

Targeted disruption of β 1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction

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Summary

Despite evidence demonstrating the role of β 1-integrin in the regulation of cancer cell proliferation *in vitro*, the importance of this cell adhesion receptor during the initiation and progression of epithelial tumors *in vivo* remains unclear. Here we have used the Cre/LoxP1 recombination system to disrupt β 1-integrin function in the mammary epithelium of a transgenic mouse model of human breast cancer. Using this approach, we show that β 1-integrin expression is critical for the initiation of mammary tumorigenesis *in vivo*, and for maintaining the proliferative capacity of late-stage tumor cells. These observations provide a direct demonstration that β 1-integrin plays a critical role in both the initiation and maintenance of mammary tumor growth *in vivo*.

Introduction

The oncogenic conversion of a primary epithelial cell to a malignant tumor has been shown to involve multiple genetic events, including the activation of growth-promoting pathways and the inactivation of tumor suppressor genes and gene products (Hanahan and Weinberg, 2000). Evidence is accumulating, however, that the growth of epithelial tumors also depends on interactions between the transformed cells and the stromal microenvironment, regardless of the underlying genetic lesions (Bissell et al., 2002; Ronnov-Jessen et al., 1996; Shekhar et al., 2003; Wiseman and Werb, 2002). A growth-promoting role for the extracellular matrix (ECM) may explain the strong stromal response accompanying the formation of aggressive epithelial tumors (Ronnov-Jessen et al., 1996), as well as the synthesis of ECM components by individual tumor cells (Saulnier et al., 1996).

A critical growth-promoting role for the tumor stroma has been demonstrated experimentally, primarily through the inhibi-

tion of integrin function in 3-dimensional cell culture models of mammary tumorigenesis (Kenny and Bissell, 2003; Wang et al., 2002; Weaver et al., 1997). Although integrins have not traditionally been regarded as protooncogenes, there is accumulating data suggesting that these molecules may play an important cooperative role during tumor induction by activated oncogenes. A potential tumorigenic role for the β 1-integrin subunit, for example, has been attributed to reciprocal signaling between β 1-integrin-containing receptors and activated growth factor receptors (Adelsman et al., 1999; Falcioni et al., 1997; Moro et al., 1998; Wang et al., 1998). Indeed, reversion of the transformed phenotype by blocking β 1-integrin binding activity in human breast cancer cells was shown to be accompanied by downregulation of EGFR signaling (Wang et al., 2002; Weaver et al., 1997).

Lateral signaling between activated growth factor receptors and integrins is associated with a number of distinct integrin-associated signaling molecules, including integrin-linked kinase (ILK), focal adhesion kinase (FAK), and the c-Src family of tyro-

SIGNIFICANCE

Crosstalk between integrin receptors and activated growth factor receptors has been hypothesized to play a critical role in the initiation and progression of human cancer. Here we provide direct demonstration that the β 1-integrin adhesion receptor is required for transformation *in vivo* by an activated oncogene. The mechanistic explanation for this result suggests that the requirement for β 1-integrin signaling pathways may apply to a variety of activated oncogenes known to be involved in human cancers. In addition, a proliferative block induced by the suppression of FAK phosphorylation in primary tumor cells lacking β 1-integrin resembles the clinical phenomenon of tumor dormancy. These experiments therefore provide a novel model for the therapeutic intervention of human breast cancer.

sine kinases (Dedhar et al., 1999; Hanks and Polte, 1997; Howe et al., 1998; Sieg et al., 1999, 2000). In the case of c-Src, the potent transforming properties of this molecule have been associated with the phosphorylation and activation of FAK (Gabarra-Niecko et al., 2003; Xing et al., 1994). After binding to an auto-phosphorylation site on β 1-integrin-associated FAK, c-Src phosphorylates several tyrosine residues along the carboxyl terminus of the FAK molecule, which subsequently provide binding sites for molecules such as Grb2. The phosphorylation of FAK has been shown to be important for several aspects of c-Src signaling, including cell cycle progression and the regulation of focal adhesion turnover during cell migration (Oktay et al., 1999; Webb et al., 2004), as well as playing a critical role during the induction of oncogenic transformation by constitutively activated versions of c-Src (Xing et al., 1994).

Activation and elevated expression of β 1-integrin-coupled signaling effectors have been implicated in the induction of a wide variety of human cancers, including those of the breast, colon, prostate, and ovaries (Ahmed et al., 2003; Cance et al., 2000; Graff et al., 2001; Marotta et al., 2001; Oktay et al., 2003; Owens et al., 1995). In addition, the overexpression of β 1-integrin-associated molecules such as ILK can result in the induction of mammary tumors in experimental mouse models (White et al., 2001). Despite the demonstrated importance of these β 1-integrin-associated signaling molecules during tumor progression, however, the role of the β 1-integrin molecule itself remains unresolved. Unfortunately, addressing the role of the β 1-integrin subunit in tumorigenesis and postnatal development has been precluded primarily by the embryonic lethality resulting from germline ablation of the β 1-integrin gene in mice (Fassler and Meyer, 1995). The recent development of mice bearing a conditional allele of the β 1-integrin gene, however, now enables investigators to circumvent this limitation, facilitating the analysis of β 1-integrin function in tissues of the postnatal mouse (Graus-Porta et al., 2001).

Given the potential importance of β 1-integrin in mammary tumorigenesis, we have used this conditional β 1-integrin allele to generate a mammary-specific knockout of β 1-integrin in a mouse model of human breast cancer. To accomplish this, we introduced the conditional β 1-integrin allele into transgenic mice expressing the polyomavirus (PyV) middle T (MT) oncogene and the Cre recombinase, both under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter/enhancer. The MMTV/PyV MT mammary tumor model affords several important advantages. Firstly, mammary tumors that arise in this strain appear with complete penetrance, and after a relatively short latency period (Guy et al., 1992). Secondly, tumor progression in the MMTV/PyV MT strain recapitulates many aspects of human breast cancer progression, providing a model of both atypical mammary gland hyperplasia as well as papillary and scirrhous carcinomas of humans (Lin et al., 2003; Maglione et al., 2001). The recapitulation of human breast carcinomas in this mouse model likely reflects the activation of downstream signaling pathways shared by the HER2/neu/erbB2 oncogene, such as those involving c-Src, Shc, Grb2, PI3'K, and the Ras-MAPK pathway (Ichaso and Dilworth, 2001).

Although ablation of β 1-integrin expression from cells of the mouse mammary epithelium did not impair mammary gland development during puberty, deletion of the conditional β 1-integrin allele dramatically impaired mammary tumorigenesis in the MMTV/PyV MT mice. Importantly, molecular analysis of the

few tumors arising in these crosses revealed that they still retained a functional β 1-integrin allele, revealing selective pressure for β 1-integrin expression during tumorigenesis. In addition, the deletion of β 1-integrin in cultured mammary tumor cells was found to be associated with a decrease in the phosphorylation of FAK tyrosine residues, including the c-Src binding site. The deletion of β 1-integrin from these PyV MT-induced tumor cells also inhibited the capacity of these cells to proliferate and form tumors *in vivo*. Taken together, these observations suggest that retention of β 1-integrin expression is required for oncogene-induced mammary tumorigenesis and mammary tumor growth *in vivo*.

Results

A conditional β 1-integrin allele can be efficiently excised from the murine mammary epithelium

To determine whether β 1-integrin is required for mammary tumorigenesis *in vivo*, we obtained mice carrying a conditional β 1-integrin allele in which the second coding exon, containing the ATG translational start site of the β 1-integrin gene, was flanked with LoxP1 recombination sites (β 1^{LoxP1}) (Figure 1A) (Graus-Porta et al., 2001). Previously, neural-specific expression of Cre has been shown to result in complete ablation of β 1-integrin protein in this tissue (Feltri et al., 2002; Graus-Porta et al., 2001). To facilitate excision of the β 1^{LoxP1} allele in the epithelial compartment of the mouse mammary gland, we bred the conditional β 1-integrin strain with a separate transgenic strain expressing Cre in the mammary epithelium (MMTV/Cre) (Figure 1B) (Andrechek et al., 2000). Targeted excision of the β 1^{LoxP1} allele in the mammary glands of β 1^{LoxP1/LoxP1} MMTV/Cre mice was confirmed using a combination of molecular and immunofluorescent approaches. As shown in Figure 1C, targeted recombination of the β 1^{LoxP1} allele could be demonstrated by PCR analysis of mammary gland DNA from β 1^{LoxP1/LoxP1} MMTV/Cre animals, using primers which amplify both the intact and Cre-deleted forms of the β 1^{LoxP1} allele (Feltri et al., 2002; Graus-Porta et al., 2001). Amplification of the unexcised form of the β 1^{LoxP1} allele likely reflects the presence of stromal cell DNA in the preparation. A corresponding reduction in total β 1-integrin protein in mammary glands of β 1^{LoxP1/LoxP1} MMTV/Cre mice, relative to control FVB mice, was confirmed by immunoblot analysis of mammary epithelial cell lysates (Figure 1D).

