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Mammary epithelial-specific disruption of the focal adhesion kinase blocks mammary tumor progression

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Elevated expression and activation of the focal adhesion kinase (FAK) occurs in a large proportion of human breast cancers. Although several studies have implicated FAK as an important signaling molecule in cell culture systems, evidence supporting a role for FAK in mammary tumor progression is lacking. To directly assess the role of FAK in this process, we have used the Cre/loxP recombination system to disrupt FAK function in the mammary epithelium of a transgenic model of breast cancer. Using this approach, we demonstrate that FAK expression is required for the transition of premalignant hyperplasias to carcinomas and their subsequent metastases. This dramatic block in tumor progression was further correlated with impaired mammary epithelial proliferation. These observations provide direct evidence that FAK plays a critical role in mammary tumor progression.

Results

Mammary-Specific Disruption of FAK Does Not Interfere with the Initial Stages of Mammary Ductal Outgrowth. Given the potential importance of FAK in mammary tumor progression, we first assessed whether mammary epithelial-specific ablation of FAK would impact normal mammary gland development. To accomplish this, we interbred conditional FAK knockout mice (10) to MMTV-Cre mice (5, 16), in which the Cre recombinase is under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter/enhancer. We have confirmed that the MMTV/Cre transgene is specific to the luminal epithelial compartment by breeding these mice to a Cre-inducible GFP reporter strain (17, 18). Immunofluorescence analyses of whole mounts derived from 6- to 8-week-old female mice with antibodies directed against myoepithelial keratin 14 or luminal keratin 8 markers revealed that GFP expression was detected solely in the luminal epithelial compartment (supporting information (SI) Fig. 6). Whole-mount analyses on mammary glands from 6- to 8-week-old MMTV-Cre virgin mice heterozygous or homozygous for a floxed fak allele (FAKlox/wt or FAKlox/lox) were then performed. Examination of mammary glands from these animals revealed that ablation of one or both fak alleles had no effect on the initial mammary ductal outgrowth (SI Fig. 7). To ensure that the absence of a mammary gland phenotype was not due to the lack of efficient Cre-mediated FAK deletion in the mammary epithelium, we performed both PCR and immunoblot analyses on whole mammary gland tissues from 6- to 8-week-old virgin animals (SI Fig. 8). The results demonstrate that mice carrying the MMTV-Cre transgene and one or both conditional floxed fak alleles exhibited evidence of fak excision (SI Fig. 8 A) that correlated with a decrease in FAK tyrosine phosphorylation (5). FAK is one of the major components of focal adhesion complexes. It regulates integrin-mediated cell adhesion and migration and is involved in the control of cell proliferation and survival (6–8). In a normal physiological context, engagement of integrin receptors by extracellular matrix proteins results in the rapid activation of Src and tyrosine phosphorylation of FAK (3, 4). FAK, in turn, phosphorylates downstream substrates, such as paxillin, on tyrosine residues and facilitates the recruitment of scaffold proteins like p130Cas (6).

