced adhesion to collagen I, an extracellular matrix component that is abundant in the mammary tumor stroma [40–42]. In line with this, it has been shown that deletion of the collagen receptor integrin α2β1 increases intravasation, but not extravasation of tumor cells, which results in strongly increased metastases formation in HER2/Neu overexpressing mouse model [43]. The reduced adhesion to collagen I that we have observed in MDA-MB-231, SKBR3 and AU565 ITGA3 KO mammary carcinoma cells is not due to the changes in the α2β1 expression, but likely due to the reduced clustering of α2β1. Indeed, the absence of α3 affected the size and area of FAs during

initial adhesion of SKBR3 cells to the collagen I. That the presence of α3β1 can affect formation and/or dynamics of other adhesion complexes has long been established [44,45]. Furthermore, clustering of integrins is connected to their increased activity and therefore increased outside-in signaling, which, among others, leads to changes in the actomyosin contractility [46].

However, no α3-dependent differences in adhesion to collagen I were observed in HER2+ BT474 cells, even though they showed an increased invasiveness in collagen I-rich matrix under flow conditions upon α3 depletion. Therefore, α3-mediated changes in cell adhesion cannot fully explain the observed differences in the invasion potential. One important difference between Matrigel and the mixture of collagen and Matrigel is their degree of stiffness. The addition of collagen I to Matrigel results in an increased stiffness, which can strongly impact invasion and migration through alterations in mechanosensing and mechanotransduction [47,48]. Furthermore, collagen I-rich, stiff and dense tissue is a known risk factor for developing breast carcinoma and metastases [42,49]. In such environment Rho-driven actomyosin contractility plays an important role in migration and invasion [42,48,50]. As already mentioned, the downregulation of Rho by α3β1-CD151 complexes is well established [8,12], therefore it is possible that α3 depletion promotes invasion also through increased Rho activity of cells in vivo and in vitro.

Finally, our experiments confirmed the previously reported pro-invasive role of α3 in MDA-MB-231 triple negative breast cancer [19–21]. Invasion assays performed in the presence or absence of interstitial fluid flow demonstrated different invasive behaviors of triple-negative MDA-MB-231, compared to HER2+ AU565, SKBR3 and BT474 cells even when α3 was not deleted. Furthermore, expression analysis of two different datasets of breast cancer cell lines suggests cancer subtype-dependent regulation of α3 expression, with its downregulation in invasive HER2+ luminal-like carcinoma cells. As it is increasingly evident that the existing classifications of breast cancer subtypes often overlap and struggle to classify the heterogeneity of the disease [51], it may be naïve to expect that we can predict the function of α3β1 in breast cancer only by HER2 status. However, striving to understand the impact of α3β1 and other similar markers under specific and defined conditions of the disease, such as HER2-overexpression, composition of the extracellular matrix and luminal cell origin, can help us towards its better clinical definition and consequently more efficient treatment strategies.

CONCLUSION

This study shows that the downregulation of α3β1 in HER2-driven mouse model and in HER2+ human mammary carcinoma cells promotes tumor progression and invasiveness of the cells. It demonstrates that the collagen I-rich extracellular matrix and interstitial fluid flow define the invasive potential of α3-depleted HER2+ cells *in vitro,* implying that such environmental factors may mediate invasiveness of cells in highly vascularized tumors in the absence of α3β1 and laminin-332. Importantly, the observed role of α3β1 in HER2-driven mammary tumorigenesis was not observed in triple-negative MDA-MB-231 cells, where the downregulation of α3β1 resulted in the opposite effect on invasiveness, which clearly demonstrates the tumor type specific function of α3β1 in breast cancer.

MATERIALS AND METHODS

Generation of mice

According to Mouse Genome Informatics (Jackson Laboratory), the names of MMTV-Cre; MMTV-cNeu (Itga3 WT) mice are Tg(MMTV-cre)4Mam; Tg(MMTV-Erbb2)NK1Mul. MMTV-Cre; MMTV-cNeu; *Itga3*$^{fl/fl}$ (Itga3 KO) mice were generated by intercrossing MMTV-Cre; MMTV-cNeu mice with *Itga3* $^{fl/fl}$ (i.e. Itga3$^{tm1Son/tm1Son}$ according to Mouse Genome Informatics). Mice were bred onto an FVB/N background.

In Vivo Tumor Analysis

Mice were examined twice a week for the presence of palpable mammary tumors and tumor sizes were measured using calipers. Mice were sacrificed when the total tumor mass per mouse reached 4 cm^3. At the end of the *in vivo* experiments, full necropsies were performed, tumor tissues and all the organs were collected, fixed in 10% neutral formalin, embedded into paraffin blocks and subsequently sectioned and stained for hematoxylin and eosin and/or immunohistochemistry analysis. Alternatively, tumors were embedded in Tissue-Tek OCT (optimal cutting temperature) cryoprotectant for immunofluorescent analysis of cryo-preserved material.

Cell culture

MDA-MB-231, SKBR3, AU565, BT-20, BT474 and Hs 578T carcinoma cell lines were obtained from the research group of L. F. A. Wessels and were authenticated by suppliers using Short Tandem Repeat profiling [26]. MDA-MB-231, Hs 578T, SKBR3 and AU565 were cultured in RPMI, BT474 in Advanced DMEM F12 and BT-20 in MEM culture medium. All cell lines were cultured with 10% heat-inactivated FCS and antibiotics. Hs

578T were additionally cultured with 10 µg ml^{-1} insulin. All cells were cultured at 37°C in a humidified, 5% CO_2 atmosphere.

Generation of integrin α3-deficient cells

The target sgRNA against ITGA3 (exon 1; 5'CGGTCGCGAGCTGCCCGCGA-3') was cloned into pX330-U6-Chimeric_BB-CBh-hSpCas9 (a kind gift from Feng Zhang [52]); Addgene plasmid #42230). MDA-MB231, SKBR3, AU565 and BT474 cells were transiently transfected with this vector using lipofectamine® 2000 (Invitrogen). Lipofectamine (20 µl ml^{-1}) and vector solution (3 µg) in Opti-MEM were mixed (1:1) and incubated for 20 min at room temperature. Cells were incubated with the transfection solution overnight. Integrin α3-deficient cells were selected by Fluorescent activated cell sorting.

Immunohistochemistry

After deparaffinization of the samples and antigen retrieval, tumor and lung tissue sections were consecutively stained with primary antibodies (see table 1) and biotin-conjugated secondary antibodies, followed by incubation with streptavidin/HRP (DakoCytomation; P0397) and detection and visualization with DAB tablets (Sigma; D-5905). 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) or with the Aperio ScanScope (Aperio, Vista, CA, USA), using ImageScope software version 12.0.0 (Aperio).

Immunofluorescence

Cryosections of tumors were prepared, fixed in ice-cold acetone, and blocked with 2% bovine serum albumin (BSA, Sigma) in PBS for 1h at room temperature. Tumor samples were incubated with the indicated primary antibodies in 2% BSA in PBS for 60 min, washed in PBS three times and further incubated with secondary antibodies diluted 1:200 for 60 min. All samples where counterstained with DAPI for 5 min at room temperature and mounted in Vectashield (Vector Laboratories H-1000). Samples were analyzed by Leica TCS SP5 confocal microscope with a 20x (NA 1.4) objective and processed using ImageJ [53, 54]. SKBR3 cells were fixed with 2% paraformaldehyde for 10 min, permeabilized with 0.2% Triton-X-100 for 5 min, and blocked with PBS containing 2% BSA for 1h at room temperature. Cells were further incubated with the primary antibodies (see **table 1**) for 1 h at room temperature, washed three times with PBS and incubated with the secondary antibodies for 1 h. For integrin α3 and α2 staining, additional incubation with biotin-conjugated antibody was performed after primary antibody staining, which was followed by incubation with fluorophore-conjugated streptavidin. Additionally, the nuclei were stained with DAPI and filamentous actin was visualized using Alexa Fluor 488-conjugated phalloidin (Invitrogen). After three washing

steps with PBS, the coverslips were mounted onto glass slides in Mowiol. Images were obtained using a Leica TCS SP5 confocal microscope with a 63x (NA 1.4) oil objective and processed using ImageJ [53, 54]. Focal adhesion size and amount were calculated using the Analyze Particle function, after drawing a region of interest (ROI) at the cell periphery (based on actin staining). The total cluster area was divided by the total ROI area to define focal adhesion area per cell.

Western Blot

Protein lysates for Western Blot analysis of tumors were obtained from FFPE tumor tissue samples by using Qproteome FFPE Tissue Kit (Qiagen) following the instructions of the manufacturer. Protein lysates of carcinoma cells were obtained from subconfluent cell cultures, washed in cold PBS and lysed in RIPA buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 4 mM EDTA (pH 7.5), 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with 1.5 mM Na_3VO_4, 15 mM NaF (Cell Signaling) and protease inhibitor cocktail (Sigma). Lysates were cleared by centrifugation at 14.000 x g for 20 min at 4°C and eluted in sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 12.5 mM EDTA, 0.02% bromophenol blue) containing a final concentration of 2% β-mercaptoethanol and denatured at 95°C for 10 min. Proteins were separated by electrophoresis using Bolt Novex 4–12% gradient Bis-Tris gels (Invitrogen), transferred to Immobilon-P transfer membranes (Millipore Corp) and blocked for 1h in 2% BSA in TBST buffer (10 mM Tris (pH 7.5), 150 mM NaCl, and 0.3% Tween-20). The blocked membranes were incubated overnight at 4°C with primary antibodies (see **table 1**) diluted 1:1000 in TBST containing 2% BSA, after which they were washed twice with TBST and twice with TBS buffer. Next, membranes were incubated 1 h hour at room temperature with horseradish peroxidase–conjugated goat anti-mouse IgG or goat anti-rabbit IgG (diluted 1:5000 in 2% BSA in TBST buffer). After washing, the bound antibodies were detected by enhanced chemiluminescence using or Clarity™ Western ECL Substrate (Bio-Rad) or Amersham ECL Western Blotting Detection Reagent (GE Healthcare) as described by the manufacturer. Signal intensities were quantified using ImageJ [53,54]).

