



## Deciphering the Mammary Stem Cell Niche: A Role for Laminin-Binding Integrins

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## Deciphering the Mammary Stem Cell Niche: A Role for Laminin-Binding Integrins

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### SUMMARY

Integrins, which bind laminin, a major component of the mammary basement membrane, are strongly expressed in basal stem cell-enriched populations, but their role in controlling mammary stem cell function remains unclear. We found that stem cell activity, as evaluated in transplantation and mammosphere assays, was reduced in mammary basal cells depleted of laminin receptors containing  $\alpha 3$ - and  $\alpha 6$ -integrin subunits. This was accompanied by low MDM2 levels, p53 stabilization, and diminished proliferative capacity. Importantly, disruption of p53 function restored the clonogenicity of  $\alpha 3/\alpha 6$ -integrin-depleted mammary basal stem cells, while inhibition of RHO or myosin II, leading to decreased p53 activity, rescued the mammosphere formation. These data suggest that  $\alpha 3/\alpha 6$ -integrin-mediated adhesion plays an essential role in controlling the proliferative potential of mammary basal stem/progenitor cells through myosin II-mediated regulation of p53 and indicate that laminins might be important components of the mammary stem cell niche.

### INTRODUCTION

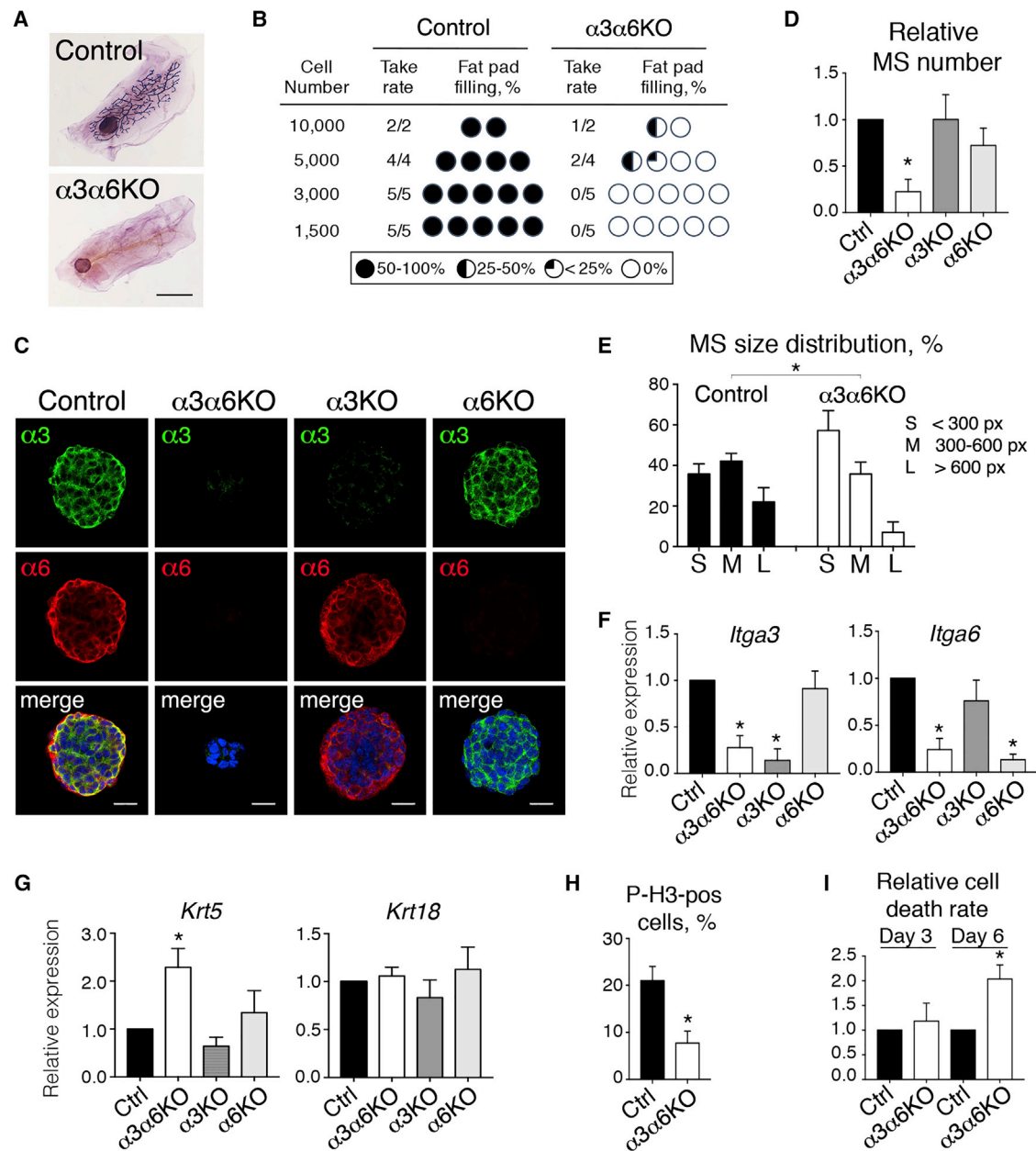
The mammary epithelium is organized as a bilayer, with a luminal layer of steroid hormone receptor-positive and -negative epithelial cell populations and an underlying layer of basal myoepithelial cells (Macias and Hinck, 2012). The extraordinary proliferative potential of the mammary epithelium, replenishing the gland during lobulo-alveolar development in successive pregnancies and repopulating the cleared mammary fat pad following the transplantation of tissue fragments or dispersed cells, strongly suggests that stem and progenitor cells are present in this tissue (Visvader and Smith, 2011). Only basal cells can regenerate bilayer mammary ducts and alveoli following the transplantation of sorted epithelial cell populations from adult mouse mammary gland (Shackleton et al., 2006; Sleeman et al., 2007; Stingl et al., 2006). These observations suggested that the mammary basal cell layer harbors multipotent stem cells able of generating various mammary cell types during lobulo-alveolar development and mature gland homeostasis. However, many lineage-tracing studies have provided evidence refuting this hypothesis and strongly indicating that, in adult glands, the luminal and the basal mammary epithelial compartments are maintained by unipotent stem cells (Davis et al., 2016; Prater et al., 2014; Van Keymeulen et al., 2011, 2017). The possible presence of functional multipotent

basal stem cells in adult glands therefore remains a matter of debate (Rios et al., 2014; van Amerongen et al., 2012).

Integrins are heterodimeric transmembrane extracellular matrix (ECM) receptors consisting of an  $\alpha$  and a  $\beta$  subunit. To date, eight  $\beta$  and 18  $\alpha$  subunits have been described. These subunits can assemble into 24 integrins with different ligand specificities (for review see Barczyk et al., 2010; Hynes, 2002). Associations between the cytoplasmic tail of the integrin and various intracellular adaptor proteins control integrin ligand-binding activity. Conversely, ligand binding triggers intracellular signaling events and extracellular force sensing increases integrin clustering, thereby amplifying biochemical cascades (Barczyk et al., 2010; DuFort et al., 2011).

Integrins have been implicated in the control of stem cell maintenance and progenitor cell differentiation in various embryonic and adult tissues (Brizzi et al., 2012; Chen et al., 2013; Glukhova and Streuli, 2013; Raymond et al., 2012). The  $\alpha 2$ -,  $\alpha 6$ -,  $\beta 1$ -, and  $\beta 3$ -integrin subunits serve as surface markers for the isolation of stem cell-enriched populations from basal and luminal mammary epithelial layers. The ECM may, therefore, be an important component of the mammary stem cell microenvironment, the stem cell niche (Asselin-Labat et al., 2007; Shackleton et al., 2006; Shehata et al., 2012; Stingl et al., 2006).