The specificity of MMTV/Cre-mediated excision of the β 1^{LoxP1} allele was demonstrated by immunofluorescence microscopy of frozen mammary gland sections, using anti- β 1-integrin polyclonal antisera. Examination of the mammary epithelium derived from wild-type virgin FVB animals revealed that β 1-integrin is normally expressed along the basolateral surface of the ductal epithelium in the mouse mammary gland (Figure 1E, red staining). In glands prepared from age-matched β 1^{LoxP1/LoxP1} MMTV/Cre animals, however, β 1-integrin could no longer be detected in many of the sections examined (Figure 1F).

Ablation of β 1-integrin expression does not interfere with the initial stages of mammary ductal outgrowth

Prior to the introduction of this conditional β 1-integrin allele into a mouse tumor model, it was necessary to determine whether targeted ablation of β 1-integrin was compatible with normal mammary gland development. For this purpose, we performed wholemount analyses of mammary gland development in 4-,

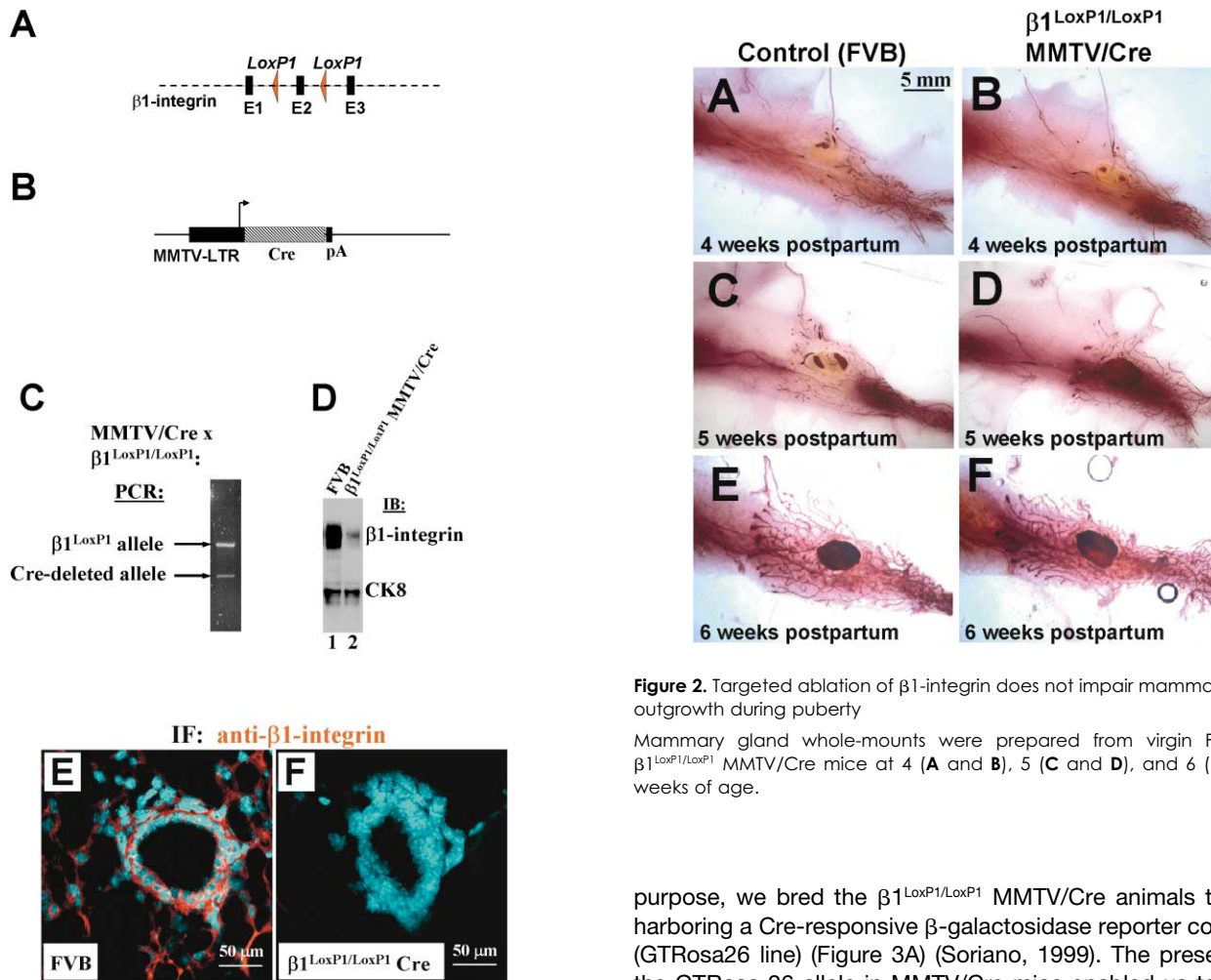


Figure 1. Targeted ablation of $\beta 1$ -integrin expression in the mouse mammary gland epithelium

A and B: LoxP1-flanked $\beta 1$ -integrin ($\beta 1^{LoxP1}$) allele (**A**) and MMTV/Cre transgene (**B**).

C: PCR analysis of mammary gland DNA from $\beta 1^{LoxP1/LoxP1}$ MMTV/Cre mice, confirming Cre-mediated excision of the $\beta 1^{LoxP1}$ allele.

D: Immunoblot analysis of $\beta 1$ -integrin expression in mammary epithelial cell lysates from FVB (lane 1) and $\beta 1^{LoxP1/LoxP1}$ MMTV/Cre (lane 2) mice.

E: IF analysis of $\beta 1$ -integrin expression (red) in a ductal cross-section from a 12-week-old FVB mouse. DAPI counterstain appears blue.

F: IF analysis of $\beta 1$ -integrin expression in a $\beta 1^{LoxP1/LoxP1}$ MMTV/Cre mouse. Note the absence of $\beta 1$ -integrin protein (red), compared to the FVB gland shown in **E**.

5-, and 6-week-old female virgin $\beta 1^{LoxP1/LoxP1}$ MMTV/Cre animals (Figure 2). Examination of glands from these animals indeed revealed normal mammary ductal outgrowth during puberty (Figures 2B, 2D, and 2F), indistinguishable from that of control FVB mice (Figures 2A, 2C, and 2E). In addition, female mice harboring this $\beta 1^{LoxP1/LoxP1}$ MMTV/Cre genetic combination were able to lactate and produce viable litters through multiple rounds of pregnancy (data not shown).

Since MMTV promoter activity has been reported to be stochastic (unpublished observations), we wanted to confirm that MMTV/Cre-expressing cells were not at a selective disadvantage in the $\beta 1^{LoxP1/LoxP1}$ MMTV/Cre background. For this

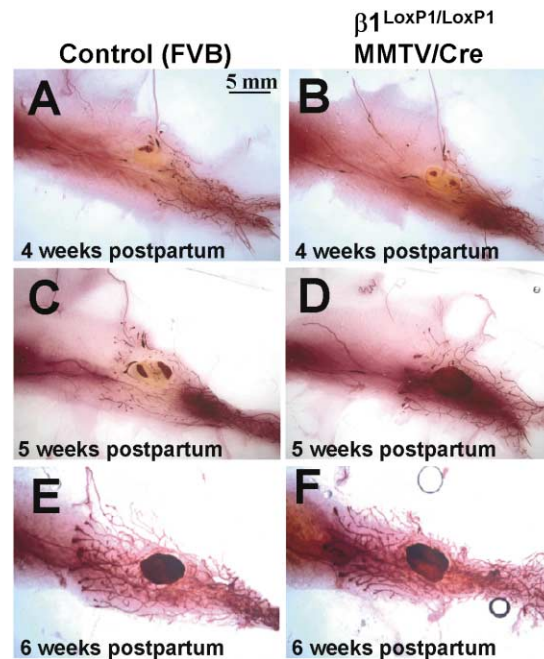


Figure 2. Targeted ablation of $\beta 1$ -integrin does not impair mammary gland outgrowth during puberty

Mammary gland whole-mounts were prepared from virgin FVB and $\beta 1^{LoxP1/LoxP1}$ MMTV/Cre mice at 4 (**A** and **B**), 5 (**C** and **D**), and 6 (**E** and **F**) weeks of age.

purpose, we bred the $\beta 1^{LoxP1/LoxP1}$ MMTV/Cre animals to mice harboring a Cre-responsive β -galactosidase reporter construct (GTRosa26 line) (Figure 3A) (Soriano, 1999). The presence of the GTRosa 26 allele in MMTV/Cre mice enabled us to monitor at the individual cell level whether a Cre-mediated excision event had occurred. When mammary glands from MMTV/Cre GTRosa26 bitransgenic animals were stained in situ with the colorimetric β -galactosidase substrate Xgal, sections of the stained glands indeed revealed stochastic expression of the MMTV/Cre transgene, ranging from 100% to 50% of the luminal epithelial cells (Figures 3B, 3C, and 3D; blue staining indicates β -galactosidase activity). Visual examination of multiple stained sections subsequently allowed us to estimate the overall proportion of Cre-expressing cells at approximately 80% (data not shown).