Flow cytometry

Cells were trypsinized, washed in PBS containing 2% FCS, and incubated for 1h at 4°C in primary antibody (1:100) in PBS 2% FCS. Cells were next washed twice in PBS containing 2% FCS and incubated with PE-conjugated donkey anti-mouse (Biolegend #406421; 1:200 dilution) or donkey anti-rat (Biolegend # 406421; 1:200 dilution) antibody for 30 min at 4°C. After subsequent washing steps, cells were analyzed on a Becton Dickinson FACS Calibur analyzer. For fluorescent activated cell sorting, α3-negative cell population was obtained using a Becton Dickinson FACSAria IIu cell sorter.

Invasion assay

Transwell inserts with 8.0 µm pore polycarbonate membrane (Corning, #3422) were coated with 150 µl of either Matrigel (Corning® Matrigel® Growth Factor Reduced Basement Membrane Matrix, 3.3 times diluted in serum-free medium), or the mixture of Matrigel (3.3 times diluted in serum-free medium) and freshly prepared collagen I solution (1,05 mg ml^{-1}), containing 20000 cells, and left incubating for 1 h at 37°C. When used, 4 µg of function blocking or control antibodies were added to the gel. Collagen I solution was prepared by mixing 10-times concentrated PBS, 1M NaOH and collagen I (2,8 mg ml^{-1}, Advanced Biomatrix #5005), after which the mixture was incubated at 4°C for 1 h. For interstitial fluid flow conditions, transwell inserts were inserted in 24-well plate, containing 280 µl of cell culture medium supplemented with 10% FCS. Next, 450 µl of serum-free medium was gently pipetted on top of the gel into transwell inserts. When used, function blocking or control antibodies were added to serum-free medium at concentration 10µg ml^{-1}. For static conditions, transwell inserts were placed in 24-well plate containing 650 µl of cell culture medium supplemented with 10% FCS and 150 µl of serum-free medium was pipetted into transwell insert. Cells were left to migrate for 21 h, after which the gel was aspirated, and the upper side of membranes cleaned with cotton swabs. Membranes were then fixed in ice-cold methanol for 10 min and washed with PBS. Invading cells were stained with DAPI for 5 min at room temperature, and total membranes were imaged with Zeiss Axio Observer Z1 inverted microscope, using automated tile imaging setting on Zeiss ZEN software and 10x objective. Images were stitched and processed with Zeiss ZEN software, and further analyzed using ImageJ [53,54]. Circular ROI was selected in the central part of the membrane (115 mm^2) and cells were quantified by counting DAPI-stained nuclei, using the Analyze Particle function.

Adhesion assay

For adhesion assays, 96-well plates were coated with 3.2 µg ml^{-1} collagen I (Advanced Biomatrix #5005) or laminin-332-rich matrix. Collagen I coating was done in PBS solution at 37°C for 1 h. Laminin-332-rich matrix was obtained by growing RAC-11P cells [55] 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 and the coated plates were kept at 4°C in PBS until they were used. Before use, coated plates were washed once with PBS and blocked with 2% BSA in PBS for 1 h at 37°C. Carcinoma cells were trypsinized and resuspended in serum-free cell culture medium. The cells were seeded at a density of $1x10^5$ cells per well and incubated for 30 min at 37°C. Nonadherent cells were washed away with PBS and the adherent cells were fixed with 4% paraformaldehyde for 10 min at room temperature, washed twice with H_2O, stained with crystal violet for 10 min at room temperature and

washed extensively with H_2O. Dried and stained cells were resuspended in 2% SDS, after which absorbance was measured at 595 nm on a Tecan infinite 200Pro microplate reader using Tecan i-control software.

Antibodies

Primary antibodies used are listed in **Table 1**. Secondary antibodies were: goat-anti rabbit Alexa Fluor 488, goat anti-mouse Alexa Fluor 568, goat anti-rat Texas FITC, goat anti-rat Alexa Fluor 647 (Invitrogen), biotin-goat anti-mouse IgG, Cy-5 streptavidin (Zymed), PE-conjugated donkey anti-mouse antibody (Biolegend #406421), PE-conjugated donkey anti-rat antibody (Biolegend # 406421), stabilized goat anti-mouse HRP-conjugated and stabilized goat anti-rabbit HRP-conjugated (Pierce).

Breast cancer cell expression data analysis

We used the RNA sequencing gene expression data from Jastrzebski et al [26] and from the Cancer Cell Line Encyclopedia [27,28]. The read counts were normalized for library size and log-transformed. HER2 and ER status were annotated according to ATTC and ExPASy Cellosaurus [60], which matched with the presence of ERBB2 gene amplification and the level of ESR1 expression in the respective datasets. Cell lines were classified as luminal, basal or post-EMT based on the annotation provided in [26]. For the cell lines not annotated in that reference, we used the same criteria to classify them as luminal, basal or post-EMT, that is, cell lines with high KRT5 expression were classified as basal, and cell lines with high expression of VIM were classified as post-EMT. Gene-level copy number estimates were obtained from segmented copy number profiles by taking the log copy number ratio of the segment containing the start of the gene.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (version 7.0c). Graphs represent the mean and error bars standard deviation (SD) or standard error of mean (SEM), as indicated per graph. Unpaired two-tailed t test was used for comparisons of experimental groups with a control group, one-way ANOVA was used to compare multiple groups across a single condition and chi-square test was used for categorical data. The statistical test used per experiment and significant values shown are described in appropriate figure legends. Results with P value lower than 0.05 were considered significantly different from the null hypothesis.

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Antigen	Name	Type	Application	Dilution	Source
β-catenin	610154	Mouse mAb	IF	1:100	BD Bioscience
actin	MAB1501R	Mouse mAb	WB	1:1000	Chemicon
Akt	9272	Rabbit mAb	WB	1:1000	Cell Signaling
Caspase3 (cleaved Asp 175)	9661L	Rabbit pAb	IHC	1:500	Cell Signaling
CD31	ab28364	Rabbit pAb	IHC	1:500	Abcam
Collagen I	A67P	Rabbit pAb	IF	1:40	Chemicon
E-cadherin	610182	Mouse mAb	IF	1:100	BD Bioscience
GAPDH	CB1001	Mouse mAb	WB	1:1000	Calbiochem
HER2	2165S	Rabbit mAb	WB	1:1000 1:200	Cell signaling
Itga2	10G11	Mouse mAb	FACS	1:100	[56]
Itga3	J143	Mouse mAb	FACS Functional assay	1:100 10 μg ml^{-1}	[57]
Itga3		Rabbit pAb	WB	1:2000	Home made
Itga3	A3-X8	Mouse mAb	Functional assay	10 μg ml^{-1}	Kind gift of C. Stipp [30]
Itga6	GoH3	Rat mAb	FACS	1:200	[58]
Itgb4	346-11A	Rat mAb	IF	1:100	BD Bioscience
Keratin 5	PRB-160P	Rabbit mAb	IF	1:100	Covance
Keratin 18	RGE53	Mouse mAb	IF	1:2	Progen
Ki67	PSX1028	Rabbit pAb	IHC	1:750	Monosan
Laminin-332	R14	Rabbit pAb	IF	1:400	Kind gift of M. Aumailey
NEU	sc-284	Rabbit pAb	IHC	1:800	Santa cruz
pAkt (Ser473)	4060	Rabbit mAb	IHC	1:10000	Cell Signaling
pAkt (Ser473)	9271	Rabbit mAb	WB	1:500	Cell lisgnaling
p4E-BP1 (Thr37/47)	2855	Rabbit mAb	IHC	1:1600	Cell Signaling
pErk1/2(Thr202/ Tyr204)	4370	Rabbit mAb	IHC	1:400	Cell Signaling
Plet1	33A10	Rat mAb	IF	1:100	[59]
Tubulin	B-5-1-2	Mouse mAb	WB	1:5000	Sigma
Vinculin	VIIF9	Mouse mAb	IF	1:5	Kind gift of M. Glukhova

