Supporting Information

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SI Text

Immunohistochemistry and Immunofluorescence. Immunohistochemical staining of lung sections was done by using anti-human Ki-67 antibody (BD PharMingen, 1:50) or cleaved caspase-3 (Asp-175) antibody (Cell Signaling, 1:100). Slides were stained with secondary antibody and developed by using either the mouse IgG or rabbit IgG Elite Vectastain ABC kit (Vector Labortories). Slides were counterstained with hematoxylin. Immunofluorescent staining of tissues and cells was performed as described (1, 2) by using antisera against proSP-C (Chemicon clone 3786 at 1:800) and Scgb1a1 (Santa Cruz T-18, 1:100). Secondary antibodies used were Alexa Fluor 594 donkey anti-rabbit and Alexa Fluor 488 donkey anti-goat at 1:200 (Molecular Probes).

Quantitative RT-PCR. RNA was prepared from total lung cells that were FACS sorted from single-cell suspensions by using the RNeasy Mini Kit (Qiagen). RNA was also prepared from ≈50,000 CD45^{neg} PECAM^{neg} Sca-1^{pos} cells, bronchiolalveolar stem cells (BASC)-enriched population, by using the Absolute RNA Nanoprep Kit (Stratagene). Reverse transcription and quantitative RT-PCR by using SYBR Green PCR Master Mix (Applied Biosystems) was performed. PCR primers used were: p19^{ARF} forward 5′-CACCGGAATCCTGGACCAG-3′ and reverse 5′-GCAGTTCGAATCTGCACCGT-3′; p16 (Cdkn2a) forward 5′-CATCTGGAGCAGCATGGAGTC-3′ and reverse 5′-GGGTACGACCGAAAGAGTTCG-3′; and ubiquitin forward 5′-TGGCTATTAATTATTCGGTCTGCAT-3′ and reverse 5′-GCAAGTGGCTAGAGTGCAGAGTAA-3′

Analysis of BASC Growth on Matrigel. BASCs (CD31- CD45- Sca-1+ cells) were isolated from Bmi1 WT or mutant mice of 5–8 weeks of age. One hundred microliters of thawed Matrigel (BD Biosciences) was plated per well of a 96-well culture dish and allowed to solidify at 37°C for 30 min. BASCs were resuspended in DMEM (GIBCO) and plated at a density of 10,000 cells per matrigel-containing well. Cells were cultured for 8–12 d. Cells were then fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences), blocked for 1 h in 10% normal donkey serum, and immunofluorescently labeled for Clara cell secretory protein (CCSP) and surfactant protein C (SP-C) (each at 1:100 dilutions). Matrigel plugs were then independently removed from each well, transferred to concave slides, treated with Vectashield + DAPI (Vector Laboratories), and imaged by using an Eclipse 90i upright fluorescence microscope (Nikon). Optical slices of cell clusters on Matrigel were imaged for deconvolution and maximum projection images are shown.

Lung Injury Analysis. Corn oil vehicle control or naphthalene (Fluka) at a dose of 200–275 mg/kg mouse weight was administered by i.p. injection to 6–8-week-old mice. Treated mice were killed at various time points, ranging from 2 d to 1 month after administering naphthalene. Lungs were initially fixed by intratracheal instillation of formalin, followed by further fixation overnight in formalin. Lungs were processed for histology by transferring to 70% ethanol, embedding in paraffin, and sectioning tissue at a thickness of 5 μm. After immunofluorescent labeling of Scgb1a1 and SP-C in lung sections, damage/repair was assessed by counting the number of cells positive for Scgb1a1 and intact DAPI-positive nuclei, both within the first 100 μm of the BADJ, and scoring all distinguishable terminal bronchiolar regions (12–30) within a given section.

RNA Interference in BASC Cultures. Retrovirus was made from murine stem cell virus mIR30-based-GFP shRNA vectors with either an empty hairpin or p16/p19^{ARF} hairpin (target sequence-CCCGCTGGGTGCTCTTTGTGTT). BASC-enriched populations were sorted by flow cytometry and plated on irradiated feeders as above. Four days after plating, cultures were infected with empty or p16/p19^{ARF} retrovirus by spinfection (cultures in 96-well plates with retrovirus plus 8 μg/ml polybrene were spun at 1,000G for 1 h). Formation of GFP-positive colonies was assessed at 1 week (3 days after infection). GFP-positive cells were replated for secondary colony forming assays.

1. Kim CF, *et al.* (2005) Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell* 121:823–835.

2. Johnson L, *et al.* (2001) Somatic activation of the K-ras oncogene causes early onset lung cancer in mice. *Nature* 410:1111–1116.

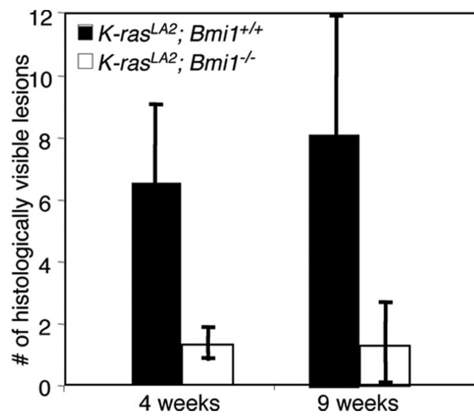

Fig. S1. Number of lesions (of all grades) visible on a single histological section per animal in 4-week and 9-week-old *K-ras*^{LA2};*Bmi1*$^{+/+}$ and *K-ras*^{LA2};*Bmi1*$^{-/-}$ mice.