Given the stochastic nature of MMTV/Cre expression, it was therefore conceivable that there was preferential retention of $\beta 1$ -integrin-positive cells in the developing glands of $\beta 1^{LoxP1/LoxP1}$ MMTV/Cre mice. As a result, we prepared wholemounts of mammary glands derived from 12-week-old virgin animals bearing GTRosa26 and MMTV/Cre alleles, and harboring either 1 (Figure 3E) or 2 (Figure 3F) copies of the $\beta 1^{LoxP1}$ allele. These glands were then stained in situ for Cre-mediated β -galactosidase activity. The results of this staining protocol revealed that the entire mammary gland tree was positive for β -galactosidase expression in both genotypes (Figures 3E and 3F). Importantly, higher magnification of mammary ducts from these Xgal-stained glands indicated that MMTV/Cre-expressing cells were present in equal proportions between the 2 genotypes (Figures 3G and 3H). In addition, an Xgal-stained section of a

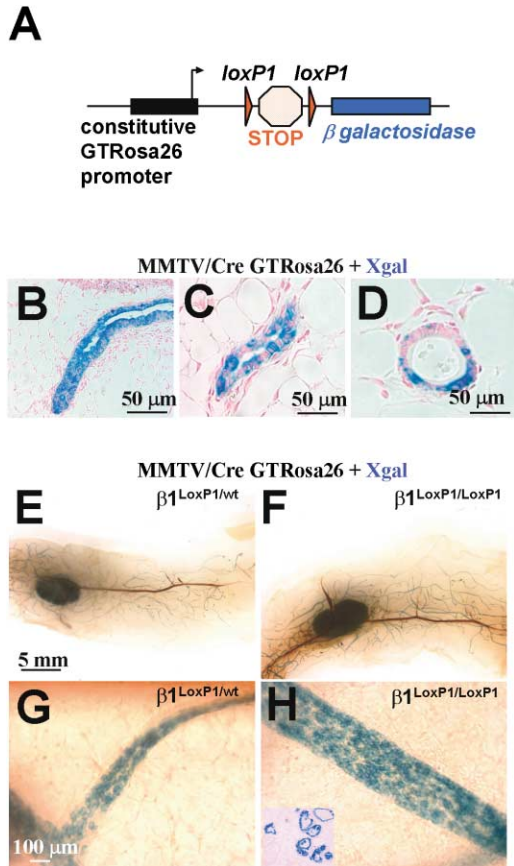


Figure 3. Mammary epithelial cells contribute to ductal outgrowth in the absence of $\beta 1$ -integrin expression

A: Cre-responsive GTRosa26 β -galactosidase reporter construct present in the GTRosa26 mouse line.

B–D: Pattern of MMTV/Cre expression (blue) in GTRosa26 MMTV/Cre mice.

E–H: MMTV/Cre expression (blue) in mammary glands of 10-week-old female GTRosa26 $\beta 1^{LoxP1/wt}$ MMTV/Cre (**E** and **G**) and GTRosa26 $\beta 1^{LoxP1/LoxP1}$ MMTV/Cre (**F** and **H**) mice. The inset in **H** shows an Xgal-stained cross-section of a gland from a lactating GTRosa26 $\beta 1^{LoxP1/LoxP1}$ MMTV/Cre mouse.

mammary gland taken from a lactating GTRosa26 $\beta 1^{LoxP1/LoxP1}$ MMTV/Cre animal revealed the presence of a $\beta 1$ -integrin-deficient differentiated epithelial population expressing the MMTV/Cre transgene (Figure 3H, inset). The presence of these β -galactosidase-expressing cells in the lactating gland suggests that there was retention of epithelial precursor or stem cell populations during development of the glands in GTRosa26 $\beta 1^{LoxP1/LoxP1}$ MMTV/Cre mice, in spite of MMTV/Cre expression. Taken together, these observations suggest that $\beta 1$ -integrin expression is not required for the initial stages of mammary gland development in these mice.

Tumorigenesis in MMTV/PyV MT mice requires $\beta 1$ -integrin expression

Immunohistochemical analysis of glands from 10-week-old MMTV/PyV MT mice revealed that $\beta 1$ -integrin is expressed throughout regions of the mammary epithelium undergoing hyperplastic expansion (Figures 4A and 4B, black asterisks). Expression of $\beta 1$ -integrin in these hyperplastic regions was markedly higher than in the adjacent, single-layered epithelium

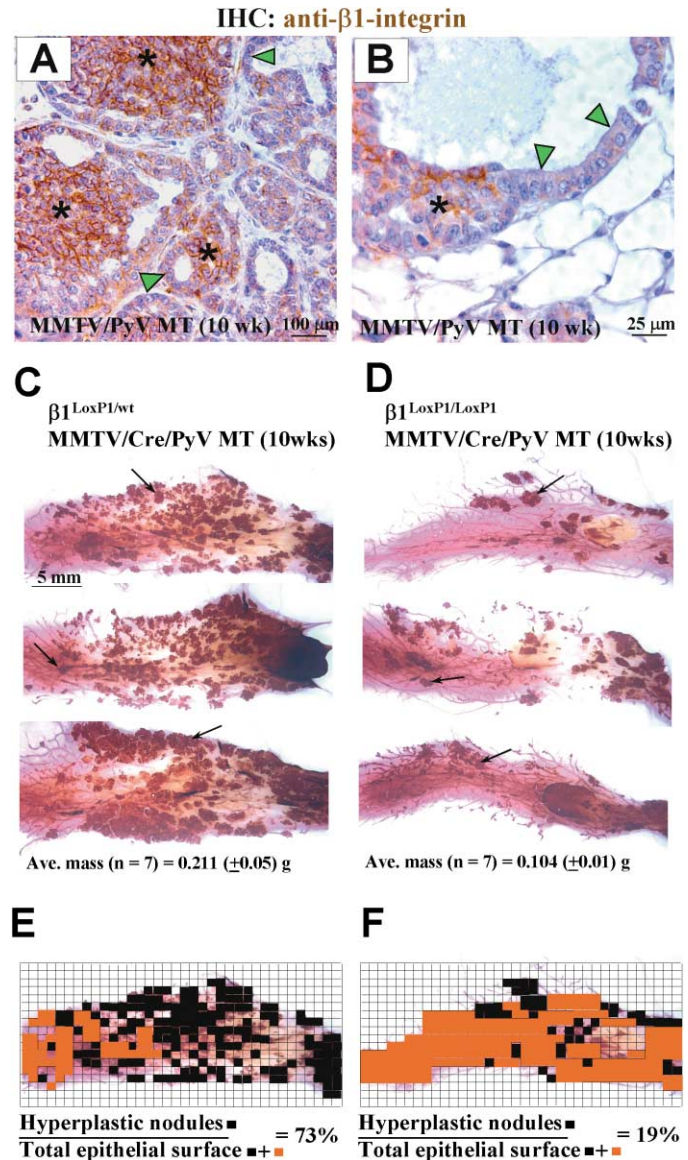


Figure 4. Ablation of $\beta 1$ -integrin expression impairs mammary tumorigenesis in MMTV/PyV MT mice

A and B: $\beta 1$ -integrin expression (brown) in mammary glands from 10-week-old female MMTV/PyV MT mice. Hyperplastic (black asterisks) and nontransformed (green arrowheads) regions of the epithelium are indicated.

C and D: Representative mammary gland whole-mounts from 10-week-old $\beta 1^{LoxP1/wt}$ MMTV/Cre/PyV MT (**C**) and $\beta 1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT (**D**) mice. Glands were prepared from 3 littermate pairs. Arrows indicate hyperplastic lesions. Average mass of 7 glands for each genotype is included.

E and F: Ratio of hyperplastic lesions (black squares) to total mammary epithelium surface area (black + red squares), for the 2 representative glands shown in the top panels of **C** and **D**.

(Figures 4A and 4B, green arrowheads), suggesting that there may be a correlation between $\beta 1$ -integrin expression and oncogenic transformation of the epithelium in the MMTV/PyV MT mice.

In order to determine if $\beta 1$ -integrin expression is required for the induction of mammary tumors in these mice, the MMTV/PyV MT transgene was introduced into the $\beta 1^{LoxP1/LoxP1}$ MMTV/Cre background. Initial comparison of 2 groups of 7 female

virgin mice carrying the MMTV/PyV MT and MMTV/Cre transgenes, and harboring either 1 ($\beta 1^{\text{LoxP1/wt}}$) or 2 ($\beta 1^{\text{LoxP1/LoxP1}}$) copies of the $\beta 1^{\text{LoxP1}}$ allele, revealed a dramatic difference in the extent of mammary tumor development. Like the parental MMTV/PyV MT strain (Guy et al., 1992), palpable mammary tumors could be detected as early as 10 weeks in the $\beta 1^{\text{LoxP1/wt}}$ MMTV/Cre/PyV MT control animals (data not shown). By contrast, mammary tumors were not palpable in $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT mice at this time point (data not shown). Consistent with these observations, whole-mount analysis of mammary glands prepared from these 2 groups of 7 animals revealed a dramatic reduction in the number of hyperplastic mammary lesions in the $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT background, as compared to the $\beta 1^{\text{LoxP1/wt}}$ MMTV/Cre/PyV MT mice (glands from 3 representative 10-week-old littermate pairs are shown in Figures 4C and 4D, with a quantitative comparison shown in Figures 4E and 4F). These observations suggest that the mammary-specific deletion of $\beta 1$ -integrin can dramatically impair mammary gland transformation and mammary tumor development in the MMTV/PyV MT mice.