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

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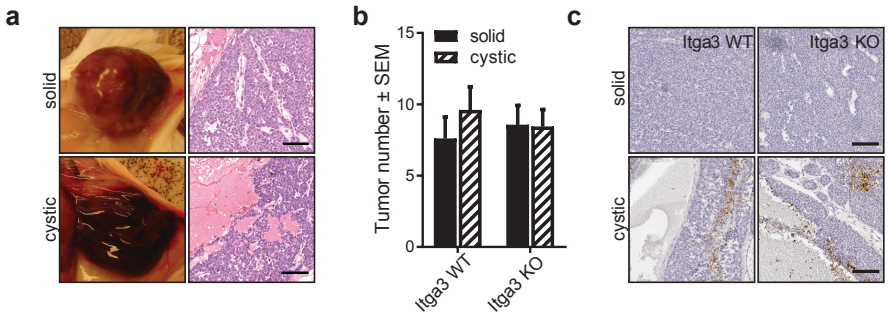
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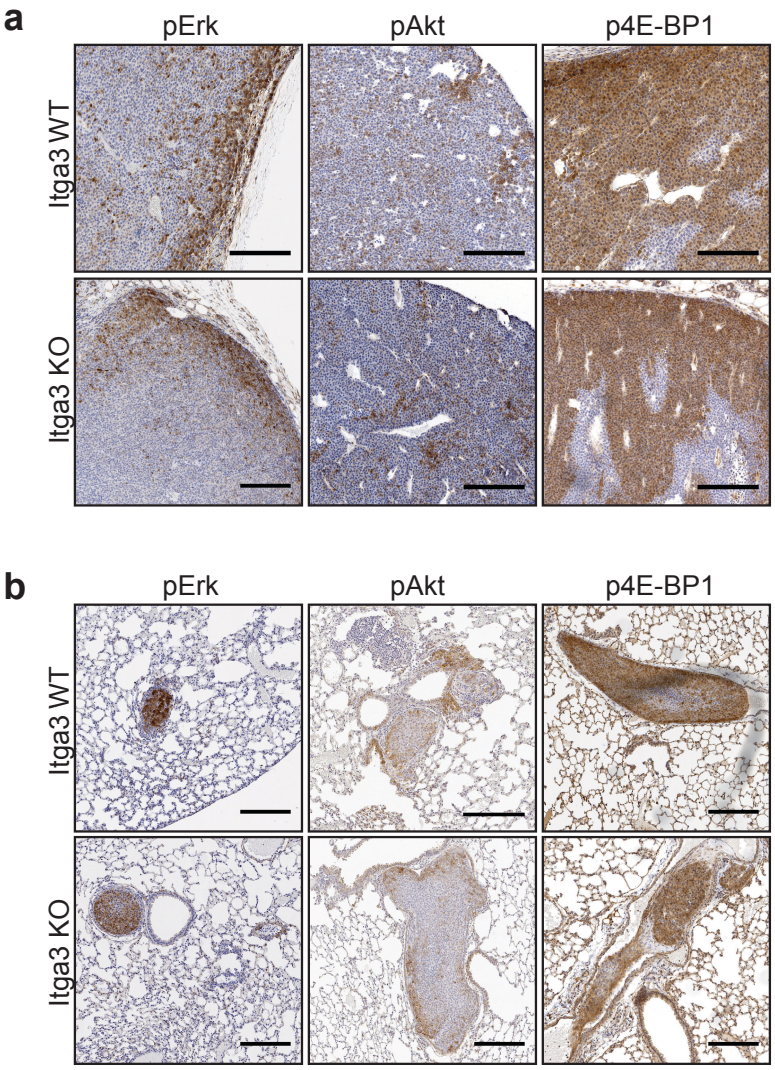
SUPPLEMENTARY FIGURES

Supplementary figure 1



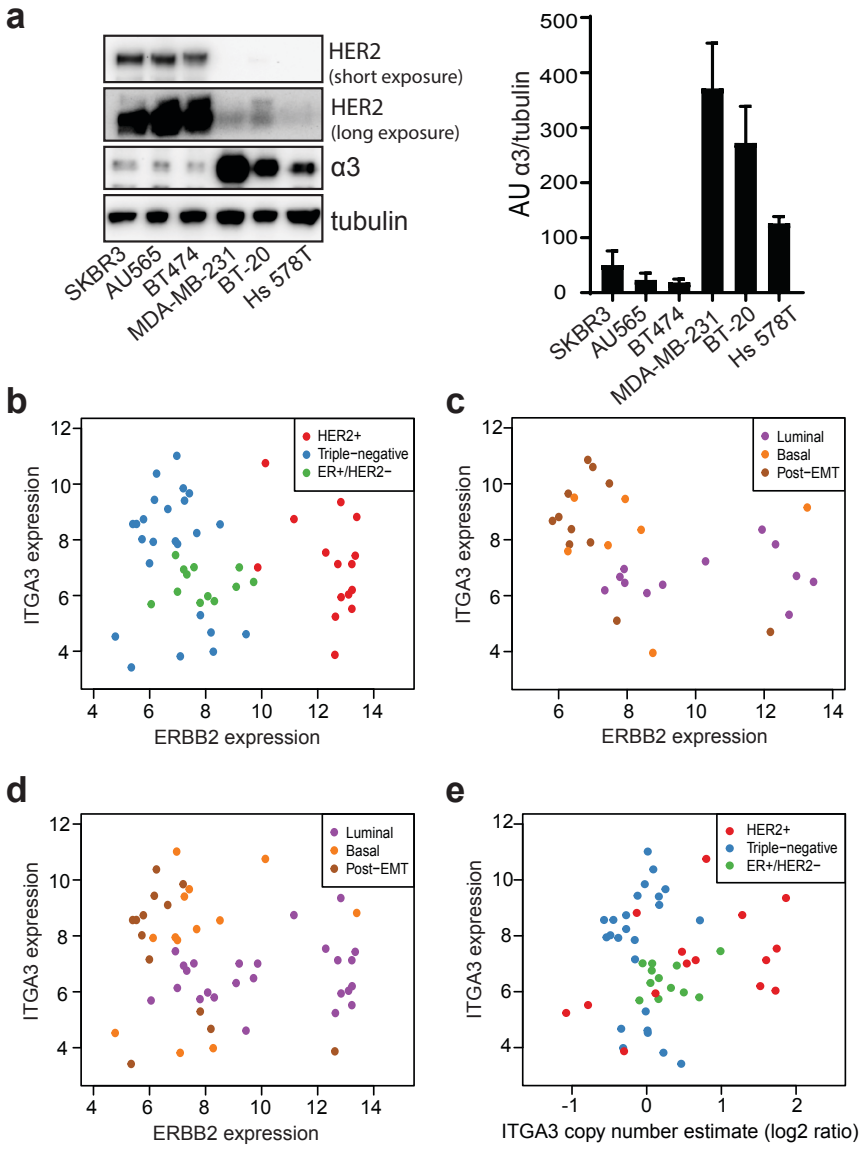
(**a**) Representative macroscopic and HE stained pictures of the two types of tumors detected: solid tumors with a high cellularity and cystic tumors which consist of multiple large and small cysts/open areas filled with blood. Scale bar: 100 μm. (**b**) Quantification of solid vs cystic tumors Itga3 KO and WT mice determined by pathological analysis of excised tumors. No significant differences between both groups could be detected. (**c**) Immunohistochemical staining for cleaved-Cas3 marker of apoptosis in representative solid (top) and cystic (bottom) tumors of Itga3 KO and WT mice. No positive staining was observed in any of the analyzed solid tumors and comparable amount of apoptosis was detected in cystic tumors of Itga3 KO and WT mice. Scale bar: 100 μm.

Supplementary figure 2



Representative images of immunohistochemical staining of (**a**) primary tumors and (**b**) metastases of Itga3 KO and WT mice. No differences were observed in activation of main HER2-mediated pathways, as seen by pAkt, pErk and p4E-BP1 staining. Scale bars: 200 µm.