We have shown that the specific deletion of  $\beta 1$ -integrins from the basal layer affects mammary stem cell self-renewal,



**Figure 1. Deletion of  $\alpha3$ - and  $\alpha6$ -Integrin Chains from Mammary Basal Cells Affects Stem Cell Activity**

Basal ( $CD24^{LOW}/ITG\alpha6^{HIGH}$ ) mammary cells were isolated from mammary tissue as described previously (Stingl et al., 2006); a typical profile is shown in Figure S1A.

(A) Recipient mouse mammary fat pads grafted with control or  $\alpha3\alpha6$ KO mammary basal cells dissected 10 weeks after transplantation and stained with LacZ and Carmine-Alum in whole mounts. Representative images. Scale bar, 5 mm.

(B) Take rate and fat pad filling in the outgrowths developed by control and  $\alpha3\alpha6$ KO mammary basal cells in limiting dilution transplantations. Pool of three independent experiments.

(C) Confocal representative images of mammospheres formed by control (Ctrl) and integrin-depleted mammary basal cells after 12 days of culture immunolabeled with anti-integrin antibodies. Nuclei were visualized with DAPI. Scale bars, 20  $\mu$ m.

(D) Mammospheres formed by integrin-depleted cells counted after 12–14 days of culture. The graph shows means  $\pm$  SD obtained in 10, 3, and 4 independent experiments for  $\alpha3\alpha6$ KO,  $\alpha3$ KO, and  $\alpha6$ KO cells, respectively;  $p < 0.0001$  for  $\alpha3\alpha6$ KO,  $p = 0.98$  for  $\alpha3$ KO, and  $p = 0.06$  for  $\alpha6$ KO.

(E) Size distribution of mammospheres formed by control and  $\alpha3\alpha6$ KO mammary basal cells. The graph shows means  $\pm$  SD from 4 independent experiments. S, small; M, medium; L, large.  $p < 0.0001$ .

(legend continued on next page)



suggesting that  $\beta 1$ -integrins play an essential role in maintaining the mammary stem cell population (Taddei et al., 2008). Other teams reported that  $\beta 1$ -integrin deletion from luminal cells reduced epithelial outgrowth development from transplanted mammary tissue (Li et al., 2005) and implicated  $\beta 1$ -integrins in the self-renewal of progenitors in mammary organoid culture (Olabi et al., 2018). However, the roles of specific integrin dimers in the maintenance of functional mammary stem cell population(s) remain unclear.

Laminins are major constituents of the basement membrane, a specialized ECM underlying the mammary epithelial bilayer, and the laminin-binding integrin dimers  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$ , and  $\alpha 6\beta 4$  are expressed by mammary basal epithelial cells (Raymond et al., 2012). We evaluated the contribution of laminin-binding integrins to the control of mammary stem cell activity, using a loss-of-function approach in which the *Itga3* and/or *Itga6* genes were deleted from the mammary basal cells with the Cre-Lox system. We show here that laminin-binding integrins are essential for mammary stem cell function, although  $\alpha 3\beta 1$ - and  $\alpha 6$ -containing integrin dimers may have at least partially redundant functions. Mechanistically, we found that lack of  $\alpha 3\beta 1$ - and  $\alpha 6$ -integrins led to increased myosin II activity and induced p53 accumulation leading to growth arrest.

## RESULTS

### Simultaneous Deletion of the $\alpha 3$ - and $\alpha 6$ -Integrin Chains Affects Mammary Basal Stem Cell Activity

Mammary epithelial cells express on their surface several integrin receptors, including those for laminins, collagens, and fibronectin (Figure S1). To study the role of laminin-binding integrins in the control of mammary stem/progenitor cell function, we deleted the *Itga3* and/or *Itga6* genes *in vitro* by transduction of mammary basal cells freshly isolated from mice carrying the corresponding conditional alleles (*Itga3<sup>F/F</sup>* and *Itga6<sup>F/F</sup>*) with an adenovirus expressing Cre recombinase (Adeno-Cre). Basal cells were sorted as described previously (Stingl et al., 2006); a typical profile is shown in Figure S1A. We used the *Rosa26LacZ*-reporter

allele (*R26R<sup>F/+</sup>*) to monitor integrin deletion. Following Adeno-Cre transduction, stem and progenitor cell activities were assessed in control (*R26R<sup>F/+</sup>*) and integrin-depleted basal cells, in limiting dilution transplantation and mammosphere assays. Basal cells depleted of *Itga3*, *Itga6*, or both of these genes, are hereafter referred to as  $\alpha 3$ KO,  $\alpha 6$ KO, and  $\alpha 3\alpha 6$ KO, respectively.

Simultaneous deletion of the *Itga3* and *Itga6* genes greatly decreased the capacity of basal cells to regenerate mammary epithelium following their transplantation into cleared mammary fat pads (Figures 1A and 1B). Deletion of the  $\alpha 3$  chain did not affect the regenerative potential of mammary basal cells, and basal cells depleted of  $\alpha 6$  presented only a moderate decrease in capacity to repopulate the fat pad (Figures S2A and S2B).

Consistent with the transplantation assay results,  $\alpha 3\alpha 6$ KO cells exhibited poor clonogenic capacity and formed few spheres, most of which were of small size, whereas  $\alpha 3$ KO and  $\alpha 6$ KO cells formed spheres of a similar size and number to those observed in control cultures (Figures 1C–1E and S2C). At least 90% of the mammospheres were devoid of integrin surface expression as assessed by immunolabeling (Figure 1C), and qRT-PCR analyses of dissociated mammospheres confirmed integrin chain deletion (Figures 1C and 1F). The addition of collagen 1 or fibronectin to the mammosphere medium did not rescue the capacity of  $\alpha 3\alpha 6$ KO cells to form spheres (Figure S2D).

Mammospheres were characterized by analyzing the expression of differentiation markers. In line with our previous studies, we found that in the control mammospheres, part of the cells, often basally located, co-expressed keratin 5 (K5) and K8, whereas the inner cells displayed a typical K5–/K8+ luminal phenotype (Chiche et al., 2013) (Figure S2E). The  $\alpha 3\alpha 6$ KO mammospheres were enriched in K5+ cells, particularly the small mammospheres (Figure S2E). Consistently, qRT-PCR analysis showed that *Krt5* expression was significantly increased in the mammospheres formed by  $\alpha 3\alpha 6$ KO cells but not in those formed by  $\alpha 3$ KO and  $\alpha 6$ KO cells, while *Krt18* levels were unchanged (Figure 1G). These data indicate that the absence of laminin-binding integrins does not completely prevent but affects the differentiation of basal cells into the luminal

(F) qRT-PCR analysis of *Itga3* and *Itga6* gene expression in mammospheres formed by integrin-depleted cells. The graph shows means  $\pm$  SD from *n* independent experiments. For  $\alpha 3\alpha 6$ KO, *n* = 6, *p* < 0.0001 for both, *Itga3* and *Itga6* genes; for  $\alpha 3$ KO, *n* = 3, *p* = 0.007 for *Itga3* and *p* = 0.2 for *Itga6*; for  $\alpha 6$ KO, *n* = 3, *p* = 0.5 for *Itga3*, *p* = 0.002 for *Itga6*.

(G) qRT-PCR analysis of *Krt5* and *Krt18* gene expression in cells obtained from mammospheres formed by integrin-depleted and control (Ctrl) mammary basal cells. The graph shows means  $\pm$  SD from three independent experiments. For  $\alpha 3\alpha 6$ KO, *p* = 0.048 for *Krt5*.

(H) The percentage of P-H3-positive cells in mammospheres formed by control or  $\alpha 3\alpha 6$ KO cells evaluated on day 3 of culture. The graph shows means  $\pm$  SD obtained in three independent experiments, *p* = 0.005.

(I) Cell death rates in mammosphere cultures of control or  $\alpha 3\alpha 6$ KO cells assessed by CellTox Green assay on days 3 and 6 after seeding. Values shown are means  $\pm$  SD from three independent experiments. 3 days, *p* = 0.5, 6 days, *p* = 0.02.