After 4 months of age, palpable mammary tumors could be detected in the $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT mice. Given the stochastic nature of MMTV/Cre expression (Figure 3), we considered the possibility that these tumors were populated by cells retaining expression of $\beta 1$ -integrin. Consistent with this hypothesis, we estimated that approximately 20% of the mammary epithelium of $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT mice has undergone transformation by 10 weeks of age (Figure 4F), which coincides with the estimated proportion of Cre-negative cells in the glands of GTRosa26 MMTV/Cre mice (Figure 3). As shown by the immunoblot analysis in Figure 5A, $\beta 1$ -integrin protein is indeed expressed in late-stage tumors derived from 5- to 6-month-old $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT mice, at levels comparable to those of control MMTV/PyV MT animals (compare lanes 1–5 with lanes 6–8). Immunohistochemical analysis of multiple sections prepared from these tumors revealed uniform expression of $\beta 1$ -integrin protein throughout the tumor tissue, indicating that $\beta 1$ -integrin expression was maintained throughout the entire tumor cell population (a representative section is shown in Figure 5B). The maintenance of $\beta 1$ -integrin protein expression in tumors from these $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT mice was found to be consistent with the relative lack of excision of the $\beta 1^{\text{LoxP1}}$ alleles in these tumors, as determined by PCR amplification of tumor-derived DNA (Figure 5C, lanes 1–5). The $\beta 1^{\text{LoxP1}}$ allele, by contrast, was found to be efficiently excised in tumors from $\beta 1^{\text{LoxP1/wt}}$ MMTV/Cre/PyV MT mice, which harbor 1 copy of the wild-type $\beta 1$ -integrin allele (Figure 5C, lanes 6–8). Importantly, the difference in the extent of excision of the $\beta 1^{\text{LoxP1}}$ allele between the $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT and $\beta 1^{\text{LoxP1/wt}}$ MMTV/Cre/PyV MT mice was found to correlate with the levels of Cre expression in these tumors, as determined by RT-PCR analysis of tumor-derived RNA (Figure 5D, compare lanes 1–5 with lanes 6–8). These results therefore support the hypothesis that tumors arising in the $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT mice are populated by cells retaining expression of $\beta 1$ -integrin, due to the absence of MMTV/Cre expression in those cells.

The retention of $\beta 1$ -integrin expression in tumors from the $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT mice suggests that there is a requirement for $\beta 1$ -integrin expression during tumorigenesis. This requirement for $\beta 1$ -integrin expression would therefore provide selective pressure against MMTV/Cre expression in cells undergoing transformation in the $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT

animals. In other words, luminal epithelial cells expressing the MMTV/Cre transgene would not be expected to undergo PyV MT-induced transformation in the $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT background, due to Cre-mediated excision of both $\beta 1^{\text{LoxP1}}$ alleles. To test this hypothesis, we introduced the GTRosa26 reporter construct into the $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT and $\beta 1^{\text{LoxP1/wt}}$ MMTV/Cre/PyV MT mice. This strategy would allow us to more accurately follow the fate of individual Cre-expressing cells during transformation of the mammary gland in these 2 genetic backgrounds. After obtaining the desired genetic combinations, mammary glands were removed from 10- and 12-week-old $\beta 1^{\text{LoxP1/wt}}$ MMTV/Cre/PyV MT GTRosa26 control mice (Figure 5E), as well as from age-matched mice of the $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT GTRosa26 background (Figure 5F). The glands were then stained in situ with Xgal and mounted, in order to visualize the distribution of Cre-expressing cells. Consistent with the results of the RT-PCR analysis (Figure 5D), Cre-expressing cells in the $\beta 1^{\text{LoxP1/wt}}$ MMTV/Cre/PyV MT GTRosa26 control background were present in both the hyperplastic lesions (Figure 5E, black arrow) and normal ductal structures (Figure 5E, green arrowhead) of the glands, suggesting that these cells were not at a selective disadvantage during tumorigenesis. The ability of Cre-expressing cells to form tumors in the $\beta 1^{\text{LoxP1/wt}}$ MMTV/Cre/PyV MT GTRosa26 background is even more apparent by the robust staining seen throughout the gland of a tumor-bearing 12-week-old animal (Figure 5E, inset). In the $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT GTRosa26 background, however, Cre-expressing cells were found to be located exclusively within the normal ductal structures (Figure 5F and inset, green arrowheads). Regions of the glands undergoing hyperplastic proliferation, by contrast, showed no evidence of Cre-mediated β -galactosidase activity (Figure 5F and inset, black arrows). When tumor sections were prepared from older (16-week-old) $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT GTRosa26 mice, Xgal staining again confirmed that β -galactosidase expression was confined to morphologically normal ductal structures in the tumors of these animals (Figure 5G, green arrowheads). The pattern of MMTV/Cre expression in Figure 5G is inversely correlated with the expression of $\beta 1$ -integrin protein in the glands of the $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT GTRosa26 mice (Figure 5H—compare $\beta 1$ -integrin expression in hyperplastic regions [black arrows] versus single-layered regions of the epithelium [green arrowheads]).

Cre-expressing cells, therefore, appear to be impaired in their ability to undergo hyperplastic proliferation in the $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT GTRosa26 background. Since the only genetic difference between the $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT GTRosa26 and $\beta 1^{\text{LoxP1/wt}}$ MMTV/Cre/PyV MT GTRosa26 mice is 2 versus 1 copy of the $\beta 1^{\text{LoxP1}}$ allele, respectively, these results strongly suggest that a functional copy of the $\beta 1$ -integrin gene is required for oncogenic transformation of individual epithelial cells in the MMTV/PyV MT mice.

Targeted disruption of $\beta 1$ -integrin in PyV MT-induced tumor cells interferes with their capacity to proliferate in vivo and in vitro

Although the above experiments suggest that $\beta 1$ -integrin is required for the initiation of PyV MT-induced mammary tumors in vivo, it is unclear whether $\beta 1$ -integrin is also required for maintenance of the transformed state in late-stage tumors. As shown by the representative section presented in Figure 6A, immunohistochemical analysis indeed revealed expression of