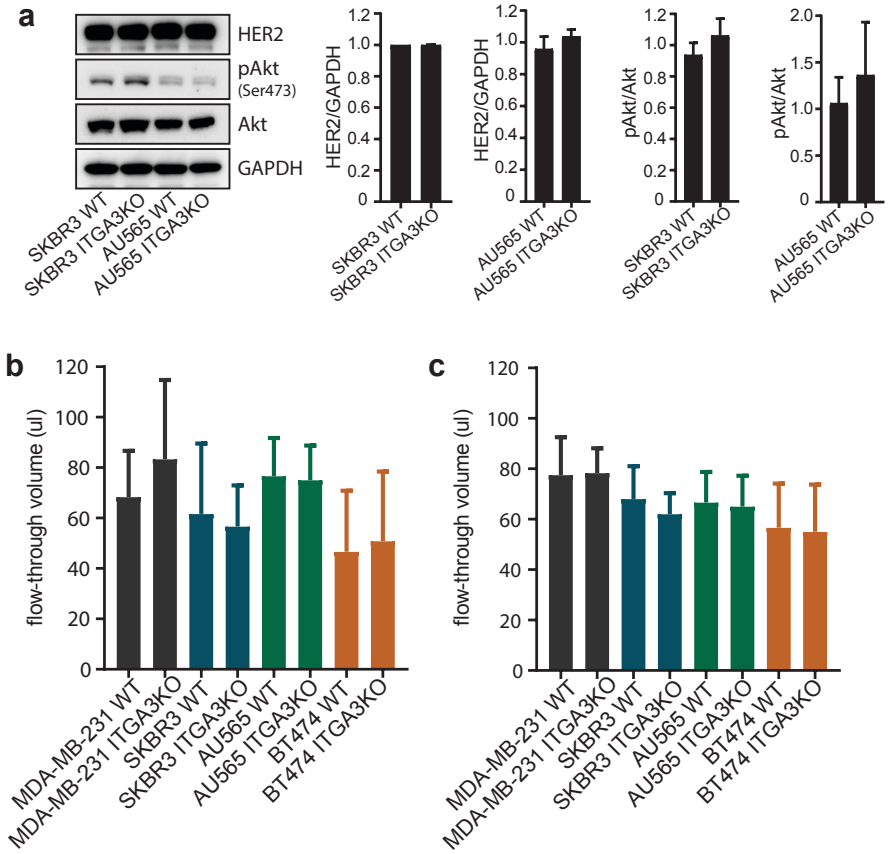
Supplementary figure 3



(**a**) Representative western blot (left) and quantification (right) of three separate experiments of whole cell lysates of triple-negative MDA-MB-231, BT-20, Hs 578T and HER2+ BT474, AU565, SKBR3 mammary carcinoma cells. HER2 overexpressing cell lines exhibit strongly reduced levels of α3 protein. (**b**) Scatter plot showing lack of correlation between ITGA3 and ERBB2 expression in CCLE breast cancer panel (Spearman's rho -0.17, P=0.22, n=51). (**c-d**) Scatter plots of ITGA3 and ERBB2 expression of breast cancer cell lines, classified as luminal-, basal- and post-EMT-like show clustering of luminal-like cell lines to low ITGA3 expression: (**c**) HER2+ and triple-negative-enriched breast cancer panel [26] (n=30), (**d**) CCLE breast cancer cell panel (n=51). (**e**) Scatter plot of ITGA3 gene copy number estimates against ITGA3 expression for CCLE dataset. Despite ITGA3 amplification in several HER2+ cell lines, their expression of ITGA3 remains

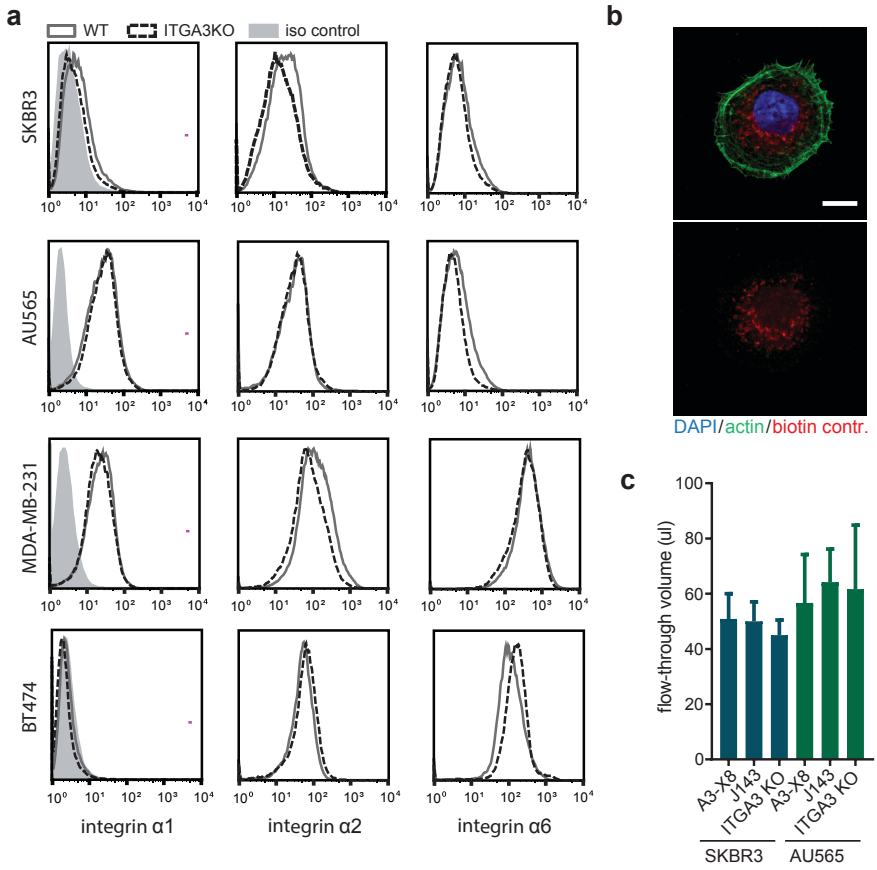
relatively low.

Supplementary figure 4



(**a**) Representative western blot (left) and quantification (right) of three separate experiments of whole cell lysates of WT and ITGA3 KO AU565 and SKBR3 mammary carcinoma cells. No differences in levels of HER2 or in Akt signaling was observed between ITGA3 KO and WT cells. (**b-c**) Volume of medium, passing the (**b**) mixture of collagen I and matrigel and (**c**) Matrigel only during the invasion assays under interstitial flow conditions (mean ± SD). No significant differences were observed (one-way ANOVA).

Supplemental figure 5



(**a**) Flow cytometry histograms of surface expression of collagen-binding integrins α1 and α2, and laminin-binding integrin α6 in ITGA3 KO and WT mammary carcinoma cells. (**b**) Representative image of SKBR3 ITGA3 WT cells, stained with biotin-conjugated secondary antibody as a background control (scale bar: 10 μm). (**c**) Volume of medium, passing the mixture of collagen I and matrigel during the invasion assays under interstitial flow conditions and with addition of α3-function blocking (J143) and control (A3-X8) antibodies (mean ± SD). No significant differences were observed (one-way ANOVA).



GENERAL DISCUSSION

The main goal of this thesis was to gain a better understanding of the conditions that are responsible for the different functions of integrin α3β1 in cancer. We aimed to achieve this by investigating the role of α3β1 in few well-defined cancer types and stages: during the initiation of non-melanoma skin cancer and during the development of invasive HER2-driven breast carcinogenesis. These cancer models present distinctive tumor environments, which differ in numerous components: extracellular matrix composition, expression of adhesion molecules, such as integrins and cadherins, expression of α3β1-associated proteins (such as tetraspanin CD151) and the nature of driving oncogenic mutation to just name a few. Furthermore, by investigating the role of α3β1 expressed in hair bulge stem cells (HB SCs) only, we gained an unexpected opportunity to shed light not only on the function of α3β1 in tumor-initiating cells, but also in cells that can regulate tumorigenesis via modulation of tumor environment. Here, we discuss the diversity of α3β1 in cancer in the light of our findings and present some preliminary data that we hope could expand the scope of this research in the future.

PRO-SURVIVAL ROLE OF α3β1 VIA ITS ADHESION TO LAMININ MATRIX

Adhesion to extracellular matrix is one of the main characteristics of integrins expressed by epithelial cells. Ligation by extracellular matrix components leads to integrin clustering and engagement of cytoskeleton, which initiates an outside-in signaling via integrin-associated kinases, such as focal adhesion kinase (FAK) and the proto-oncogene tyrosine-protein kinase Src [1,2]. As discussed in **chapter 2**, such association of integrins with extracellular matrix components can provide essential pro-survival and pro-proliferative signals during early tumorigenesis [3]. Evidently, the extracellular matrix composition of the tumor environment (i.e. the presence of laminin-332 and/or -511 isoforms which are recognized by integrin α3β1) is a primary determinant of whether the adhesion of α3β1 will be essential for tumorigenesis. Tumors, which do not originate from basal cells, such as luminal HER2-enriched cancer that we investigated in **chapter 5**, are not surrounded a by laminin-containing matrix and, therefore, do not rely on the laminin-binding α3β1 for the initial survival and growth. In addition to the presence of laminin, the importance of α3β1-mediated adhesion in promoting early tumorigenesis also depends on redundancy of its function: expression of integrins other than α3β1 that can mediate cell adhesion and thus trigger pro-survival signaling. Such redundant functions have been observed in mouse epidermis, where the deletion of individual integrins had only mild (α3β1) or no detectable effects (α2β1, α9β1 and αvβ5) on the adhesion of basal keratinocyte to the ECM, whereas that of the integrin β1 subunit or the integrin α6β4 resulted in a severe blistering phenotype [11,12]. The activity of these additional integrins could explain why FAK activation is only moderately reduced in K14 *Itgα3* KO mice and why laminin-332 organization, cell adhesion and growth of 3D spheroid culture of transformed keratinocytes, expressing laminin-binding mutant $\alpha3\beta1^{G163A}$ is seemingly unperturbed (**chapter 4**). Thus, it is conceivable that α3β1-mediated adhesion to laminin-containing matrix might not play a decisive role in the initiation of any of the cancer models presented in this thesis (**chapters 3-5**). However, studies performed with the basal mammary tumors and with epithelial bladder carcinomas indicate that α3β1-mediated adhesion of tumor cells to laminin can be crucial for tumor growth and survival in other models [13,14]. In line with this, our preliminary study showed that the growth of 3D organoids from human colorectal adenoma cells strongly depend on α3β1-mediated adhesion to secreted and deposited laminin-332 (**Fig. 1a, b**). Treatment of these organoids with the function-blocking antibody J143, which prevents ligation of α3β1 by laminin [15], resulted in concentration-dependent inhibition of organoid growth over time (**Fig. 1c**), which was not observed in organoids, treated with the function-blocking antibody GoH3 directed against α6β4 [16] (**Fig. 1d**). This preliminary data indicates that initial stages of colorectal cancer could

rely on α3β1 for growth and survival. Further studies should aim to elucidate whether such dependency is a general characteristic of early stages of colorectal cancer by testing a large panel of different adenoma organoids and investigate the mechanism behind it.