In (D), (F), (G), and (I) values obtained for control cells were set as 1 in each experiment. See also Figures S1 and S2.



lineage. Interestingly, relative expression of *Lgr5*, a basal cell marker used to enrich for mammary stem cell activity (Fu et al., 2017) is decreased in  $\alpha3\alpha6$ KO mammospheres (Figure S2F).

To understand the cellular mechanisms leading to the weak clonogenic capacity of  $\alpha3\alpha6$ KO cells, we analyzed their proliferation rates at early stages of culture. Immunolabeling with anti-phospho (ser10)-histone H3 antibody (P-H3), which specifically labels cells in mitosis (Hendzel et al., 1997), revealed that, on day 3, the percentage of cycling cells had decreased by two-thirds in  $\alpha3\alpha6$ KO cultures (Figure 1H). Bromodeoxyuridine (BrdU) incorporation assays revealed that proliferation was decreased in both basal and luminal cells (Figure S2G). Cell death rates in  $\alpha3\alpha6$ KO cultures were similar to those in control ones at day 3, not increasing until day 6 (Figures 1I and S2H). Cleaved caspase-3 staining indicated that apoptosis contributed to the observed cell death (Figure S2I). Thus, the deletion of laminin-binding integrins from mammary basal cells prevented these cells from entering the cell cycle and increased cell death rates during subsequent culture, resulting in the production of small spheres by the surviving cells.

### p53 Is Activated in $\alpha3\alpha6$ KO Mammary Basal Epithelial Cells

Earlier studies implicated p53 in cellular responses to various stress conditions (Horn and Vousden, 2007), whereas an important role of p53 in stem cell biology has emerged in the last years (Jain and Barton, 2018). In particular, p53 has been found to act as an essential regulator of mammary stem cells by restricting their proliferation and self-renewal (Chiche et al., 2013; Cicalese et al., 2009). We therefore investigated whether the p53 pathway was activated in the mammospheres derived from basal cells depleted of laminin-binding integrins. Immunolabeling showed that most cells, basal and luminal, in small  $\alpha3\alpha6$ KO-mammospheres presented nuclear p53 (Figures 2A and S3A). Moreover, qRT-PCR analyses performed with RNA samples obtained from dissociated mammospheres revealed an upregulation of p53 transcriptional targets such as *Cdkn1a* (coding for the cell cycle regulator p21) and *Mdm2* in  $\alpha3\alpha6$ KO cells, suggesting an activation of the p53 pathway in these cells (Figure 2B). Expression of *Cdkn1a* and *Mdm2* was not changed in  $\alpha3$ KO or  $\alpha6$ KO cell (Figure 2B).

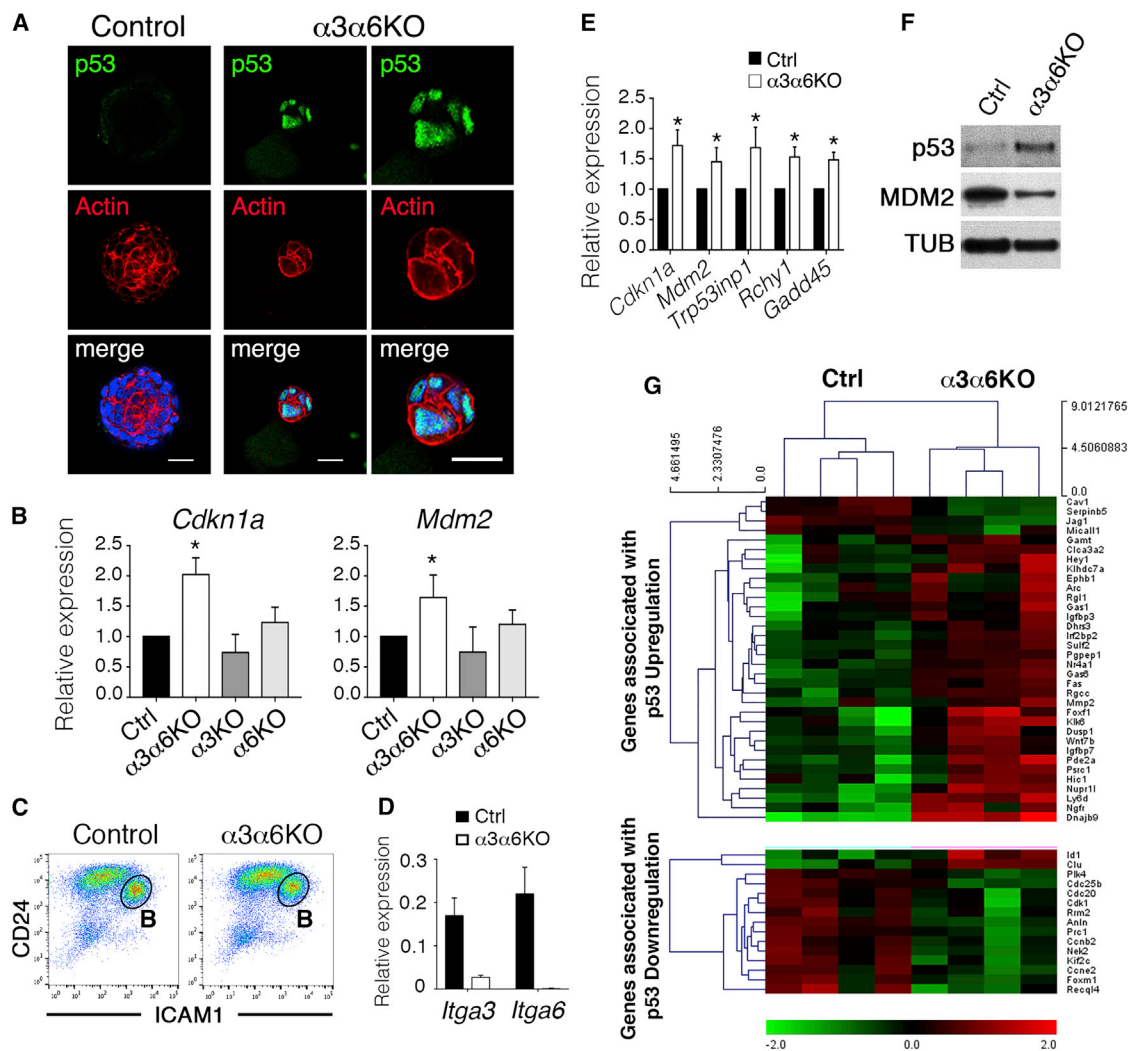
We also assessed the p53 pathway activation in cells depleted of the  $\alpha3$ - and  $\alpha6$ -integrins in explant cultures, an approach with better viable cell yields than Adeno-Cre gene deletion. Fragments of mammary ducts isolated from K5Cre-*ER*<sup>T2</sup>;*Itga3*<sup>F/F</sup>;*Itga6*<sup>F/F</sup> mice, in which Cre expression is controlled by an estrogen-responsive element, were allowed to attach to plastic, then, treated with tamoxifen to

induce Cre expression leading to integrin gene deletion. Mammary epithelial cells migrated from duct fragments onto the plastic. After several days of culture, the cells were collected, and basal populations were sorted by flow cytometry with CD24/ICAM-1 labeling, as described by Di-Cicco et al. (2015) (Figures 2C and S3B). Specific deletion of the *Itga3* and *Itga6* genes from basal cells was confirmed by qRT-PCR (Figure 2D). Consistently, immunocytofluorescence staining showed that most basal cells in the explants were devoid of  $\alpha3$ -integrin and flow cytometry analyses revealed  $\alpha6$ -integrin deletion in at least 70% of basal cells (Figures S3C and S3D). Only the  $\alpha6$ -negative-sorted basal cell population was studied further in the experiments illustrated in Figures 2E–2G. No significant changes in the surface expression of  $\alpha1$ - and  $\alpha5$ -integrins were detected;  $\alpha2$  and  $\beta4$  were moderately downregulated, whereas  $\alpha_v$  and  $\beta3$  were slightly upregulated in  $\alpha3\alpha6$ KO cells (Figure S3D). Our qRT-PCR data suggested that, as in cells from  $\alpha3\alpha6$ KO mammospheres, several p53 target genes were upregulated in freshly sorted  $\alpha3\alpha6$ KO basal cells obtained from explant cultures (Figure 2E).