Several reports have shown that FAK is overexpressed in different types of cancer, with its levels of expression often associated with advanced disease (7, 9). In vivo, the importance of FAK in tumorigenesis was established in a study in which fak was selectively deleted in the epidermis. This conditional ablation of FAK resulted in the complete block in the progression of benign papilloma lesions to malignant carcinomas in a skin carcinogenesis model (10). There is an increasing body of evidence implicating FAK activation in the pathogenesis of human breast cancer. For instance, elevated expression of FAK has been associated with malignant transformation in human breast cancers (11, 12). Also, overactivation of FAK has been observed in numerous established breast cancer cell lines (13–15). To date, however, there is no direct evidence linking FAK expression and activation to mammary tumorigenesis. For this purpose, conditional floxed (loxP-flanked) fak alleles were introduced into MMTV-Cre/PyVmT mice. This strategy allowed us to further elucidate the relative contribution of FAK as a downstream effector of integrins in PyVmT-induced mammary tumorigenesis. By contrast to mammary-specific deletion of β1-integrin, mammary hyperplasias lacking FAK expression could be readily detected. However, targeted disruption of FAK prevented progression of these hyperplastic lesions to carcinomas. Our results suggest that FAK function plays a critical role in mammary tumor progression.
significant reduction in FAK protein levels (SI Fig. 8B). Moreover, excision of one floxed \textit{fak} allele is sufficient to cause a reduction in FAK protein expression. Residual FAK expression reflects the stromal contribution to FAK, given that the mammary epithelial constitutes 20% of virgin gland. To confirm that the loss of expression was restricted to the mammary epithelium, we performed immunohistofluorescence analyses on mammary gland paraffin sections using both Cre and FAK-specific antisera (SI Fig. 8C). In FAK\textsuperscript{lox/lox}/MMTV-Cre mice, mammary epithelial cells that were positive for Cre expression completely lacked detectable FAK expression. As expected, mice heterozygous for the floxed \textit{fak} allele still retained some FAK protein staining. Careful quantification of the number of ductal cells positive for Cre and lacking FAK expression in FAK\textsuperscript{lox/lox}/MMTV-Cre mice revealed that 64.3 ± 15.6% (n = 5) of the luminal epithelial cells had undergone functional excision. Consistent with FAK immunostaining, introduction of the GTRosa26 Cre-responsive \(\beta\)-galactosidase allele (19, 20) in FAK\textsuperscript{lox/lox} and FAK\textsuperscript{lox/lox}/MMTV-Cre mice revealed that the majority of the mammary epithelium in mice homozygous for the conditional floxed \textit{fak} allele had undergone functional excision as evidenced by robust X-Gal staining (SI Fig. 9). Taken together, these observations suggest that FAK expression is dispensable for the initial stages of normal mammary ductal outgrowth.

**Targeted Disruption of FAK in Mammary Epithelium Impairs Mammary Tumor Development in the MMTV-PyVmT Mouse Tumor Model.** We next investigated whether mammary-specific disruption of FAK would impact mammary tumor development. To accomplish this, we interbred the conditional floxed FAK strain with mice expressing the PyVmT oncogene and Cre, both under the transcriptional control of the MMTV promoter.

To determine whether a functional FAK was required for tumor development, the MMTV-PyVmT transgene was introduced into MMTV-Cre mice harboring either one or two copies of the floxed \textit{fak} allele (FAK\textsuperscript{lox/lox}/MMTV-Cre/-PyVmT and FAK\textsuperscript{lox/lox}/MMTV-Cre/-PyVmT, respectively). Cohorts of female mice bearing these different genotypic combinations were subsequently monitored for mammary tumor development by physical palpation. Compared with MMTV-Cre/-PyVmT mice bearing wild-type \textit{fak}
alleles, initial development of mammary tumors appeared to be marginally delayed in MMTV-Cre/PyVmT mice homozygous for the floxed fak allele by ~1 week (Fig. 1A). Mice heterozygous for the conditional fak allele also developed tumors with delayed onset, albeit at an intermediate point between control and homozygous floxed fak carriers (Fig. 1A). Consistent with these observations, whole-mount analyses of mammary glands derived from these cohorts at 6 and 10 weeks of age revealed that excision of both floxed fak alleles resulted in a dramatic reduction in the number of PyVmT-induced mammary hyperplastic lesions (SI Fig. 10B and Fig. 1B). Quantitative measurements revealed that, compared with the FAK^{wt/}MMTV-Cre/-PyVmT control animals, there was a dramatic reduction both in the mammary gland average mass (SI Fig. 10A and Fig. 1B) and in the percentage of the total mammary gland area occupied by hyperplastic nodules in the FAK^{lox/lox}/

Fig. 3. FAK deletion is restricted to MMTV-PyVmT hyperplasias. (A) Immunohistochemistry analysis of Cre and FAK expression on paraffin sections of mammary glands from 10-week-old FAK^{wt/} (A Upper), FAK^{lox/} (A Lower), and FAK^{lox/}MMTV-Cre/-PyVmT (B) mice. Data are representative of at least five animals of each genotype. FAK is coexpressed with Cre throughout the MMTV-PyVmT-derived solid tumor tissue (A Upper). Note the reduced level of FAK expression in the Cre-positive/FAK^{lox/} tumoral cells. In FAK^{lox/}MMTV-PyVmT glands, Cre-positive/FAK-negative cells are detectable only in hyperplastic (B Upper) or nontransformed ductal structures (B Lower, arrows). FAK expressing cells are present in solid nests of tumor cells (B Lower, asterisk). (Scale bars: 20 μm.)

MMTV-Cre/-PyVmT/ mice (SI Fig. 10B and Fig. 1C). Taken together, these observations suggest that mammary-specific ablation of FAK has an important impact on mammary tumor progression in the MMTV-PyVmT mice.