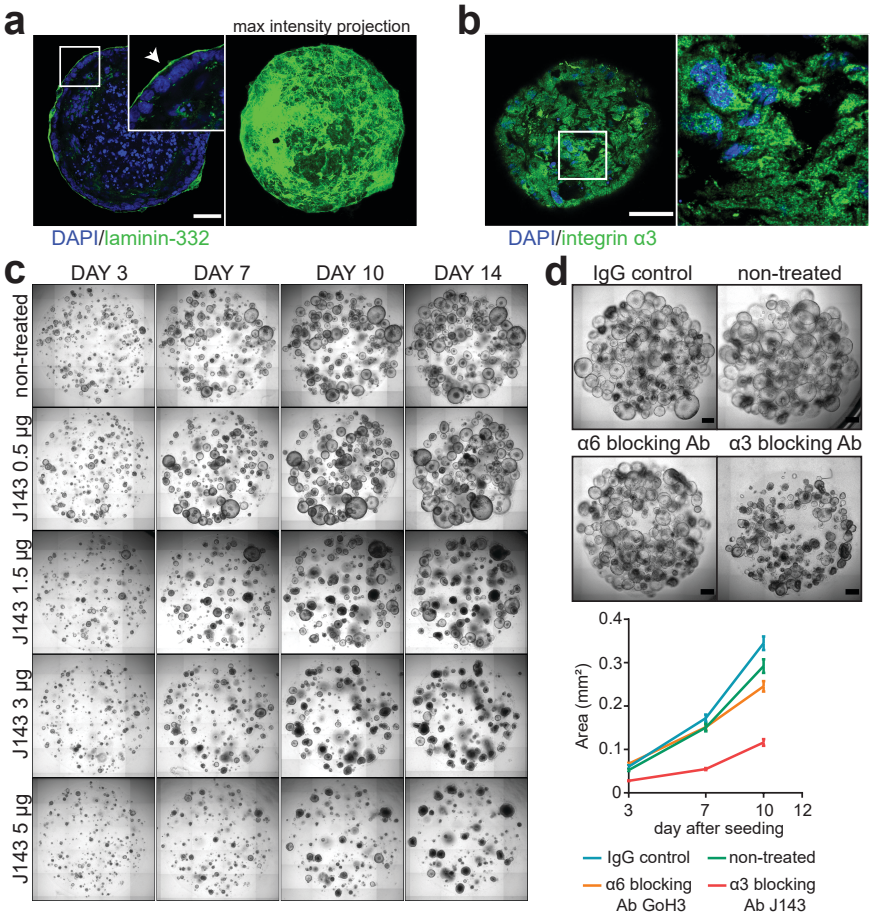


Figure 1: Integrin α3β1-mediated adhesion promotes growth of colorectal adenoma organoids. (**a-b**) Immunofluorescent staining for (**a**) laminin-332 (Ab: R14) and (**b**) integrin α3β1 (Ab: J143). Laminin-332 is deposited by basal cells (arrow) into the extracellular matrix that surrounds the organoids in 3D culture. Integrin α3β1 is expressed by basal cells. Scale bar: 40 µm. (**c**) Concentration-dependent inhibition of the growth of organoids, treated with α3β1 function-blocking antibody J143 and grown for 14 days. (d) Blocking of α3β1, but not α6β4 results in strong inhibition of organoid growth. Organoids were treated with 3 µg of control IgG, J143 or GoH3 antibodies. The area of 60-120 organoids form 3 independent experiments was quantified (mean ±SEM). Colorectal adenoma organoids were obtained and cultured from patient's resected adenoma by Magriet Lemmens, Anne Bolijn, Marianne Tijssen and Pien Delis-van Diemen (research group of dr. G. Meijer). This work was done in collaboration with dr. Beatriz Carvalho and dr. Sanne R Martens-de Kemp from the research group of dr. G. Meijer (the Netherlands Cancer Institute).

EXPANDING THE ROLE OF INTEGRIN α3β1 IN CANCER BEYOND CELL-MATRIX ADHESION

Over the last decade, the function of integrins beyond mediating cell-matrix adhesion has become more apparent, bringing new complexity and interest to the integrin field [17,18]. In **chapter 3,** we describe that in HB SCs α3β1 regulates the expression of 15 protein-coding genes during the initiation of DMBA/TPA-mediated tumorigenesis, particularly the expression of matricellular protein connective tissue growth factor (CCN2), which *in vitro* promotes clonogenic potential and 3D growth of transformed keratinocytes. We were not the first to demonstrate that α3β1 can influence cancer progression via changes in gene transcription: in transformed keratinocytes, α3β1 stimulated the expression of fibulin-2 and other genes involved in matrix remodeling and invasion [19], in basal breast carcinoma cells the expression of cyclooxygenase-2 [20] and in mammary carcinoma cells and transformed keratinocytes the expression and activity of matrix metalloproteinase MMP-9 [21–24]. Even though the exact mechanism responsible for these changes in gene expression remains to be determined, several studies demonstrated the ability of α3β1 to promote the stability of mRNA [23–25]. For a comprehensive understanding of how α3β1-regulated gene expression can influence tumor initiation and progression, it is important to note that α3β1-regulated proteins are often part of the cell secretome. The research group of DiPersio has made a notable contribution towards the recognition of α3β1 as a regulator of paracrine signaling, demonstrating that α3β1-regulated secretome can increase angiogenesis during tumor growth [20] and promotes angiogenesis and differentiation of dermal fibroblasts during wound healing [26–28]. Our findings described in **chapter 3** reaffirm the notion that the expression of α3β1 can influence cell secretome, and go a step further by suggesting that α3β1 can in such way influence tumor formation even when it is not expressed by tumor-initiating cells.

The ability of α3β1 to regulate gene expression might still dependent on its signaling function upon ligation by laminin. The fact that the application of function-blocking anti-α3β1 antibody perturbed the expression of MMP-9 in breast carcinoma cells [21] and that paracrine signaling in keratinocytes depended on α3β1-medaited FAK phosphorylation [26] supports this notion. It would be interesting to investigate whether the expression of CCN2 in our model of transformed keratinocytes also depends on association of α3β1 with laminin-containing matrix. This would not be unexpected, as the laminin-rich basement membrane underlies HB SCs. Furthermore, in 3D culture of transformed keratinocytes CCN2 typically localizes to the outer layers of spheroids, where laminin-332 is deposited. However, our observation of an increased angiogenesis in the absence of α3β1 in luminal HER2-driven tumors described in **chapter 5** suggests

that α3β1 can influence tumor stroma also independently of its ligation by laminin. That the function of α3β1 is likely not restricted to its adhesion to laminin is implied already by its localization in the epidermis: apart from the expression at the basal cell surface, α3β1 localizes also to the apical and lateral cell membranes, at the sites of cell-cell contacts. Very little is known about the role of integrins at cell-cell contacts; when associated with CD151, α3β1 is known to form a complex with E-cadherin (23), which can mediate TGFβ signaling [30]. In **chapter 4** we build on this knowledge and demonstrate that when stable cell-cell contacts are intact, i.e. when CD151 is present and E-cadherin function is unperturbed, α3β1 can promote pro-tumorigenic Stat3 and Akt signaling, therefore driving the 3D growth of transformed keratinocytes even without its ligation by laminin. This provides a new insight into the signaling function of α3β1 that is dependent on cell-cell contact integrity rather than the presence of extracellular matrix and as such can be relevant for the survival, growth and collective invasion of tumor cells that are not in contact with laminin.

Even when expressed at the basal cell surface, α3β1 can influence progression of cancer independently of its adhesion to laminin; in **chapter 5** we demonstrate that non-ligated α3β1 suppresses the invasive potential of luminal HER2-driven carcinoma cells. Such laminin-independent role of α3β1 was possible due to the ability of α3β1 to influence the clustering of collagen-binding integrin α2β1, a known suppressor of HER2-driven carcinogenesis [31], which lead to reduced accumulative surface of focal adhesions, reduced cell adhesion to collagen I matrix and increased passive invasion. Therefore, apart from its role in cell-cell contacts, α3β1 can have laminin-independent functions also through the crosstalk, i.e. cross-suppression and transdominant inhibition of other integrins. The notion that integrins in adhesion clusters can assert dominance over each other has been suggested before [26,32,33] and adds another level of complexity to roles of integrins in pathological conditions.

MECHANISMS UNDERLYING THE TUMOR-PROMOTING ROLE OF INTEGRIN α3β1 AND TETRASPANIN CD151 IN DMBA/TPA-INDUCED SKIN CARCINOGENESIS

Integrin α3β1 as a mediator of pro-tumorigenic signaling

Previous studies from our group showed that while the deletion of *Itga3* in mouse epidermis results in a strong reduction of tumorigenesis, knockout of *Cd151* (*Cd151* KO mice) has a relatively moderate effect on the formation of tumors in two stage skin carcinogenesis model [34,35]. These findings indicate that the function of α3β1, but not that of CD151 is critical for the initiation stage of DMBA/TPA-driven tumorigenesis. Similarly, we found that the growth of *Hras1* transformed keratinocytes into spheroids

depends on α3β1 but not on CD151 (**chapter 4**). Integrin α3β1 promotes the spheroid growth of these cells through its ability to activate and/or support the activation of FAK/Src, PI3K/Akt and Stat3 signaling. In line with these findings, both FAK and Stat3 have been shown to play a critical role in the initiation of DMBA/TPA-driven tumorigenesis [36,37]. Whether Akt is also needed for the formation of papillomas is not clear from the existing *in vivo* studies [38]. However, the fact that papillomas have high levels of phosphorylated Akt [39] and that active Akt is indispensable for the 3D growth of transformed keratinocytes (**chapter 4**) strongly suggest that Akt activation is needed for tumor outgrowth. Taking into consideration also our observation of reduced pro-oncogenic signaling in DMBA/TPA-primed K14 *Itgα3* KO epidermis (**chapter 4**), it can be concluded that DMBA-initiated basal keratinocytes depend on α3β1 for activation of signaling that enables their clonal outgrowth into papillomas.