Finally, we analyzed the levels of p53 and MDM2 proteins in control and  $\alpha3\alpha6$ KO basal cells by western blotting. The E3 ubiquitin-protein ligase MDM2 plays a central role in the control of p53 stability, and its binding to p53 favors the proteosomal degradation of this protein (Haupt et al., 1997; Kubbutat et al., 1997). We found that p53 protein levels were notably increased in  $\alpha3\alpha6$ KO cells in explant culture (Figure 2F). By contrast, MDM2 protein levels were decreased, providing conditions for p53 stabilization and nuclear accumulation (Haupt et al., 1997; Kubbutat et al., 1997). *Mdm2* gene is positively regulated by p53 at the transcriptional level; therefore, increased p53 activity is expected to be accompanied by higher *Mdm2* transcript levels. However, additionally, MDM2 protein expression is regulated by posttranscriptional mechanisms, including control of protein stability. Previous studies have shown that, p53 activation in stress response occurs due to MDM2 degradation (Carr and Jones, 2016).

RNA sequencing (RNA-seq) analysis on freshly sorted  $\alpha3\alpha6$ KO mammary basal cells confirmed that the p53 pathway was activated and revealed the perturbation of several other pathways including those associated with G2/M transition, mitosis, Rho GTP-ases, collagen and keratan sulfate biosynthesis, and cell surface integrin interactions (Figure 2G; Table S1). Notably, we observed altered expression of integrin subunits from dimers interacting with ECM proteins other than laminin (Figure S3E). Consistent with the flow cytometry data, RNA-seq analysis revealed that *Itga2* expression was downregulated, whereas, *Itga11* (encoding a collagen receptor subunit), *Itga9*, and *Itgb5* genes (encoding subunits of RGD-binding integrins, i.e., receptors for ECM components, such as





**Figure 2. The p53 Pathway Is Activated in Mammary Basal Cells Depleted of  $\alpha3$ - and  $\alpha6$ -Integrin Chains**

(A) Confocal representative images of mammospheres formed by control and  $\alpha3\alpha6$ KO mammary basal cells after 12 days of culture immunolabeled with anti-p53 antibody. Phalloidin served to visualize F-actin, nuclei were labeled with DAPI. Scale bar, 20  $\mu$ m.

(B) qRT-PCR analysis of *Cdkn1a* and *Mdm2* gene expression in cells obtained from mammospheres formed by integrin-depleted and control (Ctrl) mammary basal cells. The graph shows means  $\pm$  SD from n independent experiments. For  $\alpha3\alpha6$ KO, n = 6, p = 0.0003 for *Cdkn1a* and p = 0.008 for *Mdm2*; for  $\alpha3$ KO, n = 3, p = 0.3 for *Cdkn1a* and p = 0.4 for *Mdm2*; for  $\alpha6$ KO, n = 3, p = 0.25 for *Cdkn1a* and p = 0.27 for *Mdm2*.

(C) Dot plot showing flow cytometry separation of basal and luminal cells from mammary explants from *Itga3<sup>F/F</sup>;Itga6<sup>F/F</sup>;Rosa26<sup>LacZ</sup>* (control) or *K5-Cre<sup>ERT2</sup>+/+;Itga3<sup>F/F</sup>;Itga6<sup>F/F</sup>;Rosa26<sup>LacZ</sup>* mice, treated with 4-OHT (4-hydroxytamoxifen). B, basal cell population.

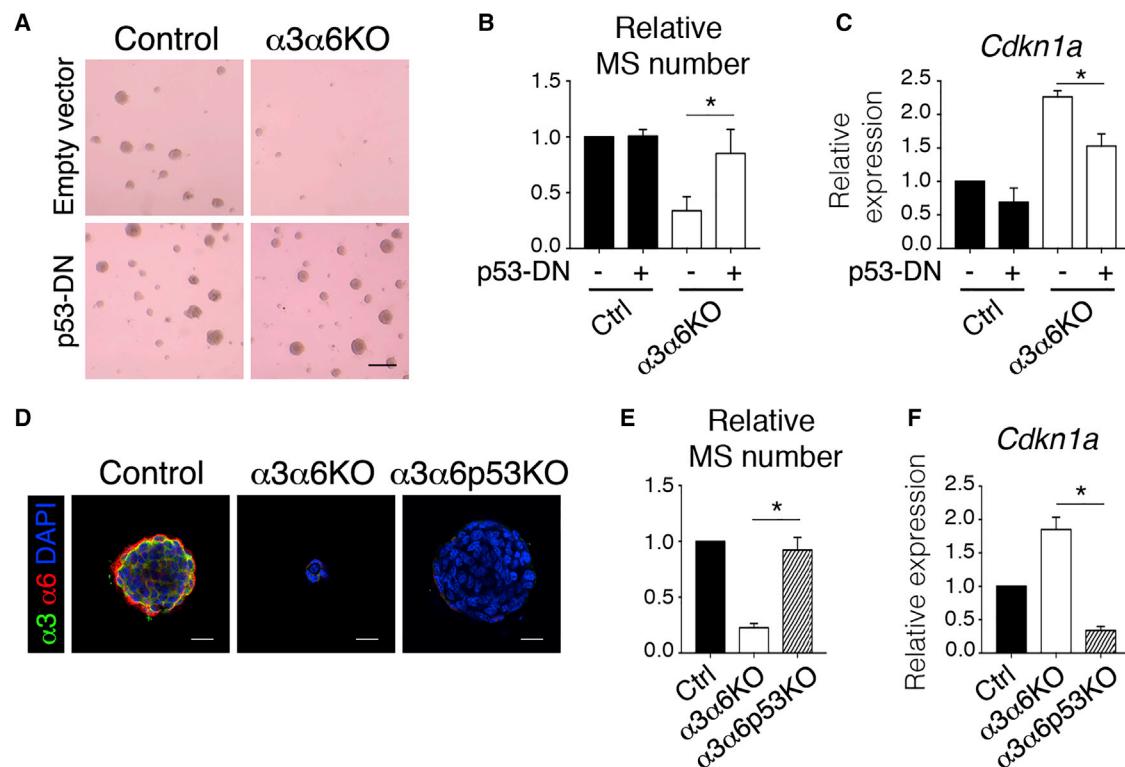
(D) qRT-PCR analysis of *Itga3* and *Itga6* gene expression in  $\alpha3\alpha6$ KO basal cells isolated from mammary explants (n = 3). p = 0.0038 for *Itga3* and p = 0.0036 for *Itga6*.

(E) qRT-PCR analysis of p53 target gene expression in  $\alpha3\alpha6$ KO basal cells isolated from mammary explants. The graph shows means  $\pm$  SD from three independent experiments. p = 0.008 for *Cdkn1a*; p = 0.027 for *Mdm2*; p = 0.025 for *Trp53inp1*; p = 0.005 for *Rchy1*; p = 0.003 for *Gadd45*.

(F) Western blotting analysis of p53 and MDM2 protein levels in extracts of control and  $\alpha3\alpha6$ KO mammary basal cells isolated from 4-OHT-treated explants. Tubulin served as a loading control.

(G) Heatmap based on RNA-seq analysis of freshly sorted control and  $\alpha3\alpha6$ KO basal cells isolated from mammary explants.

In (B) and (E), values obtained for control cells were set as 1 in each experiment. See also Figure S3 and Table S1.