One potential explanation for the emergence of mammary tumors in the “FAK-deficient” strain is that, because of the stochastic expression of the MMTV-Cre transgene, there is a selection for mammary epithelial that retain functional fak alleles due to Cre expression. Indeed, in our previous studies of mammary-specific deletion of β1-integrin in the PyVmT mouse model, tumors that eventually arose were derived from this “escape” population of Cre-negative mammary epithelial cells (5). To test this possibility, we introduced the GTRosa26 Cre-inducible β-galactosidase reporter (5, 19, 20) into the different fak genetic backgrounds. This strategy allowed us to follow the fate of individual Cre-expressing cells during mammary tumorigenesis by staining mammary gland whole mounts from 10-week-old virgin mice with X-Gal. As expected, the results revealed that in the FAK^{lox/lox}/MMTV-Cre/-PyVmT/GTRosa26 background, Cre-expressing cells were present in the untransformed ductal structures (Fig. 2A). However, both unstained (Fig. 2B, arrow) and X-Gal-stained (Fig. 2B, arrowhead) mammary hyperplastic lesions were found in these mice. Histological analyses allowed us to determine more precisely the pattern of MMTV-Cre expression in the mammary glands of the FAK^{lox/}/MMTV-Cre/-PyVmT/GTRosa26 mice. Mammary gland sections obtained from 10-week-old control FAK^{wt/}/MMTV-Cre/-PyVmT/GTRosa26 mice revealed cells stained with X-Gal in both hyperplastic structures (Fig. 2C, arrowheads) and solid nests of tumor cells (Fig. 2C, asterisks). By contrast, mammary-specific deletion of FAK in age-matched glands resulted in induction of mammary tumors that lacked robust X-Gal staining in solid tumor regions (Fig. 2D, asterisks) but retained β-galactosidase expression in hyperplastic lesions (Fig. 2D, arrowheads). Consistent with these observations, examination of mammary-gland sections obtained from 12-week-old FAK^{lox/}/MMTV-Cre/-PyVmT/GTRosa26 females (4- to 6-week tumor-bearing animals) showed that, whereas late-stage tumors were uniformly β-galactosidase-negative (Fig. 2E and F, asterisks), only untransformed ductal structures (Fig. 2E, arrows) or hyperplastic lesions (Fig. 2F, arrowhead) exhibited evidence of homozygous deletion of FAK. Thus, these observations suggest that emergence of the early hyperplastic lesions can occur in the absence of FAK. However, given the lack of β-galactosidase expression in the carcinoma regions, these results suggest that FAK
expression is required for the conversion of these early lesions into a fully malignant phenotype.

**FAK-Deleted Cells Do Not Contribute to Either Mammary Carcinoma Formation or Lung Metastasis.** To confirm that FAK expression was confined to hyperplastic lesions, we performed immunohistochemistry (SI Fig. 11) and immunofluorescence analyses on sections of transformed mammary gland from mice wild-type, heterozygous, or homozygous for the floxed fak allele using both FAK-specific antisera. Consistent with the X-Gal staining pattern obtained (Fig. 2F), only early hyperplastic lesions retained expression of Cre in the samples from FAK\textsuperscript{flox/flox} mice (Fig. 3B Upper and SI Fig. 11C Left). Moreover, coimmunostaining (Fig. 3) or staining of sequential sections for FAK expression (SI Fig. 11) revealed that these lesions have undergone efficient excision of the floxed fak alleles. By contrast, examination of solid carcinoma areas revealed an absence of Cre expression that was correlated with retention of FAK staining (Fig. 3B Lower, asterisk, and SI Fig. 11C, asterisk). Moreover, in the control FAK\textsuperscript{wt/wt} and heterozygous FAK\textsuperscript{flox/wt} samples, these solid tumoral areas retain both Cre and FAK expression although with a reduced level of FAK expression in the FAK\textsuperscript{flox/wt} mice (Fig. 3A and SI Fig. 11 A and B). These observations indicate that mammary hyperplastic cells lacking functional copies of the fak allele could not contribute to carcinoma formation.