However, further tumor growth and the expansion of suprabasal transformed keratinocytes depends on both, α3β1 and its binding partner CD151. Despite their initial outgrowth, papillomas lacking CD151 are smaller and display decreased keratinocyte proliferation. Interestingly, in the CD151-deficient papillomas, proliferation of keratinocytes is restricted to the laminin-adherent basal cell layer, whereas in WT papillomas scattered Ki67-positive keratinocytes can be observed also in suprabasal layers [34]. These observations can be explained by our finding that the survival and expansion of suprabasal differentiating transformed keratinocytes depends on the ability of α3β1-CD151 complex to promote Stat3 and Akt signaling. Remarkably, this function can be supported by α3β1 that is not ligated by laminin (**chapter 4**). Whether these findings translate *in vivo* should be investigated by assessing the activation of FAK/Src, Stat3 and Akt in *Cd151* KO papillomas. If CD151 is not needed for α3β1-mediated FAK/Src signaling during the initiation stage of tumorigenesis, but together with α3β1 mediates tumor expansion by promoting Stat3 and Akt-mediated survival of differentiating suprabasal keratinocytes, we would expect to detect a marked reduction in Stat3 and Akt, but not FAK/Src phosphorylation in the absence of CD151.

The role of α3β1 in maintenance of tumor-initiating cell population

The outgrowth of papillomas from DMBA-initiated keratinocytes depends on the ability of these cells to persist long enough in the epidermis during the initiation stage of chemically induced skin carcinogenesis to acquire the necessary genetic alterations. Slow-cycling basal keratinocytes have long been recognized as the tumor-initiating cells [40]. Even though some of the previous studies, including a study by our group, indicated that slow-cycling HB SCs represent such reservoir of tumor-initiating cells [35,41,42], our investigations described in **chapter 3** contests this hypothesis. Instead, several epidermal stem cell populations, including those in the interfollicular epidermis

and isthmus in hair follicles likely exert this function [43]. The genetic deletion of *Itga3* causes an increased epidermal turnover and loss of slow-cycling basal keratinocytes, which is further increased upon TPA-treatment. Thus, the tumor-promoting role of α3β1 has been previously attributed to its ability to retain HB SCs in their niche and protect them from premature loss through terminal differentiation [35]. Putting aside the assumption that HB SCs crucially contribute to the formation of papillomas, the loss of slow-cycling tumor-initiating cells in hair follicles and interfollicular epidermis remains the mechanism underlying the absence of tumor formation upon the deletion of α3β1. This is likely caused by a combination of a reduced adhesion strength of *Itga3* KO basal keratinocytes, reflected in reduced FAK activation (4,28, **chapter 4**) and an increased rate of terminal differentiation, promoted by decreased Akt and Stat3 activation in α3β1-depleted suprabasal keratinocytes (**chapters 3 and 4**).

In contrast, no clear defects could be observed in CD151-depleted epidermis and epidermal turnover was only slightly increased upon TPA-treatment (i.e. to a lesser extend compared to K14 *Itga3* KO mice) [44]. Similarly, the loss of slow-cycling basal keratinocytes was more prominent upon the deletion of *Itga3,* compared to that of *Cd151* [34,35]. Therefore, contrary to the loss of α3β1, tumor-initiating cells lacking CD151 persist for longer periods of time in their niches, which results in only moderate decrease in tumor formation upon DMBA/TPA-treatment [34]. It is thus likely that the TPA-driven increase in epidermal turnover of *Cd151* KO mice depends exclusively on deregulated differentiation of the TPA-induced hyperplastic skin. This is in line with our findings that CD151 together with α3β1 promotes Akt and Stat3 activation and regulates the survival of differentiating keratinocytes (**chapter 4**). Inspection of short-term DMBA/TPA-treated *Cd151* KO epidermis for differentiation markers, active Akt and nuclear Stat3 could provide a firmer ground for this hypothesis.

Integrin α3β1 as a promoter of pro-tumorigenic cell environment

In addition to its crucial role in promoting pro-tumorigenic signaling in tumor-initiating cells and retaining them in their niches, α3β1 affects tumor formation and tumor growth also when expressed by keratinocytes that do not (significantly) contribute to the tumor cell mass, such as HB SCs (K19 *Itga3* KO and WT mice) (**chapter 3**). We were not the first to report that gene modification of HB SCs can affect two-stage carcinogenesis model, although other studies attributed this effect to the now disputed role of HB SCs as tumor-initiating population [41,42]. During the submission of our manuscript, a research paper was published by the group of Valentina Greco, in which they describe that HB SCs show tolerance to *Hras* mutation, as well as that they affect neighboring epithelial and stromal cells [45]. As far as we are aware, this is the first study (apart from ours) that demonstrates that transformed keratinocytes in HBs can

directly affect their environment. We were not able to elucidate whether α3β1 in HB SCs affects tumor formation through changes in neighboring epithelial cells or stromal cells, such as fibroblasts, endothelial cells or immune cells, which are known to play an important part in development and progression of DMBA/TPA-initiated tumors [46,47]. Immunohistochemical staining for markers of macrophages, T-cells and myofibroblasts showed no obvious differences between their expression or localization in the skin of short-term DMBA/TPA-treated K19 *Itgα3* KO and WT skin (data not shown), however, a more detailed study of several markers using flow cytometry would be needed to properly asses this.

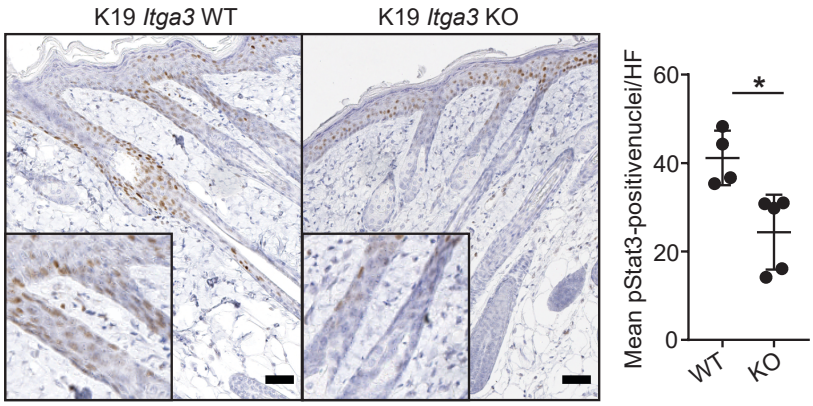


Figure 2: Reduction of nuclear phosphorylated Stat3 in the hair follicles of K19 *Itgα3* KO mice. IHC images stained for pStat3 (Y705, Ab D3A7 Cell Signaling) were quantified manually. Each dot on the graph represents a mouse and is an average of quantification of 30-50 hair follicles, longitudinally cut and in anagen hair cycle stage. (mean ± SD, student t test, $p<0.05$). Scale bars: 50 μm.

In **chapter 3** we provide evidence that suggests that α3β1 might affect the tumor environment through the secretion of the matricellular protein CCN2. We show that the tumorigenic potential of transformed keratinocytes can be enhanced by extracellular CCN2 *in vitro* when α3β1 is present. However, we were not able to determine the mechanism behind this process. Several options seem likely: Firstly, because CCN2 is known to bind different cell surface receptors, such as receptor tyrosine kinases and integrins, it may impact the tumorigenic potential of cells by altering the function of these receptors. Furthermore, CCN2 can directly bind cytokines, some of which are known to play a crucial role during two stage carcinogenesis (e.g. TGFβ), and thus regulates their availability and activity [48]. How α3β1 regulates CCN2 expression remains to be investigated. α3β1 regulates activation of several known promoters of CCN2 expression, such as FAK, Smad2/3 and Stat3 [48]. Since we observed IL6-mediated upregulation of CCN2 in 2D culture of transformed keratinocytes, the possibility that α3β1 promotes CCN2 expression via Stat3 activation is particularly attractive (**chapter 3**). This hypothesis

is additionally supported by the finding that deletion of Stat3 in HB SCs strongly reduced tumorigenesis [41] and our observations of reduced active Stat3 in the hair follicles of K19 *Itgα3* KO mice (**Fig. 2**). However, α3β1 also promotes CCN2 expression in the absence of IL6 stimulation, which in 2D culture of transformed keratinocytes is needed for Stat3 activation (data not shown). Therefore, it is likely that α3β1-mediated expression of CCN2 is not exclusively regulated by α3β1-mediated activation of Stat3.