**Figure 3. Inhibition of p53 Rescues Clonogenic Capacity of Mammary Basal Cells Depleted of  $\alpha 3$ - and  $\alpha 6$ -Integrins**

(A) Microphotographs of mammospheres formed by control or  $\alpha 3 \alpha 6$ KO mammary basal cells expressing p53-dominant-negative (p53-DN) mutant lentivirus. Representative images. Scale bar, 200  $\mu$ m.

(B) Quantification of mammosphere formation by mammary basal cells expressing p53-DN. Values shown represent means  $\pm$  SD from three independent experiments,  $p = 0.03$ .

(C) qRT-PCR analysis of *Cdkn1a* gene expression in mammospheres formed by  $\alpha 3 \alpha 6$ KO mammary basal cells. The graph shows means  $\pm$  SD from three independent experiments,  $p = 0.008$ .

(D) Confocal representative images of mammospheres formed by  $\alpha 3 \alpha 6$ KO and  $\alpha 3 \alpha 6$ p53KO cells double immunolabeled with anti- $\alpha 3$  and anti- $\alpha 6$  antibodies. Scale bars, 30  $\mu$ m.

(E) Quantification of mammosphere formation by  $\alpha 3 \alpha 6$ KO and  $\alpha 3 \alpha 6$ p53KO mammary basal cells. Values shown represent means  $\pm$  SD from three independent experiments,  $p = 0.005$ .

(F) qRT-PCR analysis of *Cdkn1a* gene expression in mammospheres formed by  $\alpha 3 \alpha 6$ KO and  $\alpha 3 \alpha 6$ p53KO mammary basal cells. The graph shows means  $\pm$  SD from four independent experiments,  $p = 0.0002$ .

In (B), (C), (E), and (F) values obtained for control cells were set as 1 in each experiment. See also Figure S4.

tenascin-C or osteopontin), were upregulated. In addition, *Arhgap11a* and *Racgap1*, coding for two RhoGAPs inactivating, respectively, Rho and Rac, were downregulated in integrin-depleted cells (Figure S3E).

These data strongly indicate that alteration of cell-ECM interactions in mammary basal cells depleted of laminin-binding integrins triggers p53 activation, preventing their entry into the cell cycle and leading to a lack of clonogenic capacity.

#### Suppression of p53 Function Restores Clonogenic Potential in $\alpha 3 \alpha 6$ KO Mammary Basal Cells

We investigated whether p53 activation was responsible for the low clonogenic potential of  $\alpha 3 \alpha 6$ KO cells, by intro-

ducing a dominant-negative p53 mutant (p53-DN) into sorted mammary basal cells carrying conditional alleles for *Itga3* and *Itga6* genes and then treating the cells with Adeno-Cre virus to delete integrin genes. The expression of p53-DN had no significant effect on control cultures, but restored the capacity of  $\alpha 3 \alpha 6$ KO basal cells to form mammospheres (Figures 3A and 3B). Consistently, expression of *Cdkn1a* was diminished in  $\alpha 3 \alpha 6$ KO spheres expressing p53-DN (Figure 3C), whereas expression of *Krt5* was similar to that found in control spheres, indicating that downregulation of p53 function re-establish normal differentiation of  $\alpha 3 \alpha 6$ KO basal cells (Figure S4A).

We then simultaneously deleted *Trp53*, *Itga3*, and *Itga6* genes from mammary basal cells isolated from mice



carrying the corresponding conditional alleles. *Trp53* deletion restored the number and size of the spheres formed by  $\alpha 3\alpha 6$ KO cells and, as expected, downregulated the expression of *Cdkn1a* and *Mdm2* genes (Figures 3D–3F, S4B, and S4C).

Altogether these results indicate that increased p53 activity is responsible for the perturbed clonogenic capacity induced by the loss of laminin-binding integrins in basal mammary cells.

### Inhibition of Myosin II Function Restores Clonogenic Capacity in $\alpha 3\alpha 6$ KO Mammary Basal Cells by Suppressing p53

The activity of the small Rho GTPases RHOA was particularly high in  $\alpha 3\alpha 6$ KO cells (Figure 4A). RHOA activates ROCK, and an inhibitor of ROCK, Y27632, is often used to improve the viability of embryonic stem cells or mammary stem cells in culture (Guo et al., 2012; Watanabe et al., 2007). We therefore tested whether ROCK inhibition by Y27632 could improve the sphere-forming capacity of mammary basal cells depleted of the  $\alpha 3$ - and  $\alpha 6$ -integrin chains. Decreased phospho-MLC levels were detected in Y27632-treated cultures, confirming ROCK inhibition (Figure S5A). Strikingly, in the presence of Y27632, the clonogenic capacity of  $\alpha 3\alpha 6$ KO cells was restored (Figures 4B and 4C) and the formed mammospheres were of similar size to that of control spheres (Figure S5B). ROCK controls the phosphorylation/activation status of the regulatory myosin light chain essential for myosin II interaction with actin required for ATPase activation and contraction. Another drug, blebbistatin, inhibits myosin II activity downstream of ROCK. Blebbistatin had similar effects to Y27632 on mammary basal cells: it restored the number and size of mammospheres formed by  $\alpha 3\alpha 6$ KO cells and enhanced sphere formation by control cells (Figures 4B, 4C, and S5B). Interestingly, *Lgr5* expression levels were restored by these drugs in the integrin-depleted mammospheres (Figure S5C).

We next studied the effects of Y27632 and blebbistatin on p53 activity in control and integrin-depleted cells. Expression of the p53 target genes *Cdkn1a* and *Mdm2* was downregulated in the presence of the drug, suggesting an inhibition of p53 transcriptional activity (Figures 4D and 4E). In line with these data, p53 protein levels were significantly lower in the presence of Y27632 or blebbistatin (Figure 4F). Phosphorylation of MDM2 favors its binding to p53 facilitating its proteosomal degradation (Carr and Jones, 2016). Both phosphorylated and total MDM2 levels were decreased in  $\alpha 3\alpha 6$ KO cells, whereas the inhibition of myosin II by Y27632 or blebbistatin was accompanied by an increase in phosphorylated and total MDM2 protein levels in control and mutant cells (Figure 4F). Taken together, these data strongly indicate that myosin II inhibi-

tion favors p53 degradation enabling the cell to enter the cell cycle.

The scheme presented in Figure 5 summarizes the results of this study, implicating laminin receptors,  $\alpha 3$ - and  $\alpha 6$ -integrins, in the myosin II-mediated control of p53 activity and, thereby, regulation of mammary basal stem cell function.