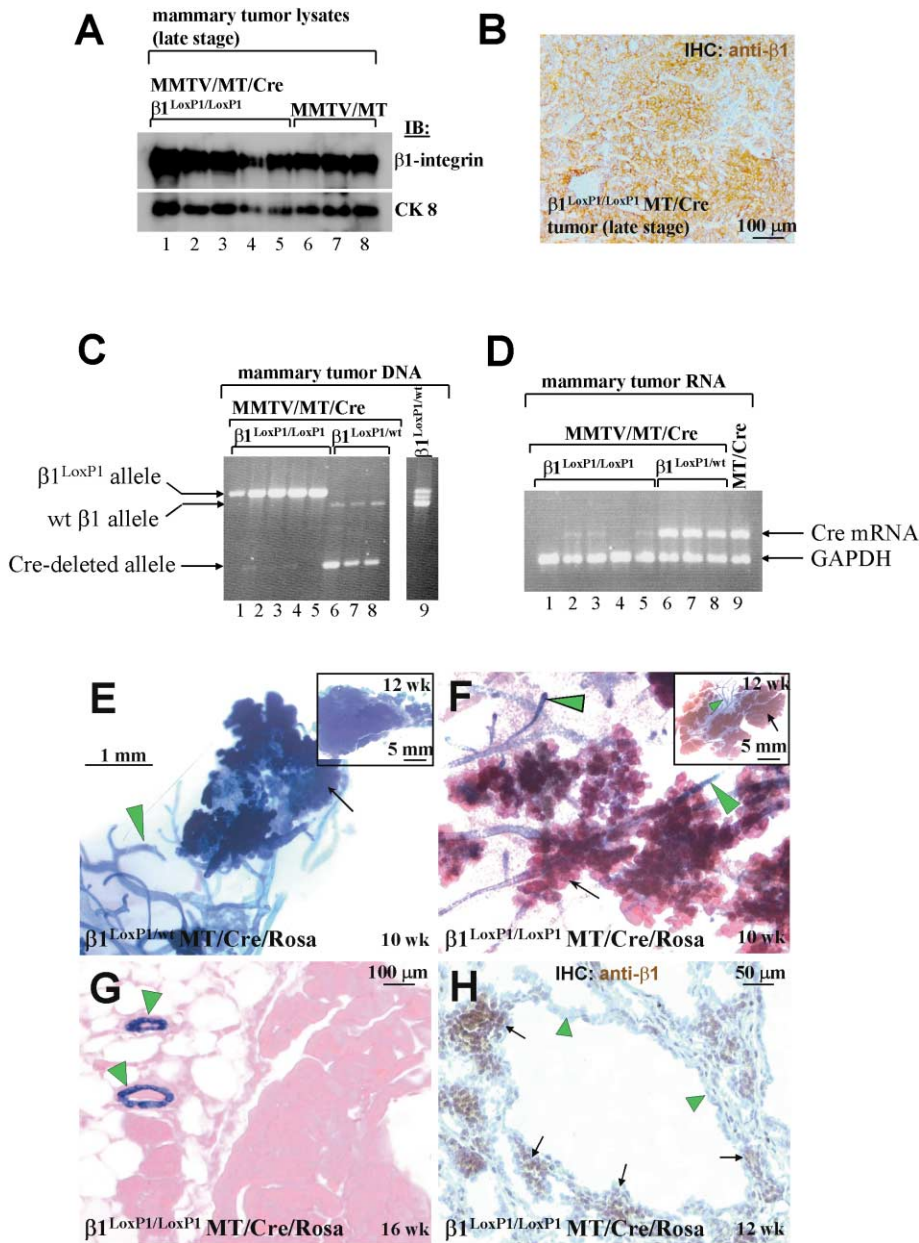


Figure 5. Cre-expressing cells do not contribute to tumorigenesis in the $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT mice

A: $\beta 1$ -integrin expression is maintained in tumors of $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT mice (lanes 1–5). Tumor lysates from MMTV/PyV MT control mice were included for comparison (lanes 6–8).

B: $\beta 1$ -integrin (brown) is expressed throughout $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT-derived tumor tissue. The staining pattern is representative of several late-stage tumors.

C: PCR amplification of tumor DNA from $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT (lanes 1–5) and $\beta 1^{\text{LoxP1/wt}}$ MMTV/Cre/PyV MT (lanes 6–8) mice, using primers which amplify the intact and Cre-deleted forms of the $\beta 1^{\text{LoxP1}}$ allele, as well as the wild-type $\beta 1$ -integrin gene. Mammary gland DNA from a $\beta 1^{\text{LoxP1/wt}}$ mouse was amplified as a reference for PCR fragment size (lane 9).

D: RT-PCR analysis of tumor RNA from $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT (lanes 1–5), $\beta 1^{\text{LoxP1/wt}}$ MMTV/Cre/PyV MT (lanes 6–8), and $\beta 1^{\text{wt/wt}}$ MMTV/Cre/PyV MT (lane 9) mice, using Cre-specific PCR primers.

E and F: Xgal-stained mammary gland whole-mounts from 10-week-old $\beta 1^{\text{LoxP1/wt}}$ MMTV/Cre/PyV MT GTRosa26 (**E**) and $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT GTRosa26 (**F**) mice. Normal ducts (green arrowheads) and hyperplastic nodules (black arrows) are indicated. **E** is representative of 6 stained glands from 3 mice. **F** is representative of 8 stained glands from 4 mice. Insets show stained glands prepared from older (12-week-old) animals.

G: Representative section of an Xgal-stained tumor from a $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT GTRosa26 mouse. Normal ductal structures are indicated by green arrowheads.

H: $\beta 1$ -integrin protein expression (brown) in a $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/Cre/PyV MT-derived mammary gland section. Hyperplastic (black arrows) and nontransformed (green arrowheads) regions are indicated. This staining pattern is representative of multiple fields from 3 animals.

$\beta 1$ -integrin protein throughout solid, late-stage mammary tumors taken from 4- to 5-month-old MMTV/PyV MT mice. Expression of $\beta 1$ -integrin protein in these tumors was confirmed by immunoblot analysis, as shown in Figure 6B (lanes 1–3). The levels of $\beta 1$ -integrin expression in PyV MT-induced mammary tumors were comparable to those induced by expression of an MMTV/erbB2 (*neu*) transgene (Figure 6B, lanes 4–6), suggesting that $\beta 1$ -integrin expression may be common to oncogene-induced mouse mammary tumors (levels of *neu* protein are shown in the middle panel). The proportion of epithelial cells in these tumors was normalized by blotting with an antibody recognizing the cytokeratin-8 (CK8) luminal epithelial protein (Figure 6B, bottom panel).

In order to determine if $\beta 1$ -integrin expression is required for maintenance of the tumor phenotype in MMTV/PyV MT mice, we developed a strategy to excise the $\beta 1^{\text{LoxP1}}$ alleles from primary

tumor cells cultured from $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/PyV MT and $\beta 1^{\text{LoxP1/wt}}$ MMTV/PyV MT mice (Figure 6C). This strategy involves infection of the culture monolayers with an adenovirus vector expressing the Cre recombinase (AdCre). Inclusion of the GTRosa26 reporter in the donor mice subsequently facilitates identification of individual cells undergoing Cre-mediated excision. As shown by the results of Xgal staining, approximately 30% of the cultured tumor cells can be infected in this way (Figures 6D and 6G). Despite a corresponding reduction in $\beta 1$ -integrin protein levels, the AdCre-infected $\beta 1^{\text{LoxP1/LoxP1}}$ MMTV/PyV MT GTRosa26 culture did not exhibit an increase in apoptotic cell death, compared to AdCre-infected control cultures (data not shown).

The AdCre-infected cultures, from both genotypes, were subsequently injected into the cleared fat pads of 10 syngeneic FVB mice (1.5×10^5 cells/gland). After 3 to 4 weeks, solid tumors could be palpated at the site of injection in 5 mice of the control

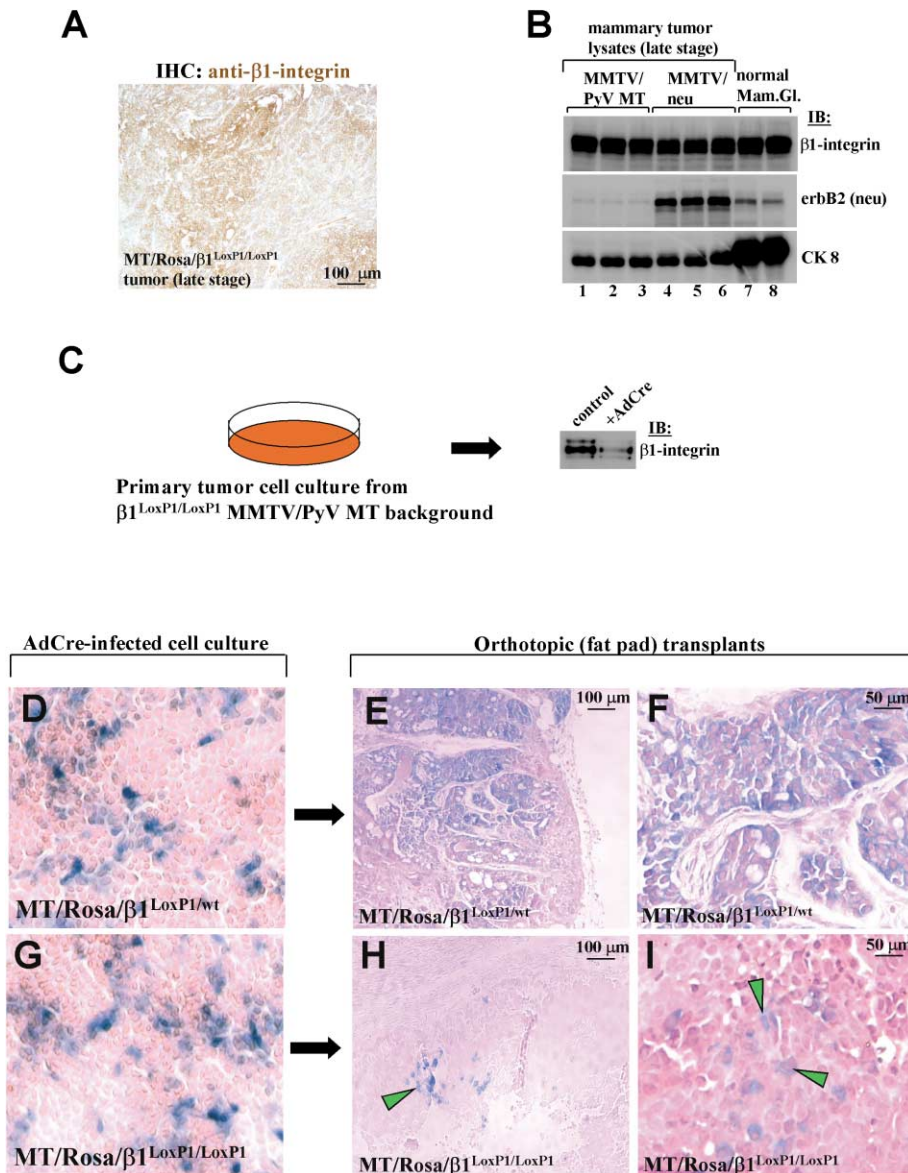


Figure 6. Cells expressing PyV MT are unable to contribute to tumor growth following loss of β 1-integrin

A: β 1-integrin (brown) is expressed throughout late-stage PyV MT-induced mammary tumors. This staining pattern is representative of multiple sections from PyV MT-induced tumors, prepared after 4 months of age.