Despite a strong reduction of tumorigenesis, K14 *Itgα3* KO mice still developed some sporadic, small papillomas of 3 mm in diameter or less [35]. Since the tumor-promoting role of α3β1 is well established and we know that α3β1 is not absent from all keratinocytes in K14 *Itgα3* KO mice (**chapter 3**), it seems likely that these papillomas originated from the cells that have escaped Cre-mediated recombination. During the studies described in this thesis, we mostly worked with short-term DMBA/TPA treated skin of K14 *Itgα3* KO mice and thus did not verify this hypothesis. However, provided it withstands scrutiny, it would offer an additional substantial evidence for the role of α3β1 in the promotion of pro-tumorigenic environment, as it would confirm that α3β1-expressing tumors still depend on the expression of α3β1 by their neighboring keratinocytes in order to grow efficiently.

Role of integrin α3β1 in human non-melanoma skin cancer

Whether our findings in mice would hold true in human patients should be an interesting next step for future research endeavors. Our preliminary data on the expression of α3β1 in biopsies of different stages of the disease looks promising (**Fig. 3**). For the presented pilot experiment, we collected biopsies of five benign non-melanoma lesions (keratoacanthoma and Bowen's disease) and five progressed tumors (invasive squamous cell carcinomas) with matching normal skin and assessed them for the expression of α3β1. 60% of the squamous carcinomas displayed lower levels of α3β1 compared to normal skin. This is in line with findings of the two-stage carcinogenesis model, in which α3β1 integrin is crucial for the initial stages of tumorigenesis as discussed above, but suppresses progression of tumors towards invasive phenotype [35]. The analysis of the expression of α3β1 in biopsies of Bowen's disease also nicely correspond with the role that α3β1 has in early skincarcinogenesis: out of four analyzed samples, 80% had higher α3β1 expression compared to normal skin. Similarly, the only sample of keratoacanthoma that we obtained revealed a much higher level of expression of α3β1 compared to normal skin. Of course, larger sample groups are needed to properly assess the expression levels of α3β1 at different stages of non-melanoma skin cancer in patients. Furthermore, inspecting the benign tumor samples for activation of Akt, Stat3 or FAK, and the expression of CCN2, which we all found to be controlled by α3β1 during two-stage skin carcinogenesis in mice should be of high interest.

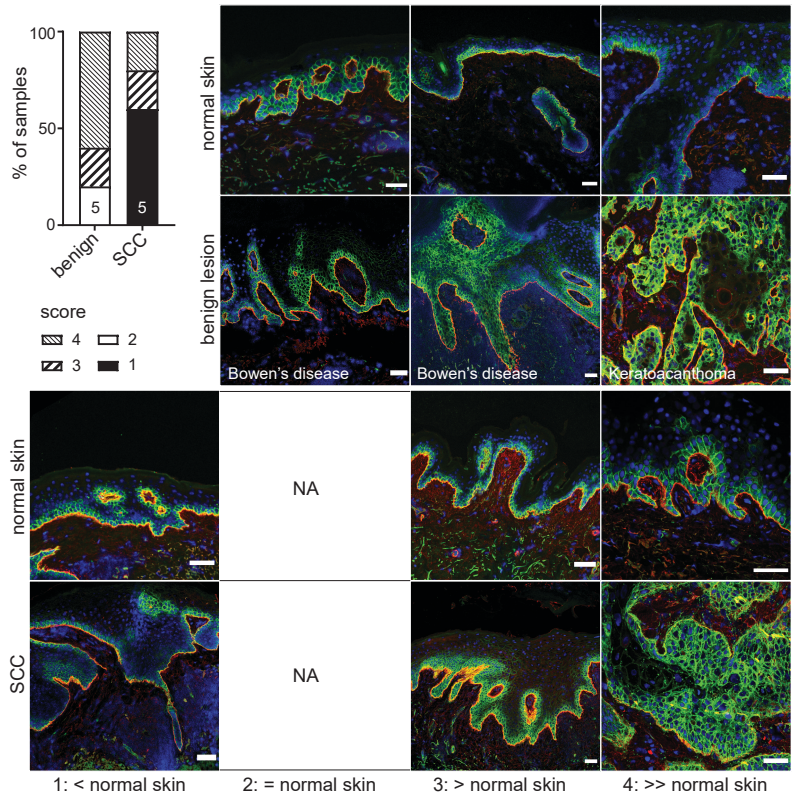


Figure 3: An increased expression of α3β1 in benign, but not progressed human skin lesions. Biopsies of five patients with benign Bowen's disease and keratoacanthoma and five patients with invasive squamous cell carcinomas (SCC) with matching normal skin were stained for integrin α3β1 (Ab: J143) and laminin-332 (Ab: R14) and the surface of α3β1 staining in lesional skin was scored based on its relative expression compared to normal skin (1: less than normal skin, 2: comparable to normal skin, 3: more than normal skin, 4: much more than normal skin). Scale bars: 50 μm.

CONCLUSIONS

The work presented in this thesis focuses on the role of α3β1 in HER2-driven breast carcinogenesis and non-melanoma skin cancer. Presented studies showed that integrin α3β1 can take on diverse roles, depending on the type and stage of cancer. We show that α3β1 can regulate pro-tumorigenic signaling pathways and promote formation of pro-tumorigenic tumor environment during the initiation phase of two-stage skin carcinogenesis, and can promote clustering and adhesion strength of other integrins to suppress invasive potential of HER2-overexpressing mammary carcinoma cells. We hope that demonstrating such complexity will prevent an oversimplistic and generalistic view on the function of integrins in cancer, which is essential for their applicational potential in the treatment of this disease.

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APPENDICES

SUMMARY

NEDERLANDSE SAMENVATTING

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SUMMARY

Cells in epithelial tissues, such as skin, are tightly bound to each other and to the underlying extracellular matrix. A family of proteins that plays a crucial role in this process is integrins, transmembrane proteins that connect extracellular environment (they are ligated by the extracellular matrix proteins) with the intracellular cytoskeleton (e.g. actin). As such they play a crucial role in maintenance of the integrity of epithelial tissues. However, the roles of integrins expand beyond a simple adhesion – they can translate mechanical signals into biochemical, and thus modulate mechanisms driving numerous aspects of the cell behavior, ranging from cell migration and spreading to cell survival and proliferation. Considering this, it is not unexpected that integrins play a role in development and progression of numerous types of cancers.

Our group has previously demonstrated the prominent role of integrin α3β1, which is ligated by extracellular matrix proteins laminins-332 and -551, in development of non-melanoma skin tumorigenesis in mice, exposed to pro-tumorigenic DMBA/TPA treatment. Interestingly, whereas α3β1 was crucial for the development of benign skin tumors, its expression inhibited tumor progression in later stages of skin carcinogenesis. Such diverse and even opposing roles of α3β1 have been reported before, indicating that there is a need for a better understanding of the function of this integrin in well-defined histopathological types and stages of cancer. In this thesis we uncover the mechanisms behind the role of integrin α3β1 during the initiation of non-melanoma skin tumorigenesis and explore its role in HER2-driven breast carcinogenesis.

In **chapter 1** we briefly introduce the topic and the scope of the thesis. We focus on the non-melanoma skin and HER2-driven breast cancer and highlight the available knowledge on the role of α3β1 in both cancer types.

In **chapter 2** we summarize the main findings on the roles that laminin-binding integrins, especially α3β1 can have in cancer. We dive into the diversity of the functions of α3β1 in different stages and types of the disease and provide insight into the underlying mechanism and the role of the cell environment the mechanisms and the cell environment that defines them.

Chapters 3 and **4** are focused on the mechanisms behind the tumor-promoting role of α3β1 in the DMBA/TPA-mediated non-melanoma skin tumorigenesis. In **chapter 3** we start by building on the hypothesis, formed by the previous members of our group, which suggests that the absence of the formation of skin tumors in mice, lacking α3β1 in epidermis is due to the changes in the behavior of the main hair follicle stem cell

population – hair bulge stem cells (HB SCs). They suggested that HB SCs represent the main population of the tumor cells-of-origin and that that they egress from their niche in the absence of α3β1. This leads to their loss due to the terminal squamous differentiation before they could outgrow as tumors. We test this hypothesis by generating mice with HB SC-specific deletion of α3β1 and by tracking α3β1-deficient HB SCs over time. Our findings reject the original hypothesis: HB SCs remain confined within their niche regardless and hardly contribute to the tumor mass regardless of the α3β1 expression. However, a deletion of α3β1 in HB SCs nonetheless reduces the number of tumors in mice, suggesting that the contribution of HB SCs to tumorigenesis is indirect, via the promotion of a tumor permissive environment. We uncover that α3β1 can modulate the expression of connective tissue growth factor (CCN2) in HB SCs and propose that the secretion of CCN2 promotes tumorigenic potential and tumor growth of transformed keratinocytes.

As the deletion of α3β1 in HB SCs resulted in only moderate reduction of tumorigenesis, we re-examined the mechanisms behind the essential role of epidermal α3β1 in DMBA/TPA-driven tumorigenesis in **chapter 4.** We uncovered that during the initiation of tumorigenesis, α3β1 plays a central role in promoting the activation FAK/Src, Akt and Stat3 – all members of signaling pathways that are crucial for tumor formation in skin. Furthermore, α3β1 together with its binding tetraspanin CD151 regulates signaling molecules that control the survival of differentiating keratinocytes.