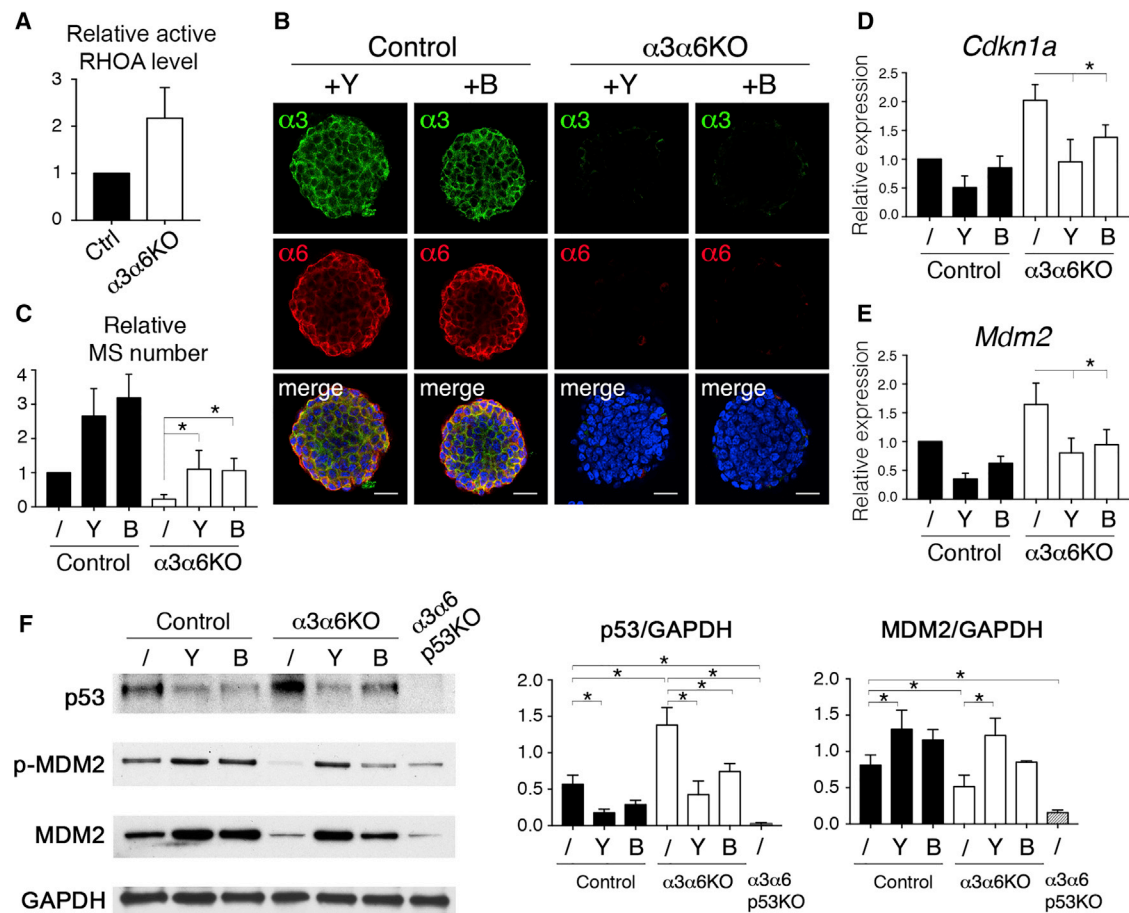
## DISCUSSION

Together with hormone and soluble growth factor signaling, integrin-mediated adhesion to ECM plays an important role in the maintenance of the mammary stem cell population (Taddei et al., 2008; Visvader and Stingl, 2014). However, knowledge of the mechanisms controlling mammary stem cell functions and the composition of their niche remains incomplete. Laminins are major components of the mammary basement membrane, and the integrin receptor subunits  $\beta 1$  and  $\alpha 6$  serve as markers for isolating stem cell-enriched preparations from mammary epithelium (Raymond et al., 2009; Shackleton et al., 2006; Stingl et al., 2006). Laminins have been identified as components of the stem cell microenvironment in other tissues, such as skeletal muscle, the mammalian neural central system, and mouse testis (Chen et al., 2013; Rayagiri et al., 2018; Raymond et al., 2009). We therefore reasoned that signaling via integrins interacting with laminins might contribute to the control of mammary stem function.

The *in vivo* transplantation model remains the gold standard assay for stem or progenitor cell activity. In addition, high proliferative potential, one of the essential characteristics of stem cells and their progeny can be assessed *in vitro*, in clonogenicity assay, i.e., mammosphere test in case of mammary stem cells. Here, we show that deletion of  $\alpha 3$ - and  $\alpha 6$ -integrins affects stem cell activity. Mammary basal cells depleted of laminin-binding integrins failed to regenerate mammary epithelium upon transplantation into cleared mouse mammary fat pads and displayed poor clonogenic activity in culture, due to the cell-cycle arrest associated with p53 activation.

Strikingly, the clonogenic activity of mammary basal cells depleted of  $\alpha 3$ - and  $\alpha 6$ -integrins was rescued by the myosin II inhibitors Y27632 and blebbistatin. Fuchs' laboratory previously showed that, in squamous cell carcinoma, MYH9, a non-muscle myosin IIA, regulates the posttranscriptional stabilization of p53, acting as a tumor suppressor (Schramek et al., 2014). A connection between functional myosin IIA and p53 accumulation associated with tumor suppressor activity has also been reported for head and neck carcinomas (Coaxum et al., 2017). The exact molecular mechanisms underlying p53 activation by myosin II remain to be unraveled. Consistent with





**Figure 4. Inhibition of ROCK or myosin II Leads to MDM2 Activation, Prevention of p53 Accumulation and Restores Mammary Stem/Progenitor Cell Activity**

(A) Relative activated RHOA levels in  $\alpha 3\alpha 6$ KO cells. Activated RHOA, evaluated by ELISA assay, was normalized to total RHOA levels measured by western blotting and represent means  $\pm$  SD from three independent experiments,  $p = 0.03$ .

(B) Confocal representative images of mammospheres formed by control or  $\alpha 3\alpha 6$ KO mammary basal cells grown in the presence of Y27632 or blebbistatin double immunolabeled with anti- $\alpha 3$  and anti- $\alpha 6$  antibodies. DAPI served to visualize nuclei. Scale bars, 30  $\mu$ m.

(C) Quantification of mammosphere formation. The values shown represent means  $\pm$  SD obtained in 10 independent experiments for untreated and Y27632-treated cells and 4 independent experiments for blebbistatin-treated cells. For  $\alpha 3\alpha 6$ KO mammospheres,  $p = 0.001$  for Y27632-treated cells compared with non-treated cells,  $p = 0.01$  for blebbistatin-treated cells compared with non-treated cells.

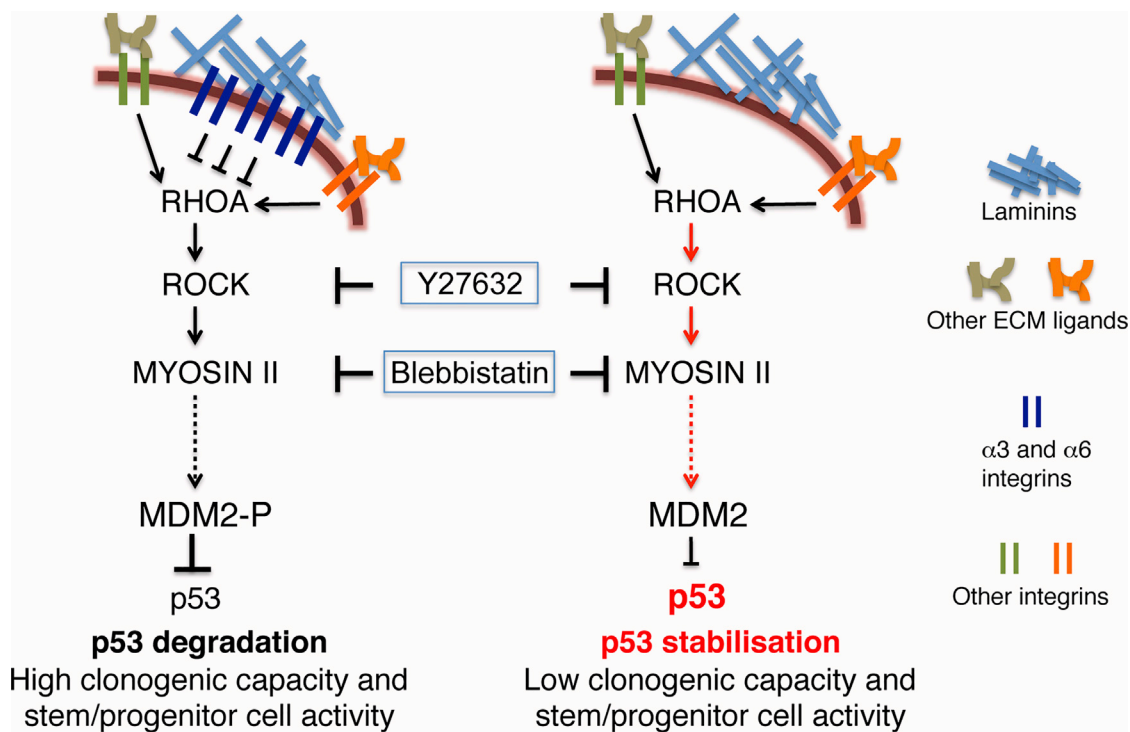
(D and E) qRT-PCR analysis of *Cdkn1a* and *Mdm2* gene expression in cells obtained from mammospheres formed by integrin-depleted mammary basal cells in the presence of Y27632 or blebbistatin. The graph shows means  $\pm$  SD from  $n$  independent experiments. For Y27632 treatment,  $n = 6$ ; for blebbistatin treatment,  $n = 3$ . (D) for  $\alpha 3\alpha 6$ KO mammospheres, Y27632-treated versus untreated,  $p = 0.0004$ , blebbistatin-treated versus untreated,  $p = 0.01$ ; (E) Y27632-treated versus untreated,  $p = 0.001$ , blebbistatin-treated versus untreated,  $p = 0.02$ .