B: β 1-integrin expression in MMTV/PyV MT-induced tumors (lanes 1–3), MMTV/erbB2 (neu)-induced tumors (lanes 4–6), and normal mammary glands (lanes 7 and 8). Levels of erbB2 (neu) protein are shown in the middle panel. CK8 was used as a control for protein loading and epithelial cell content.

C: Strategy for excising the β 1^{LoxP1} alleles from late-stage tumor cells, by infection with an AdCre vector.

D and G: Tumor cells were cultured from GTRosa26 β 1^{LoxP1/wt} MMTV/PyV MT (**D**) and GTRosa26 β 1^{LoxP1/LoxP1} MMTV/PyV MT (**G**) mice, infected with AdCre, and stained for Cre-induced β -galactosidase expression 3 days later (β gal-expressing cells appear blue). Eosin counterstain appears pink.

E, F, H, and I: Cells from **D** (**E** and **F**) and **G** (**H** and **I**) were injected into the cleared fat pads of syngeneic FVB mice. Resulting tumors were stained for β -galactosidase expression and sectioned (β gal-expressing cells appear blue). Eosin counterstain appears pink.

group, and 7 mice of the AdCre-infected β 1^{LoxP1/LoxP1} MMTV/PyV MT GTRosa26 culture. To assess the tumorigenic capacity of the AdCre-infected cells from either genotype, sections of tumor material from both sets of injections were stained for β -galactosidase activity. As expected, tumors derived from the AdCre-infected β 1^{LoxP1/wt} MMTV/PyV MT GTRosa26 control cells exhibited robust staining for β -galactosidase activity, indicating that mammary tumor cells retaining one functional copy of the β 1-integrin allele could contribute equally to the tumor mass in vivo (Figures 6E and 6F, blue staining). In contrast, very few β -galactosidase-positive cells were detected in animals transplanted with AdCre-infected β 1^{LoxP1/LoxP1} MMTV/PyV MT GTRosa26 tumor cells (Figures 6H and 6I, blue cells indicated by green arrowheads). The small patches of Cre-expressing cells present in these tumors, rather, seemed to delineate the site of inoculation (Figure 6H, green arrowhead). At higher magnification (Figure 6I), the Cre-expressing cells could be identified as single, isolated entities, showing no evidence of having undergone prolif-

eration during growth of the tumor (green arrowheads). These observations suggest that while tumor cells can survive in the absence of β 1-integrin expression, expression of the β 1-integrin subunit is essential for the proliferative expansion of PyV MT-induced tumor cells in vivo.

The impaired proliferative capacity of the AdCre-infected β 1^{LoxP1/LoxP1} MMTV/PyV MT tumor cells could be confirmed in vitro, by costaining an infected culture with antisera to both the Cre recombinase and the Ki67 proliferation marker (Figure 7A). As shown by the immunofluorescent staining pattern in Figure 7A, expression of Cre and of Ki67 was found to be mutually exclusive in the AdCre-infected cells. The absolute segregation of Cre and Ki67 in these cells was confirmed by the examination of multiple fields (Figure 7D). Parallel analysis of AdCre-infected β 1^{LoxP1/wt} MMTV/PyV MT control cells, however, revealed coexpression of Cre and Ki67 in a large proportion of the AdCre-infected cells (Figures 7B and 7D). Similarly, the nuclear localization of cyclin D1 was found to be impaired in

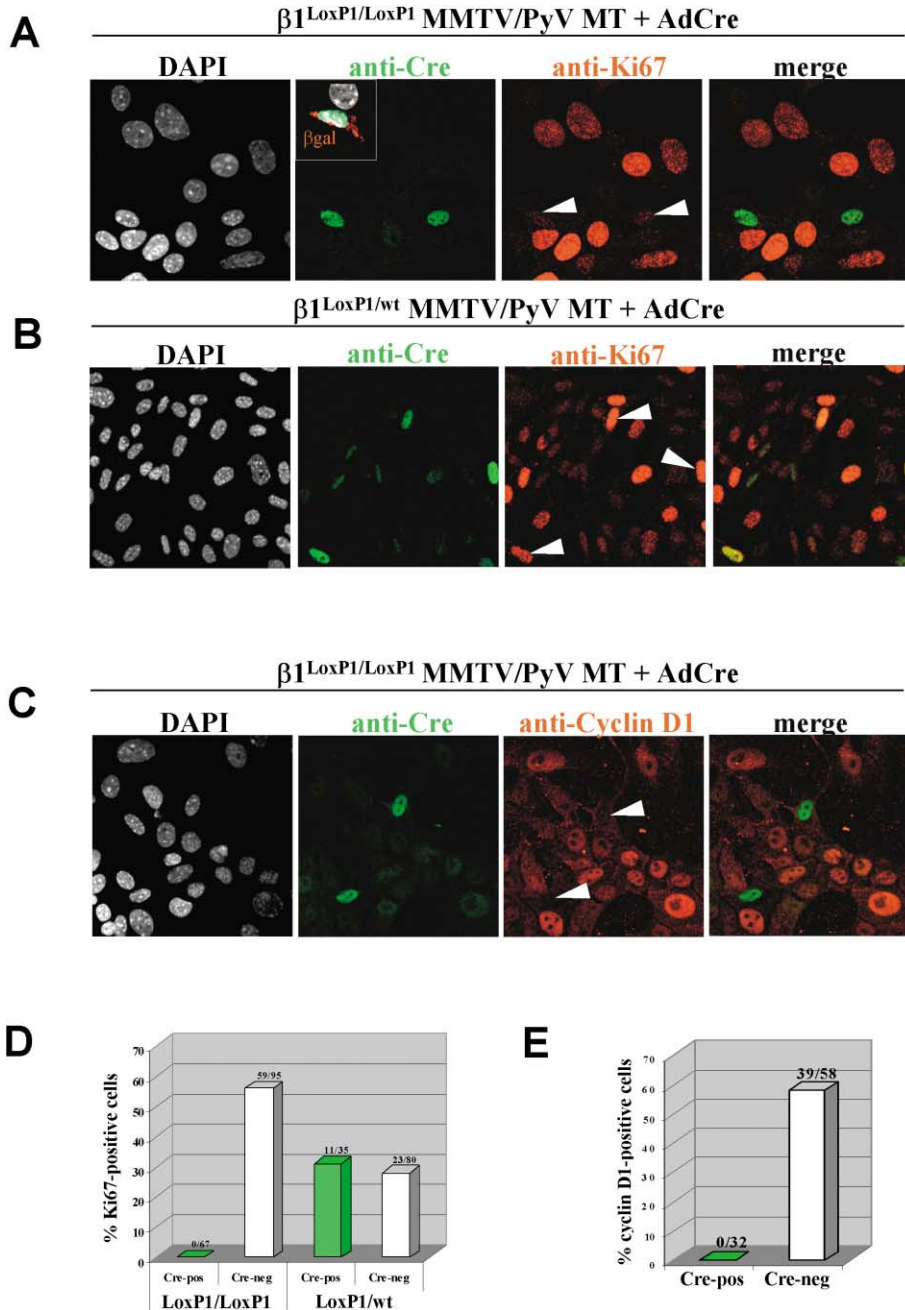


Figure 7. $\beta 1^{LoxP1/LoxP1}$ MMTV/PyV MT tumor cells infected with AdCre fail to express markers of cell proliferation

A: $\beta 1^{LoxP1/LoxP1}$ MMTV/PyV MT tumor cells were infected with AdCre, and stained for Cre (green) and Ki67 (red) expression. The locations of Cre-positive cells in the third panel are indicated by white arrowheads.

B: $\beta 1^{LoxP1/wt}$ MMTV/PyV MT control cells were infected and stained as above. The locations of Cre-positive cells in the third panel are indicated by white arrowheads.

C: AdCre-infected $\beta 1^{LoxP1/LoxP1}$ MMTV/PyV MT tumor cells were stained for Cre (green) and Cyclin D1 (red) expression. The locations of Cre-positive cells in the third panel are indicated by white arrowheads.

D and E: Graphical representation of the results shown in **A–C**, following analysis of multiple fields.

AdCre-infected tumor cells from $\beta 1^{LoxP1/LoxP1}$ MMTV/PyV MT mice (Figures 7C and 7E). Functional excision in Cre-expressing cells was confirmed by staining infected MMTV/PyV MT GTRosa26 cultures with antibodies to the β -galactosidase reporter protein (Figure 7A, second panel, inset). Taken together, the results of these experiments suggest that $\beta 1$ -integrin expression is required for the proliferation of PyV MT-induced tumor cells both in vivo and in vitro.