An important characteristic of PyVmT-induced mammary tumors is that they frequently metastasize to the lung (21). Moreover, FAK expression and/or activity have been associated with increased invasion and metastatic potential (6, 7, 22–24). To investigate whether mammary-specific ablation of FAK can affect the metastasis of PyVmT-induced mammary tumors, we first evaluated the number of metastatic lesions per lung in MMTV-Cre/-PyVmT mice homozygous for either the wild-type or floxed fak allele. The percentage of 8-week tumor-bearing mice that developed lung metastases was not significantly different between wild-type and FAK\textsuperscript{flox/flox}/MMTV-Cre/-PyVmT mice [93.3% (n = 15) and 83.3% (n = 18), respectively]. Furthermore, the number of metastatic lung lesions per lobe between the two groups was the same (8.3 ± 2.8 and 7.5 ± 2.3, respectively). To assess whether these metastatic lesions could derive from primary hyperplastic lesions lacking FAK, we next examined both Cre and FAK expression patterns using X-Gal and immunohistochemical staining of lungs harvested from wild-type, heterozygous, or homozygous floxed fak PyVmT mice. β-Galactosidase assay on lung lobes from FAK\textsuperscript{wt/wt}/MMTV-Cre/
PyVmT/GTRosa26 mice revealed that these lung metastatic lesions readily stained blue in the presence of X-Gal (Fig. 4 Left). Indeed, 62.5 ± 28.6% (n = 5) of the lung lesions from control animals expressed β-galactosidase. Consistent with this finding, immunohistochemical staining of lung sections with specific antibodies revealed both Cre and FAK expression among the metastatic lesions in the wild-type animals (SI Fig. 12 A and B Left). By contrast, none of the lung metastases from mice bearing both conditional floxed fak alleles (n = 8) exhibited either X-Gal staining or Cre antibody labeling. Consistently, the metastatic lesions lacking Cre expression retained robust FAK antibody labeling (Fig. 4 Right and SI Fig. 12 A and B Right). However, lung lesions from mice bearing a single conditional floxed fak allele displayed both Cre and FAK immunostaining (SI Fig. 12 A and B Center). Thus, just as the PyVmT-induced mammary carcinoma development depended on the presence of FAK, the formation of lung metastases in mice homozygous for the floxed fak allele also requires the retention of functional FAK alleles.

**Mammary-Specific Disruption of FAK Results in a Dramatic Reduction in the Proliferative Potential of PyVmT Hyperplastic Cells.** To investigate the observed defect in mammary tumor progression in the FAK-deficient strains, we measured the proliferative potential of hyperplastic mammary cell aggregates by performing immunofluorescence staining of mammary gland sections using antibodies against both Ki67, a conventional proliferation marker, and Cre. To ensure that the mammary glands examined correspond to the early phases of oncogenic transformation, we used only nontumor-bearing mice in this analysis. We first assessed whether lack of FAK expression had an impact on the proliferative potential of the MMTV-PyVmT hyperplastic cells. The results showed that >50% of the Cre-positive hyperplastic cell aggregates arising in wild-type mammary glands exhibited robust Ki67 communostaining (Fig. 5 A Top and C). However, the number of Cre and Ki67 double-positive cells was significantly lower in FAK1/–/–MMTV-Cre+/PyVmT mammary glands (Fig. 5 A Middle and C). Moreover, staining of sections prepared from “FAK-deficient” glands exhibited a dramatic reduction (i.e., 16.5%) in the number of Cre-positive cells that also expressed Ki67 (Fig. 5 A Bottom and C).

Using an identical approach, we looked at the apoptotic status of these mammary glands by performing activated caspase-3 immunostaining analyses. Unlike the strong defect in Ki67 staining, the Cre-positive hyperplastic cell aggregates observed on FAK1/–/– and FAK1/–/–/MMTV-Cre+/PyVmT cross-sections failed to exhibit evidence of enhanced apoptotic rate (Fig. 5 B and D). Taken together, these observations suggest that the dramatic defect in FAK1/–/–/MMTV-Cre+/PyVmT tumor progression is caused by a lack of FAK expression leading to a reduced proliferative capacity of PyVmT tumor cells.

**Discussion**

Activation of FAK has been associated with breast cancer progression. Indeed, a large proportion of primary human breast cancers possess elevated FAK expression that is further correlated with malignant transformation (11, 12, 24) and poor clinical outcome (22, 23). We demonstrate that targeted disruption of FAK in mammary tumor cells can block progression of hyperplasias to full carcinomas. We further show that this block in tumor progression is correlated with a dramatic reduction in the ability of the mammary tumor cells to proliferate. These observations provide compelling evidence that activation of FAK plays a critical role in mammary tumorigenesis.