(F) Western blotting analysis of p53, MDM2, and Phospho-MDM2 protein levels in extracts of mammosphere formed by control,  $\alpha 3\alpha 6$ KO and  $\alpha 3\alpha 6$ p53KO cells. Y27632 or blebbistatin were present, where indicated. GAPDH was used as a loading control. The graphs show the means  $\pm$  SEM from at least three independent experiments. For all indicated comparisons,  $p < 0.05$ .

In (A), (C), (D), and (E) values obtained for control cells were set as 1 in each experiment. Through the whole figure: Y, Y27632, B, blebbistatin. See also Figure S5.

our findings, in keratinocytes treated with doxorubicin to induce DNA damage response, the inhibition of myosin II with Y27632 or blebbistatin prevents p53 stabilization (Schramek et al., 2014). By contrast, the inhibition of actin polymerization by latrunculin did not interfere

with p53 accumulation, indicating that the effects of myosin II on p53 were not relevant to its role in stimulation of actin polymerization (Schramek et al., 2014). Accordingly, in our model, the treatment of  $\alpha 3\alpha 6$ -integrin-depleted cells with latrunculin did not rescue their



**Figure 5. Intracellular Signaling Events Underlying Regulation of Mammary Basal Stem Cell Activity by Laminin-Binding Integrins: Hypothetic Scheme**

Left: adhesion of mammary basal cells to laminin via  $\alpha 3$ - and  $\alpha 6$ -integrins restricts RHOA activation, thereby limiting myosin II contractility. These events serve for MDM2 phosphorylation/activation thus favoring p53 degradation and providing clonogenic capacity to stem cells. Right: mammary basal cells depleted of laminin-binding integrins display elevated active RHOA triggering activation of myosin II accompanied by low MDM2 activity allowing p53 stabilization and suppression of clonogenicity in mammary basal stem cell. Inhibition of myosin II activity by Y27632 or blebbistatin rescues clonogenic activity of integrin-depleted cells. Red color indicates abnormally activated state of the molecule/pathway in  $\alpha 3/\alpha 6$ -integrin-depleted cells.

clonogenic capacity, which was abolished by p53 accumulation (data not shown).

A pioneering study by the Damsky laboratory showed that cell interactions with fibronectin provide survival signals that suppress a p53-regulated cell death pathway (Ilic et al., 1998). The involvement of p53 in cell death induced by detachment from ECM, anoikis, was subsequently studied mostly in the context of cancer (Grossmann, 2002; Horbinski et al., 2010). By contrast, in this study, rather than focusing on anoikis-associated events, we analyzed the signaling mechanisms activated in the surviving mammary basal cells after the depletion of laminin-binding integrins.

Streuli's laboratory has shown that mammary epithelium from late (embryonic day [E] E16.5–E17.5) embryos with germline inactivation of *Itga3*, *Itga6*, or *Itgb4* may develop normally following transplantation into cleared mammary fat pads (Klinowska et al., 2001). However, this study provided no quantitative evaluation of stem cell activity in the mutant epithelium studied. By contrast, we report here the results of limiting dilution transplantations and

clonogenicity assays performed with sorted  $\alpha 3\alpha 6$ -integrin-depleted cells, allowing a quantitative characterization of stem cell function. We found that only simultaneous deletions of both  $\alpha 3$ - and  $\alpha 6$ -integrin subunits resulted in p53 activation leading to a complete loss of stem/progenitor cell activity. Our data indicate that the functions of  $\alpha 3\beta 1$ - and  $\alpha 6$ -containing integrins in the control of stem/progenitor cell proliferative potential may overlap at least partially. However, the possibility that  $\alpha 3\beta 1$ - and  $\alpha 6$ -integrins are involved in different signaling events that, when combined, lead to p53 activation and stem cell perturbation cannot be excluded.

Previous studies have revealed non-redundant cellular functions of these integrins in different physiological contexts. We recently showed that the  $\alpha 3\beta 1$ -integrin is essential for the development of mammary tumors with basal-like characteristics in K5 $\Delta$ N $\beta$ cat mice presenting a constitutive activation of WNT signaling in the basal cell layer (Cagnet et al., 2014; Moumen et al., 2013; Teuliere et al., 2005). Further, loss of  $\alpha 3$ -integrin prevented tumor formation in the two-stage skin carcinogenesis mouse



model (Sachs et al., 2012). Of note, those studies have analyzed the effects of *Itga3* deletion in tumor context, where ECM composition and organization are severely perturbed, involving integrin receptors different from those acting in normal homeostasis. Another recent report provided evidence that *Itgb4* gene deletion *in vivo* in an MMTV-Cre model, leading to embryonic  $\alpha 6 \beta 4$ -integrin depletion, affected early mammary development, including basal cell differentiation (Li et al., 2015). However, adult basal stem cell activity was not examined in these mutant mice.

Our current knowledge of laminin variant diversity in mammary basal membrane remains incomplete, but several laminin chains, including  $\alpha 1$ ,  $\alpha 5$ ,  $\beta 1$ -3,  $\gamma 1$ , and  $\gamma 2$ , have been identified in mammary ducts and alveoli by immunolabeling (Prince et al., 2002). Stem/progenitor cells can alter their proximal ECM composition, thereby regulating their function (Rayagiri et al., 2018). Thus, the diverse ligand specificity of laminin-binding integrins, with a preference of  $\alpha 3 \beta 1$  and  $\alpha 6 \beta 4$  for laminins 511/521 and laminin 332, and a broader binding specificity for  $\alpha 6 \beta 1$ , which can also interact with laminin 111 (Yamada and Sekiguchi, 2015), appears to be of great importance, as it can provide highly specific responses to a complex ECM. Thus, it will be interesting to determine which combination of integrins ( $\alpha 3 \beta 1$  plus  $\alpha 6 \beta 1$  and/or  $\alpha 6 \beta 4$ ) are involved in stem cell regulation.

RNA-seq-based comparative analysis of  $\alpha 3 \alpha 6$ -depleted and control mammary basal cells revealed perturbation of several important intracellular pathways including those associated with the control of proliferation and cell-ECM interactions. Notably, altered expression of several integrins interacting with ECM proteins other than laminins, such as collagens, fibronectin, and osteopontin, were observed in  $\alpha 3 \alpha 6$ -integrin-depleted cells. Thus, alterations of interactions with the ECM may underlie the stronger RHOA activation observed in  $\alpha 3 \alpha 6$ -deficient mammary basal cells. It has been shown previously that laminins preferentially trigger RAC1 and CDC42 activation, while other ECM components, such as fibronectin or collagen, sustained strong RHO activation (Gu et al., 2001; Zhou and Kramer, 2005). Interestingly, the transcripts of two RhoGAPs, ARHGAP11A and RACGAP1, inactivating RHO and RAC, respectively, and recently implicated in the control of proliferation (Lawson et al., 2016), were found downregulated in integrin-depleted cells. The reduced *Arhgap11A* expression may account for the higher levels of RHO activation in  $\alpha 3 \alpha 6$ KO cells. Consistently, laminin-binding integrins in association with tetraspanins have been proposed to generate RHO-suppressive signals (Novitskaya et al., 2014).