Excision of $\beta 1$ -integrin suppresses the tyrosine phosphorylation of FAK in PyV MT-transformed tumor cells

The ability of PyV MT to transform mammary epithelial cells has been shown to be dependent on its capacity to activate the

c-Src tyrosine kinase (Guy et al., 1994). As a result, we assessed the state of c-Src phosphorylation in PyV MT tumor cells carrying the $\beta 1^{LoxP1}$ alleles, following infection with the AdCre virus. Immunofluorescence microscopy of infected cells revealed that Cre expression did not impact on either PyV MT expression (Figure 8A) nor the distribution of tyrosine phosphorylated c-Src (compare Figures 8B and 8C, white arrowheads). In addition, immunoblot analysis using phosphospecific antibodies directed to tyrosine 416 (Y416) of c-Src revealed comparable levels of c-Src phosphorylation between control and AdCre-infected cells, in spite of reduced $\beta 1$ -integrin protein levels (Figure 8D). These results suggest that the suppression of proliferation in AdCre-infected $\beta 1^{LoxP1/LoxP1}$ MMTV/PyV MT tumor cells was not

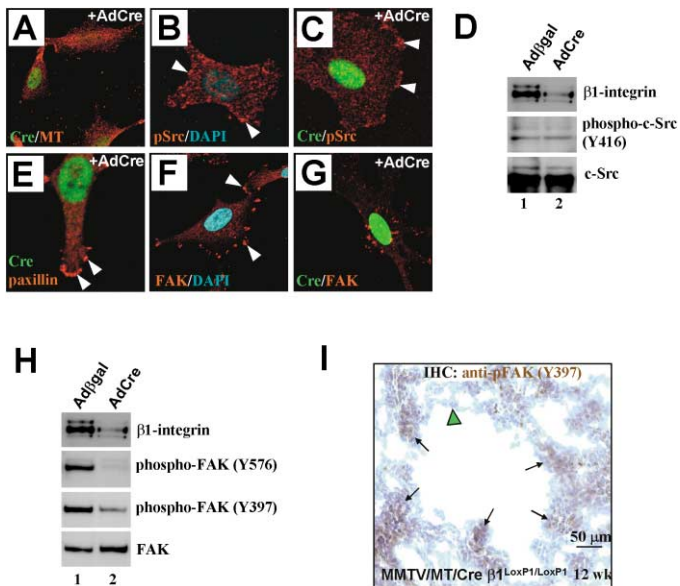


Figure 8. Ablation of $\beta 1$ -integrin expression suppresses FAK phosphorylation in PyV MT-induced tumor cells

A: Cre (green) and PyV MT (red) expression in AdCre-infected $\beta 1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumor cells.

B and C: Distribution of phospho-c-Src (Y416) (red) in uninfected (**B**) and AdCre-infected (**C**) $\beta 1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumor cells.

D: Phosphorylation status of c-Src (Y416) in Ad β gal-infected (lane 1) and AdCre-infected (lane 2) $\beta 1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumor cells.

E: Focal distribution of paxillin (red) in AdCre-infected $\beta 1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumor cells.

F and G: Distribution of FAK (red) in uninfected (**F**) and AdCre-infected (**G**) $\beta 1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumor cells.

H: Immunoblot analysis of Ad β gal-infected (lane 1) and AdCre-infected (lane 2) $\beta 1^{LoxP1/LoxP1}$ MMTV/PyV MT-derived tumor cells, using antibodies for $\beta 1$ -integrin (top panel), phospho-FAK (Y576), phospho-FAK (Y397), and FAK.

I: Distribution of phospho-FAK (Y397) (brown) in a mammary gland section from a 12-week-old $\beta 1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT mouse. Hyperplastic (black arrows) and nontransformed (green arrowhead) regions of the epithelium are indicated. This staining pattern is representative of multiple fields from 3 animals.

due to the inability of PyV MT to recruit and activate the c-Src tyrosine kinase.

We therefore decided to examine the phosphorylation status of FAK, which has been shown to be important for mediating the biological effects of both c-Src and $\beta 1$ -integrin (Gabarra-Niecko et al., 2003). In response to integrin engagement, FAK is phosphorylated at tyrosine residue 576 (Y576) in its kinase domain, followed by phosphorylation at the c-Src binding site (Y397) (Kornberg et al., 1992). We therefore examined the phosphorylation status of these 2 important tyrosine residues in the FAK molecule following AdCre infection of the $\beta 1^{LoxP1/LoxP1}$ MMTV/PyV MT tumor cells, by immunoblotting with phosphospecific antibodies. As shown in Figure 8H, phosphorylation of both Y576 and Y397 on FAK were found to be reduced in AdCre infected $\beta 1^{LoxP1/LoxP1}$ MMTV/PyV MT cells (Figure 8H, lane 2), as compared to Ad β gal-infected cells (Figure 8H, lane 1). Interestingly, the targeted disruption of $\beta 1$ -integrin did not interfere with the capacity of cultured MMTV/PyV MT tumor cells to form paxillin-containing focal adhesions (Figure 8E, white arrowheads). However, in contrast to cells retaining a functional $\beta 1$ -

integrin, where FAK was localized to peripheral focal adhesions (Figure 8F, white arrowheads), there was almost no FAK protein detected in the focal contacts of AdCre-infected $\beta 1^{LoxP1/LoxP1}$ MMTV/PyV MT cells (Figure 8G). Taken together, these observations argue that ablation of $\beta 1$ -integrin directly affects the tyrosine phosphorylation of FAK without disrupting the formation of focal adhesions. Given that activation of FAK has been implicated in cell cycle progression (Zhao et al., 1998), the altered tyrosine phosphorylation of FAK may account for the block in cell proliferation that occurs as a result of targeted inactivation of $\beta 1$ -integrin.

In order to confirm in vivo that the phosphorylation of FAK is important for tumorigenesis in $\beta 1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT mice, we performed immunohistochemistry on sections of glands from these animals, using antibodies specific for phosphotyrosine 397 of FAK (Figure 8I). Positive staining for phosphorylated FAK was clearly visible in regions of the glands undergoing hyperplastic proliferation (Figure 8I, black arrows), whereas phospho-FAK could not be detected in nontransformed regions of the epithelium (Figure 8I, green arrowhead). Since the distribution of phospho-FAK was found to overlap with the expression of $\beta 1$ -integrin in the glands of $\beta 1^{LoxP1/LoxP1}$ MMTV/Cre/PyV MT mice (compare with Figure 5H), these results argue that $\beta 1$ -integrin-mediated FAK phosphorylation may be necessary for PyV MT-induced mammary tumorigenesis in vivo.

Discussion

$\beta 1$ -integrin is dispensable for the initial stages of mammary ductal outgrowth

There is an increasing body of evidence implicating members of the integrin family as important signaling components involved in promoting the malignant growth of tumor cells (Wang et al., 2002). Here, we demonstrate that the functional integrity of $\beta 1$ -integrin is absolutely required for the induction of mammary tumors in a well-characterized transgenic mouse model of human breast cancer. We further demonstrate that ablation of $\beta 1$ -integrin in mammary tumor cells is associated with a proliferative block resulting in viable but dormant cancer cells. These observations provide compelling evidence that $\beta 1$ -integrin plays a critical role in tumor progression and dormancy.

The ablation of $\beta 1$ -integrin expression in the mammary epithelium, however, did not impair the initial stages of mammary gland outgrowth during puberty. In contrast to PyV MT-induced mammary tumors, whole-mount analyses of normal glands harboring the Cre-responsive GTRosa26 reporter construct suggested that $\beta 1$ -integrin-negative mammary epithelial cells were not subjected to a selective disadvantage in the mammary epithelium of these mice. The presence of β -galactosidase-positive cells in the lactating glands of these mice suggests that a $\beta 1$ -integrin-deficient stem cell population was maintained during development. These cells, however, seemed to be at a proliferative disadvantage following repeated rounds of pregnancy and lactation (data not shown). Consistent with these observations, the mammary-specific expression of a dominant-negative allele of $\beta 1$ -integrin was reported to have no obvious effects on mammary gland outgrowth in virgin animals, while resulting in a growth disadvantage during lactation (Faraldo et al., 1998, 2002). It is conceivable that other members of the integrin family are able to functionally compensate for loss of $\beta 1$ -integrin in the mammary epithelium during development, or that mammary

epithelial proliferation during ductal outgrowth is primarily dependent on exogenous hormones such as estrogen.

β 1-integrin is required for mammary tumor progression: Implications for human breast cancer

The targeted disruption of β 1-integrin was shown to have a profound effect on oncogene-induced mammary tumor development in the MMTV/PyV MT mice. By introducing the GTRosa26 β -galactosidase reporter construct into the genetic combination, we have shown that tumors which develop in the β 1^{LoxP1/LoxP1} MMTV/Cre/PyV MT background arise from cells which fail to express the MMTV/Cre transgene, and have therefore been selected to retain a functional β 1-integrin gene. Combined with the molecular analysis of these tumors, these results strongly suggest that β 1-integrin expression is required for the initial stages of PyV MT-induced mammary tumorigenesis *in vivo*.