In **chapter 5** we investigate whether α3β1 plays a role also in HER2-driven breast cancer. We uncover that the downregulation of α3β1 in HER2-driven mouse model and in HER2-enriched human mammary carcinoma cells promotes tumor progression and invasiveness of the cells. We show that the role of α3β1 in cell invasion depends on the environmental factors, especially extracellular matrix composition and the interstitial fluid flow conditions. By comparing the role of α3β1 in HER2-positive and HER2-negative human mammary carcinoma cells we reaffirm that the role of α3β1 is cancer-type dependent.

In **chapter 6** we discuss the remaining questions, address the potential future research and provide future perspectives through the preliminary data based on human skin biopsies and human colorectal organoids.

In summary, this thesis highlights that the role of α3β1 in cancer depends on time and place: the nature of the cell environment (such as extracellular matrix composition), type of cancer and its driving mechanism, as well as the stage of the disease. We provide a new insight into the mechanisms behind the role of α3β1 in HER2-driven breast cancer and in DMBA/TPA-induced non-melanoma skin tumorigenesis.

SAMENVATTING

Cellen in epitheelweefsels, zoals de huid, hechten zich aan elkaar en aan de onderliggende extracellulaire matrix. Een familie van transmembraan eiwitten die een belangrijke rol spelen bij de hechting van cellen aan de extracellulaire matrix en de verankering van het cytoskelet (b.v. actine) aan de plasmamembraan. Het vermogen van deze eiwitten om de extracellulaire omgeving te verbinden met de binnenkant van de cel is van groot belang voor het behoud van de structurele integriteit van epitheelweefsels. Daarnaast zijn integrinen ook betrokken bij het omzetten van mechanische belasting in biochemische signalen en kunnen zij op deze manier mechanismen aandrijven die het gedrag van cellen beïnvloeden, waaronder het vermogen van cellen om te migreren, spreiden, overleven en prolifereren. Het komt dan ook niet als een verrassing dat integrinen een belangrijke rol spelen in de ontwikkeling en progressie van een groot aantal soorten kankers.

Onze onderzoeksgroep heeft eerder aangetoond dat de integrine α3β1, die bindt aan de extracellulaire eiwitten laminine-332 en -511, van essentieel belang is voor de ontwikkeling van niet-melanoom huidkanker in muizen die zijn blootgesteld aan behandeling met DMBA/TPA. Hoewel α3β1 nodig is voor de ontwikkeling van goedaardige huidtumoren, remt het integrine in een latere fase van de ziekte de progressie van tumoren. De tegengestelde functie van α3β1 in tumoren is eerder beschreven maar nooit systematisch onderzocht. Daarom hebben wij besloten om de functie van dit integrine nader te onderzoeken in twee soorten kanker die histopathologisch goed zijn gedefinieerd: niet-melanoom huidkanker en HER2-positieve borstkanker. In dit proefschrift beschrijven wij onze bevindingen betreffende het mechanisme dat ten grondslag ligt aan de essentiële rol die α3β1 speelt bij de initiatie van niet-melanoom huidkanker en de functie van dit integrine in HER-positieve borstkanker.

In **hoofdstuk 1** wordt de rol van α3β1 in beide soorten kanker kort besproken

In **hoofdstuk 2** worden de voornaamste bevindingen van diverse onderzoeken naar de effecten van laminine-bindende integrinen, in het bijzonder α3β1, in kankercellen samengevat. Wij bespreken de functie van α3β1 in verschillende stadia van en soorten kanker en geven inzicht in de mechanismen die hieraan ten grondslag liggen, alsmede de invloed die de omgeving heeft op de vorming en progressie van tumoren.

In de **hoofdstukken 3** en **4** hebben wij onderzoek gedaan naar het mechanisme hoe α3β1 de vorming van DMBA/TPA-geïnduceerde non-melanoom huidtumoren induceert. Het onderzoek dat is beschreven in **hoofdstuk 3** bouwt voort op eerdere bevindingen

die aantonen dat er geen huidtumoren worden gevormd in muizen waarin het integrine α3β1 in de huid is verwijderd. Er is gepostuleerd dat huidtumoren ontstaan uit stamcellen in de haarzakjes ("cells-of-origin") en dat de stamcellen met tumor initiërende capaciteit voortijdig hun niche verlaten als gevolg van een verminderde adhesie aan de extracellulaire matrix en vervolgens verloren gaan doordat ze terminaal differentiëren. Wij hebben deze hypothese getest door α3β1 specifiek te verwijderen in de haarstamcellen van muizen en deze cellen in de tijd te volgen. Onze bevindingen tonen aan dat, ongeacht de afwezigheid van α3β1, de stamcellen aanwezig blijven in de haarzakjes en nauwelijks bijdragen aan de tumormassa. Daarmee kunnen wij de oorspronkelijke hypothese verwerpen. Desondanks ontwikkelen de muizen zonder α3β1 minder tumoren. Dit wijst erop dat de stamcellen in de haarzakjes indirect bijdragen aan de vorming van tumoren, namelijk door het creëren van een tumor-permissieve omgeving. Wij hebben ontdekt dat α3β1 de expressie van "connective tissue growth factor" (CCN2) in de haarstamcellen kan moduleren en veronderstellen dat door het uitscheiden van CCN2 de stamcellen de groei van getransformeerde huidcellen kunnen stimuleren. Omdat de deletie van α3β1 in de stamcellen van de haarzakjes slechts een gering effect heeft op de vorming van tumoren, hebben wij het mechanisme achter de essentiële rol van α3β1 voor de initiatie van DMA/TPA geïnduceerde huidtumoren verder onderzocht in **hoofdstuk 4**. Wij hebben ontdekt dat α3β1 van groot belang is voor het activeren van FAK/Src, Akt en Stat3, eiwitten die schakels zijn in het doorgeven van signalen van buiten de cel naar de kern en die cruciaal zijn voor de vorming van tumoren in de huid. Tevens hebben wij gevonden dat α3β1, tezamen met zijn vaste partner tetraspanine CD151, de activiteit van signaaleiwitten reguleert die het overleven van gedifferentieerde keratinocyten controleren.

In **hoofdstuk 5** hebben wij onderzoek gedaan naar de rol van α3β1 in een muizenmodel voor HER2-positieve borstkanker. We laten zien dat door het verlagen van de expressie van α3β1 in dit muizenmodel alsmede in HER2-positive humane borstkankercellen, de tumorprogressie en het invasieve gedrag van deze cellen wordt vergroot. Tevens laten wij zien dat de rol van α3β1 voor de invasie van cellen afhankelijk is van omgevingsfactoren, met name de samenstelling van de extracellulaire matrix en de interstitiële vloeistofstroom. Onderzoek naar de rol van α3β1 bij een groot aantal HER2-positive en -negatieve tumoren bevestigen deze afhankelijk is van het tumortype.

In **hoofdstuk 6** bediscussiëren wij de algemene bevindingen van dit proefschrift, bespreken wij de overgebleven vragen en worden aan de hand van voorlopige bevindingen met menselijke huidbiopten en colorectale organoïden vervolgstappen en perspectieven voor toekomstig onderzoek gegeven.

Samengevat, in dit proefschrift wordt aangetoond dat de rol van α3β1 in kanker afhankelijk is van de tijd en plaats: de aard van cellulaire omgeving (zoals de samenstelling van de extracellulaire matrix), het soort kanker en de mechanismen die de transformatie vaan een normale cel tot een kankercel veroorzaken alsmede het stadium van de ziekte. We verschaffen nieuwe inzichten in het mechanisme dat ten grondslag ligt aan de rol van α3β1 in HER2-positieve borstkanker en DMBA/TPA-geïnduceerde non-melanoom huidkanker.

CURRICULUM VITAE

Veronika Ramovš was born on 30th of May 1988 in Ljubljana, Slovenia. She completed her secondary education at the high school *Gimnazija Bežigrad* in 2007 and continued with *BSc Biology* study at *Biotechnical Faculty* at the *University of Ljubljana*. During her BSc study she worked on project *Life+: Life at night*, aiming to improve the conservation status of nocturnal animals by reducing the effect of artificial lighting at cultural heritage sites and she completed a voluntary internship at a *Department of Molecular and Biomedical Sciences* at *Jožef Stefan Institute*, where she worked on investigating the role of secretory phospholipases A2 in breast cancer. During this time, she also completed studies at the *Conservatory of music and ballet Ljubljana*. In 2011, she continued her education at the *Utrecht University,* where she completed *MSc Molecular and Cellular Life Sciences* in 2013 with *cum laude* distinction. During her MSc study she worked on hypoxia-specific nanobodies for breast cancer screening in the research group of Dr. Paul van Bergen en Henegouwen at *Utrecht University* and on the mapping of the genetic diversity and populations structure in wildlife populations of Dama dama in the research group of Prof. Dr. AR Hoelzel at *Durham University*. In 2013 she joined the research group of Prof. Dr. Arnoud Sonnenberg at the *Netherlands Cancer Institute* to work as a graduate student on a role of integrin α3β1 in cancer, resulting in this thesis. Since 2020, Veronika works as a postdoctoral research fellow under the mentorship of Dr. Karine Raymond in the research group of Prof. Dr. Christine Mummery at the *Leiden University Medical Center*.

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Ramovs V, Krotenberg Garcia A, Kreft M, Sonnenberg A. Integrin α3β1 is a key regulator of several pro-tumorigenic pathways during skin carcinogenesis. J Invest Dermatol. 2020 Aug 14;S0022-202X(20)31981-3.

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