Integrins are mechanosensing receptors, and the depletion of laminin-binding integrins alters cellular responses

to external mechanical signals induced by cell confinement and ECM stiffness. Of note, the loss of  $\alpha 3$  from mammary basal cells *in vivo* impairs RAC1 activation, resulting in sustained phosphorylation of myosin light chains and a hypercontractile state (Raymond et al., 2011). Integrins are well-known modulators of the activities of Rho GTPases, which control epithelial cell polarization, lumen formation during morphogenesis, centrosome positioning, and spindle orientation during mitosis, with an impact on symmetric and asymmetric cell divisions (Akhtar and Streuli, 2013; Rodriguez-Fraticelli and Martin-Belmonte, 2014). Future studies will show whether these alterations of intracellular signaling are relevant to the impaired stem cell properties of integrin-deficient mammary epithelial cells documented here.

In brief, we show here that cell interactions involving  $\alpha 3 \beta 1$ - and  $\alpha 6$ -containing integrins binding laminins, control the proliferative potential of mammary basal stem cells by inhibiting p53 activation via mechanisms involving myosin II. These findings provide new insights into the molecular mechanisms controlling mammary stem cell activity, and indicate that laminins are highly likely to contribute to mammary stem cell niche. Future studies are required to characterize the role of specific laminin variants in the mammary stem cell niche.

## EXPERIMENTAL PROCEDURES

### Mice

The generation of *Itga6*<sup>F/F</sup>, *Trp53*<sup>F/F</sup>, and *Itga3*<sup>F/F</sup> mice has been described previously (De Arcangelis et al., 2017; Jonkers et al., 2001; Margadant et al., 2009). *K5Cre-ER*<sup>T2</sup> mice and the *Rosa26LacZ* reporter strain, were kindly provided by Dr. Metzger (Indra et al., 1999) and by Dr. Soriano (Soriano, 1999), respectively. All mice were bred in a mixed DBA/CS7Bl6 background. In Adeno-Cre infection experiments *Rosa26LacZ* mice were used as controls. The care and use of animals were conducted in accordance with the European and National Regulations for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (facility license C750517/18). All Experimental Procedures were ethically approved (ethical approval 02,265.02).

### Mammary Gland Dissociation, Cell Sorting, and Flow Cytometry Analysis

Thoracic and inguinal mammary glands of three to four 15–20-week-old virgin females were pooled, dissociated, and processed for single-cell suspension and flow cytometry as described elsewhere (Stingl et al., 2006; Taddei et al., 2008). For basal cell isolation, cells were incubated at 4°C for 20 min with the following antibodies: anti-CD45-APC (clone 30-F11), anti-CD31-APC (clone MEC13.3), anti-CD24-BViolet421 (clone M1/69), and anti-CD49f-PeCy7 (clone GoH3), or anti-ICAM1-PeCy7 (clone YN1/1.7.4), all from BioLegend. Labeled cells were analyzed and sorted out using



either a FACS Vantage flow cytometer (BD Biosciences) or a MoFlo Astrios cell sorter (Beckman Coulter). Sorted cell population purity was at least 95%. The antibodies used for the analysis of cell surface integrin expression in basal and luminal cells are presented in Table S2. Data were analyzed using FlowJo software.

### AdCre-Mediated Gene Deletion

For conditional gene deletion, freshly sorted basal cells (CD31/45<sup>-</sup>, CD24<sup>low</sup>, CD49f<sup>high</sup> population) were incubated with an adenovirus expressing the Cre recombinase under the control of the CMV promoter (AdCre, SignaGen Laboratories) at MOI of 5,000 for 1 h at 37°C. Trypan blue staining showed that at least of 90%–95% were viable after infection.

After washing, cells were resuspended in mammosphere medium and plated in low attachment 24-well plates at 5,000 cells/well in duplicate as described previously (Chiche et al., 2013). For ROCK or myosin II-ATP-ase inhibition, Y27632 (1  $\mu$ M, Millipore) or Blebbistatin (5  $\mu$ M, R&D Systems) were added to the mammosphere medium and replenished twice a week. After 12–14 days of culture, wells were photographed and the number and size of mammospheres was analyzed using ImageJ software. When indicated, collagen-I (200  $\mu$ g/mL) and fibronectin (200  $\mu$ g/mL) were added to the mammosphere medium containing 2% Matrigel. For BrdU incorporation assays, the mammospheres were incubated during 1 h with 5  $\mu$ M BrdU, before fixation in Methacarn (methanol:chloroform:acetic acid, 6:3:1) for 15 min. Quantification of RHOA activation was performed using G-LISA RhoA Activation Assay Biochem Kits, following the manufacturer's instructions (Cytoskeleton).

### Transplantation

After Adeno-Cre infection, sorted basal cells were resuspended in 10  $\mu$ L of 50% growth factor-reduced Matrigel (BD Biosciences) and injected into the inguinal fat pads of 3-week-old nude BALB/c females cleared of endogenous epithelium as described elsewhere (Taddei et al., 2008). Ten weeks after transplantation, fat pads were fixed in 2.5% paraformaldehyde for 1 h at 4°C and stained overnight at 30°C with X-gal (Biology of the Mammary Gland, <http://mammary.nih.gov>).

### Mammary Explant Culture

Mammary organoids were obtained from mammary glands dissected from K5-Cre<sup>ERT2+/-</sup>;Itga3<sup>F/F</sup>;Itga6<sup>F/F</sup>;Rosa26<sup>LacZ</sup> or K5-Cre<sup>ERT2+/-</sup>;Itga3<sup>F/F</sup>;Itga6<sup>F/F</sup>;Rosa26<sup>LacZ</sup> mice according to Ewald's protocol (Ewald et al., 2008). A total of 60,000 organoids were plated on P150 plastic plates in 15 mL of DMEM:F12 medium containing 5% fetal bovine serum, 5  $\mu$ g/mL insulin, 10 ng/mL epidermal growth factor, and penicillin/streptomycin. After 48 h, 4-hydroxytamoxifen was added at 100 nM (Sigma) and replenished 24 h later at the same concentration. The explants were trypsinized after 6 days of culture and basal cells (CD31/45<sup>-</sup>, CD24<sup>low</sup>, ICAM1<sup>high</sup>) were FACS-sorted.

### RNA Extraction and qRT-PCR

RNA was extracted with the RNeasy Micro Kit including DNase treatment (QIAGEN), and reverse-transcribed using MMLV H(-) Point reverse transcriptase (Promega). qPCR was performed using

the QuantiNova SYBR Green PCR Kit (QIAGEN) on a LightCycler 480 real-time PCR system (Roche). The values obtained were normalized to Gapdh levels. The primers used for qRT-PCR analysis were purchased from SABiosciences/QIAGEN or designed using Oligo 6.8 software (Molecular Biology Insights) and synthesized by Eurogentec. Primers used in this study are listed in Table S3.

### Statistical Analysis

All values are shown as mean  $\pm$  SD or SEM. The p values were determined using Student's t test with two-tailed distribution and unequal variance (Welch's correction). For mammosphere size comparison, a Pearson's chi-square test was applied.

### ACCESSION NUMBERS

RNA-seq data have been deposited in the GEO under accession number GEO: GSE117120.

### SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.stemcr.2019.02.008>.

### AUTHORS CONTRIBUTIONS

M.R., S.C., A.C., L.B., K.R., M.-A.D., and M.M.F. designed and performed the experiments. S.B. and P.d.I.G. performed the RNA-seq analyses. A.D.A., M.K., E.G.-L., and A.S. provided the mouse strains. M.A.G. and M.M.F. designed the experimental plan and wrote the manuscript. All authors contributed to data analysis and interpretation and final approval of the manuscript.

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