It is important to point out that an initial report by Lin et al. (2003), involving immunohistochemical analysis of PyV MT-induced mammary lesions, indicated that β 1-integrin expression was lost during PyV MT-induced transformation. Here, however, we clearly show by both immunohistochemical and immunoblotting techniques that β 1-integrin protein is indeed robustly expressed throughout multiple samples of late-stage PyV MT-induced mammary tumors. Whether this difference reflects antibody choice or sample preparation is not clear, but an extensive analysis of both MMTV/PyV MT and MMTV/erbB2 (neu)-induced tumors clearly demonstrates that β 1-integrin expression is maintained in these oncogene-induced mouse mammary tumors. In addition, the expression of β 1-integrin protein in mammary tumors induced by overexpression of ILK (unpublished data) suggests that β 1-integrin may be important for tumor induction by a variety of oncogenes.

The ablation of β 1-integrin expression in established PyV MT-induced tumor cells results in inhibition of the proliferative capacity of these cells, both *in vivo* and *in vitro*. These observations are consistent with those of previous studies involving the phenotypic reversion of malignant cells by inhibition of β 1-integrin binding activity (Wang et al., 2002; Weaver et al., 1997). In both examples, reversion of the tumorigenic phenotype was associated with a block in cell proliferation, rather than induction of apoptotic fate (Wang et al., 2002; Weaver et al., 1997). Interestingly, engagement of the β 1-integrin-associated ILK has been shown to be important in promoting cell cycle progression through upregulation of cyclin D1 expression in various cell types, both *in vitro* and *in vivo* (D'Amico et al., 2000; Radeva et al., 1997; Terpstra et al., 2003; Troussard et al., 2003). Future studies involving the conditional inactivation of ILK in mouse mammary tumor models should allow us to elucidate the relative contribution of this β 1-integrin-associated kinase to mammary tumor progression *in vivo*.

In addition to ILK, the activation of FAK following β 1-integrin engagement has been shown to be critical for promoting entry into the cell cycle (Oktay et al., 1999; Zhao et al., 1998). Consistent with these observations, we have observed a decrease in the tyrosine phosphorylation status of FAK following ablation of β 1-integrin expression in PyV MT-induced tumor cells. A critical role for FAK during tumor progression has been noted in other tumor models, such as during the induction of papilloma formation during experimental mouse skin carcinogenesis (Mc-

Lean et al., 2001). Similar to the selection for retention of β 1-integrin expression in mammary tumors of MMTV/PyV MT mice, the papillomas that arose in FAK^{+/-} mice were found to be from cells overexpressing the FAK protein from the remaining allele (McLean et al., 2001). The specific requirement for FAK signaling during mammary tumorigenesis awaits future experimentation.

The retention of β 1-integrin null tumor cells in tumor cell transplants suggests that the primary function of β 1-integrin is to promote cell proliferation. In many respects, the presence of oncogene-induced tumor cells that are unable to proliferate but still retain viability bears a remarkable resemblance to the phenomenon of tumor dormancy. In this regard, it is interesting to note that the inhibition of FAK signaling has been shown to induce dormancy in human carcinoma cells *in vivo* (Aguirre Ghiso, 2002). Similarly, erbB2-induced mammary tumors that emerge from dormancy frequently acquire a spindle cell phenotype (Moody et al., 2002), which is also characteristic of mammary tumors that have been induced by elevated expression of ILK (White et al., 2001). It is tempting to speculate that integrin-coupled pathways, such as those involving ILK or FAK, may play an important role in the regulation of tumor dormancy. Whether activation of these pathways is involved in emergence of tumors from dormancy will require further studies.

The observation that β 1-integrin is required for mammary tumor progression in the MMTV/PyV MT transgenic mouse model of human breast cancer has important therapeutic implications for the treatment of human breast cancer. Given that therapeutic inhibitory antibodies directed against β 1-integrin have already been shown to be effective in reverting the tumorigenic phenotype of breast cancer cell lines (Wang et al., 2002; Weaver et al., 1997), it is conceivable that such therapeutic agents may be ideally suited for the treatment of primary breast cancers. Other potential therapeutic targets for blocking tumor development include integrin-coupled signaling pathways such as those involving ILK and FAK. In this regard, it has recently been demonstrated that pharmacological inhibition of ILK can interfere with prostate tumor growth by inhibition of tumor angiogenesis (Tan et al., 2004). The future development of therapeutic reagents against β 1-integrin and its coupled signaling pathways has excellent potential as an effective therapy to block cancer growth and recurrence.

Experimental procedures

Animals

MMTV/PyV MT, β 1^{LoxP1}, MMTV/Cre, and GTRosa26 mice were generated and characterized as described elsewhere (Andrechek et al., 2000; Graus-Porta et al., 2001; Guy et al., 1992; Soriano, 1999). The animals were housed in the Central Animal Facility at McMaster University, under compliance with the Animal Research Ethics Board (AREB). All animals were in the FVB genetic background.

Antibodies

Primary antibodies used in these experiments include anti- β 1-integrin rabbit polyclonal (M-106) (Santa Cruz), anti-CK-8 (NCL-CK8-TS1) (Novocastra), anti-Cre mAb (MMS-106A), and rabbit polyclonal (PRB-106C) (Covance), anti-Ki67 mAb (MM1) (Novocastra), anti-cyclin D1 mAb (72-13G) (Santa Cruz), anti-PyV MT mAb (Pab 762) (gift of Dr. Steven Dilworth), anti-FAK, anti-pFAK (Y576), anti-pFAK (Y397) rabbit polyclonals (Upstate), anti-c-Src mAb (GD11) (Upstate), and anti-pSrc (PyV416) polyclonal (Cell Signaling Technology).

PCR analysis of Cre-mediated recombination

For PCR analysis of Cre-mediated recombination of the $\beta 1^{\text{LoxP1}}$ allele, the sequence of the primers, as well as the PCR reaction conditions, are described elsewhere (Graus-Porta et al., 2001).

Mammary gland whole-mounts

Glands were processed overnight in acetone, and stained in Harris' hematoxylin (Fisher Scientific) for several hours (Webster et al., 1998). After dehydration in xylenes, glands were mounted using Permount (Fisher Scientific).

IHC and IF analysis of tissue sections

For paraffin-embedded tissue, samples were first deparaffinized in 3 changes of xylenes. Sections were heated in 10 mM sodium citrate (pH 6), followed by incubation in 3% H_2O_2 for 20 min. Samples were incubated in primary antibody (1:100) for 1 hr at RT, washed in PBS, and incubated in FITC- or Cy3-labeled (Molecular Probes) (IF) or HRP-conjugated (Jackson ImmunoResearch Laboratories) (IHC) secondary antibody (1:1000) for 30 min at RT.

In situ β -galactosidase assay

Glands were spread on 35 mm culture dishes and fixed for 1 hr in 2% PFA containing 0.25% glutaraldehyde and 0.01% NP-40. The glands were then rinsed in PBS and incubated in the Xgal staining solution ($1 \times$ PBS, 2 mM MgCl_2 , 0.01% sodium deoxycholate, 0.02% NP-40, 30 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 30 mM $\text{K}_4\text{Fe}(\text{CN})_6$, 1 mg/ml Xgal) overnight at RT. For sectioning, tumors and glands were stained as above then embedded in paraffin. Sections were counterstained with eosin for clear delineation of β -galactosidase-expressing cells.

Tumor cell explants and adenovirus infection

Excised tumors were finely chopped and incubated in 2 mg/ml collagenase B (Roche) for 1.5 hr at 37°C, with constant agitation. Tumor cell aggregates were then washed in DMEM (with spins at 800 rpm) and plated in 10% FBS/DMEM. Cells were infected with Ad vector (gift of Dr. Frank Graham) at 50 moi for 30 min in PBS^{+/+}. Analysis for β -galactosidase expression, Cre-mediated excision events, and protein phosphorylation was performed at 3 to 5 days postinfection.

Tumor cell transplants

Surgeries were performed in accordance with AREB guidelines. The #4 glands of anaesthetized, 3-week-old female FVB mice were surgically exposed under sterile conditions. Cells suspended in 10 μl sterile PBS were injected into the fat pad using a Hamilton syringe and 25⁵⁸ gauge needle, at a site proximal to the abdominal wall and distal to the nipple. The entire first quadrant of the fat pad, containing the lymph node and nipple, was then removed by cauterization. Mice were sutured and allowed to recover under supervision.

IF microscopy of cultured cells

Cells were fixed with 2% PFA for 20 min, and permeabilized with 0.5% Triton X-100 for 10 min at RT. Primary antibodies (1:100) were diluted in blocking buffer (0.2% Triton X-100, 0.05% Tween-20, 3% BSA in PBS), and applied to cells for 1 hr at RT. Cells were washed in PBS and incubated with FITC- or Cy3-labeled secondary antibodies (Molecular Probes) (1:1000 in blocking buffer) for 30 min at RT. Cells were counterstained with DAPI, and photographed on a Zeiss 510 confocal microscope.

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