Whereas targeted ablation of FAK negatively impacts on malignant progression, mammary epithelial-specific loss of FAK did not affect the initial stages of mammary gland outgrowth. This latter observation contrasts a recent study where a similar mammary gland FAK conditional knockout model was used to show that fak deletion delays mammary ductal elongation and induces lactation defects (25). This divergence is plausibly due to the different background strains (C57BL/6) and MMTV-Cre transgenic mice (line F) used. In our model, we have shown that the lack of an obvious mammary ductal outgrowth defect was due to absence of efficient and functional excision of the conditional floxed fak alleles, as evidenced by PCR analysis, expression of β-galactosidase, and, finally, immunohistofluorescence analyses showing the presence of Cre and the lack of FAK expression in the mammary epithelium. Consistent with these observations, mammary-specific ablation of β1-integrin, which functions upstream of FAK, has also been shown to have little phenotypic impact on the initial stages of ductal outgrowth (5). However, mammary-specific ablation of β1-integrin during the later stages of differentiation resulted in impairment of lactation due to an alteration of mammary epithelial proliferation (26, 27). Although FAK ablation does not affect the ability of mice to lactate, the impact of FAK disruption on the proliferative potential of differentiated mammary epithelial remains to be addressed.

Mice lacking a functional FAK develop mammary tumors, yet there is a dramatic attenuation in the number of hyperplastic lesions. Significantly, those homozygous floxed fak late-stage tumors and their derived lung metastases arise from cells that do not express Cre recombinase and thus have failed to undergo functional excision of the floxed fak alleles. This suggests that FAK function is critical for mammary tumor progression in the PyVmT transgenic mouse model. Consistent with these observations, we have demonstrated that targeted disruption of β1-integrin results in a similar block in tumor progression (5). However, unlike the β1-integrin-deficient strains, mammary-specific deletion of FAK did not hinder the emergence of preneoplastic adenomas. These observations are consistent with the previous findings showing that conditional deletion of fak in the epidermis interferes with the malignant conversion of benign papillomas to carcinomas in a mouse skin carcinogenesis model (10). One key difference between the skin carcinogenesis model and PyVmT mammary tumor model is that the inability of mammary hyperplasias to progress to carcinomas was associated with a decrease in epithelial proliferation, whereas the defect in skin tumorigenesis was associated with enhanced apoptosis (10). Whether the disparity in the response of these mouse tumor models to loss of FAK reflects intrinsic tissue specificity or differences in the nature of the carcinogenic factor used (chemical vs. oncogene) remains to be elucidated. However, our data are consistent with results obtained using MTLn3 rat mammary adenocarcinoma cells expressing FAK-related nonkinase (FRNK), a dominant-negative splice variant of FAK. Indeed, in syngeneic tumors obtained after injection of these cells into the mammary fat pad, van Nimwegen et al. (23) have shown that FRNK-induced expression decreased the primary tumor growth without causing apoptosis. It would also be interesting to assess whether the observed decrease in proliferation could lead to apoptotic cell death at later time points, because apoptosis was assayed on mammary glands from 6-week-old animals.

The finding that mammary-specific deletion of fak impacts on the proliferative capacity of the PyVmT hyperplastic cells is consistent with previous observations showing that mammary-specific ablation of β1-integrin resulted in a proliferative arrest (5). Given the phenotypic similarities between β1-integrin- and FAK-deficient mammary tissues in the PyVmT mouse model, activation of FAK appears to be one of the critical signaling events immediately downstream of β1-integrin. Indeed, loss of β1-integrin is associated with dramatic down-regulation of tyrosine phosphorylation of FAK (5). However, given that the β1-integrin-deficient PyVmT mice failed to develop mammary epithelial hyperplasias, whereas FAK-deficient cells are still capable of forming hyperplastic lesions, there must be other FAK-independent β1-integrin-coupled signaling pathways that are also involved in mammary tumor formation. For instance, the ILK pathway seems to be a very good candidate (2). We have actually shown, using a mammary-specific ILK conditional
knockout mouse model, that ILK deletion has significant effects on PyVmt-induced mammary tumor progression without affecting FAK activity (unpublished data).

Another interesting observation is that FAK-deficient hyperplasias are still detectable as distinct entities in late-stage tumor samples. Remarkably, it seems the phenomenon of tumor dormancy observed in T-HeP3 human carcinoma cells after inhibition of FAK signaling (28).

The observation that FAK plays a critical role in tumor progression in the MMTV-PyVmt mouse tumor model may have important therapeutic implications for the treatment of human breast cancer. Given the large proportion of human breast cancers that express elevated levels of FAK (11, 22), inhibition of FAK catalytic activity with small-molecule inhibitors (29, 30) may be an important strategy in the management of the disease. In this regard, there have been several reports suggesting that inhibition of FAK can have a profound effect on tumor growth and invasion (23, 24, 29, 31–33). The future development of therapeutic reagents directed against FAK and its coupled signaling pathways has excellent potential for the treatment of primary breast cancers.

**Methods**

**Transgenic Mice.** MMTV-PyVmt, MMTV-Cre, and FAKlox mice were generated and characterized as described (10, 16, 21). GTRosa26 mice were purchased from The Jackson Laboratory and are described in refs. 5 and 19. The CAG-CAT-EGFP reporter mice are described in refs. 17 and 18. To preclude the possible involve-

ment of genetic background variability, all transgenic mice used were derived from the inbred FVB/N strain. Mammary tumor formation was monitored in virgin mice by biweekly palpation. All experiments involving animals were conducted in accordance with McGill University animal care guidelines.

**Antibodies.** Primary antibodies used in these experiments include rabbit poly-

clonal anti-FAK (06–543, Upstate Biotechnology), anti-cre recombinase (PRB-106C, Covance), anti-cleaved caspase-3 (9661, Cell Signaling), anti-cytokeratin 14 (clone anti-FAK (06–543, Upstate Biotechnology), anti-Cre recombinase (PRB-106C, Covance) and anti-β-actin (AC-15, Sigma). Guinea pig polyclonal anti-cytokeratin 8/18 (BD-RIDPROG11) was purchased from Fitzgerald. The Alexa Fluor goat anti-guinea pig 555 (A-21435), goat anti-mouse 555 (A-21422), 633 (A-21050), goat anti-

rabbit 555 (A-21428), and 633 (A-21070) antibodies were from Molecular Probes (see **Text**).

**Histological and Immunohistochemical Analysis of Tissue Sections.** Thoracic mammary glands or lung lobes were fixed in 10% neutral buffered formalin (Surgipath) and transferred to 70% ethanol the next day. Samples were then paraffin-embedded and sectioned at 5 μm. Endogenous peroxidase activity was blocked with 3% peroxide hydrogen in methanol. Antigen retrieval was accom-

plished in citrate buffer and by using a pressure cooker (Cuisinart). Sections were then blocked with Power Block Universal Blocking Agent (Biogenex) and incubated in primary antibody as described (5). Lungs were harvested from mice bearing tumors for 8 weeks. Pulmonary metastases were identified by micro-

scopic analyses of hematoxylin/eosin-stained lung step sections. For immunoflu-

orescence analyses, sections were incubated with secondary antibodies for 1 h at room temperature followed by incubation with DAPI (Sigma) for 10 min. They were visualized by using a Zeiss LSM 510 META confocal microscope.

**Mammary Gland Whole Mounts and β-Galactosidase Assay.** Whole mounts were prepared from the no. 4 inguinal mammary glands as described (5). For in situ β-galactosidase assay, no. 3 thoracic and no. 4 inguinal glands were processed as described (5). For X-Gal staining of tumor cryosections, small pieces of tumors were placed in plastic molds and frozen in Tissue-Tek OCT (Surgipath) by using liquid nitrogen. Samples were sectioned at 8 μm using a HM 500 OM microtome cryostat (Micron). Sections were dried for 1 h at room temperature, fixed in PBS/0.2% glutaraldehyde (Sigma) for 10 min, rinsed in PBS, and incubated in X-Gal staining solution (2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% Nonidet P-40, 5 mM K4Fe(CN)6, 5 mM K3Fe(CN)6, and 1 mM mg/mL X-Gal (Invitrogen) in PBS (pH 7.4) overnight at 37°C. Sections were fixed again, rinsed, dehydrated through a graded series of ethanol baths and xylenes, and mounted with Immu-mount (Shandon). X-Gal-stained paraffin sections or cryosections were counterstained with Nuclear Fast Red.

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