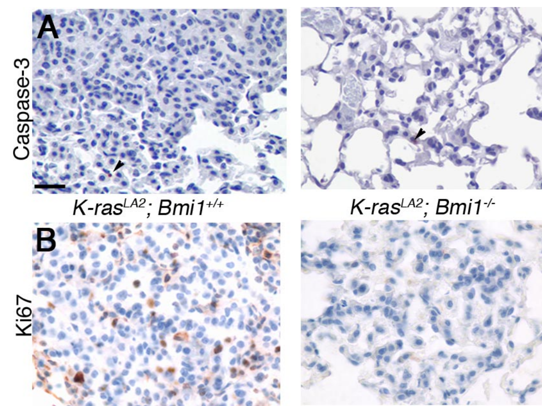

Fig. S2. Bmi1-null lungs have increased proliferation but no increased apoptosis. (*A*) Immunohistochemical staining by using an antibody against cleaved caspase-3 to measure the number of apoptotic cells (marked by arrowhead). (*B*) Immunohistochemical staining by using an antibody against Ki67 to mark cycling cells. (Scale bar, 20 μm.)

Fig. S3. Hematoxylin and eosin (H&E) images of lesions present in *K-ras*^{LA2}; *Bmi1*$^{-/-}$;*Arf*$^{-/-}$ lungs (*Top*) and *K-ras*^{LA2}; *Bmi1*$^{+/+}$;*Arf*$^{-/-}$ lungs (*Bottom*). The *Upper Left* lesion is an atypical adenomatous hyperplasia similar to those seen in Arf WT animals. The *Upper Middle* and *Upper Right* lesions are both higher grade lesions that previously seen in the Bmi1-null; Arf WT animals. The *Bottom Left* lesion is predominantly a Grade 2 adenoma (with glandular differentiation) with a smaller area of Grade 3 adenocarcinoma (note nuclear atypia) in the upper left portion of the lesion (this portion is enlarged in the *Bottom Middle* image). The *Bottom Right* lesion is an advanced adenocarcinoma.

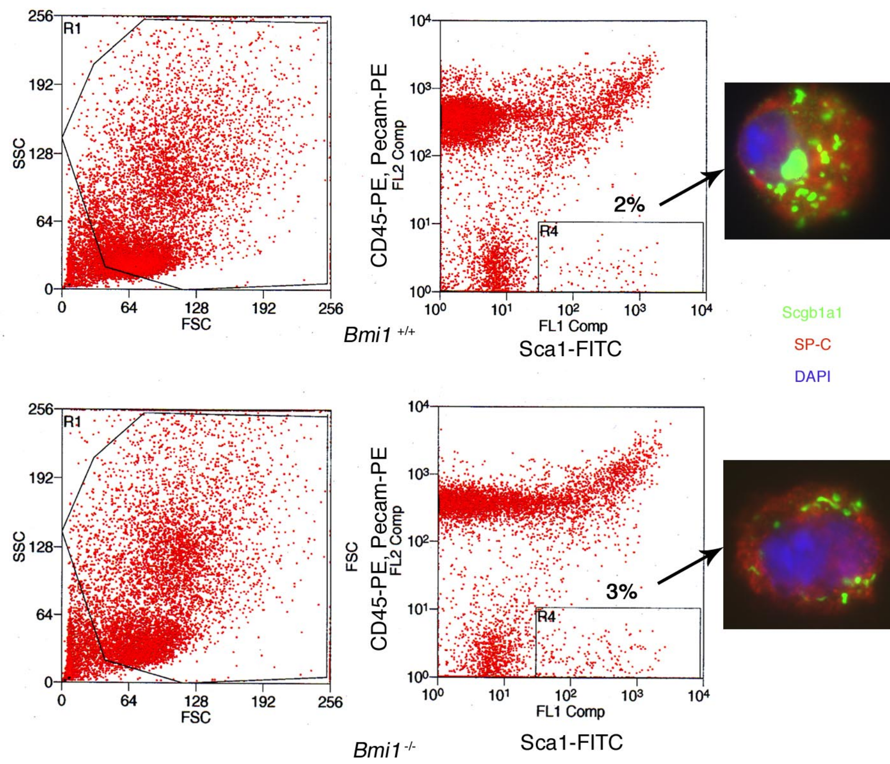

Fig. S4. FACS analysis data from the lungs of WT and *Bmi1*-null animals stained with antibodies against CD45, PECAM, and Sca-1. R4 gate marks a BASC-enriched population that is CD45^{neg}, PECAM^{neg}, and Sca-1^{pos}. Immunofluorescent staining of cytospins from this population demonstrated that ≈70% of the population was double positive for Scgb1a1 and SP-C (shown to the right).

Fig. S5. Bmi1-null BASCs have impaired proliferation and self-renewal, but normal differentiation. (*A*) Phase-contrast images of primary colonies derived from feeder-cultured BASCs (7 d after plating) from *Bmi1*$^{+/+}$, *Bmi1*$^{+/-}$, and *Bmi1*$^{-/-}$ lungs. (*B*) Limiting dilution analysis for *Bmi1*$^{+/+}$, *Bmi1*$^{+/-}$, and *Bmi1*$^{-/-}$ primary BASC colonies. The percentage of wells without colony formation for the corresponding number of plated cells is shown with the formula from regression analysis. Data from at least three independent experiments (mean ±SD) were used. (*C*) Limiting dilution analysis of secondary colonies derived from passaged primary colonies. (*D*, *Top*) Phase-contrast images of differentiated BASCs, plated on Matrigel, from *Bmi1*$^{+/+}$ and *Bmi1*$^{-/-}$ lungs. (*Bottom*) Immunofluorescent staining of Matrigelcultures showing cells in these three dimensional structures to have differentiated into single positive Scgb1a1 cells (arrows) and single positive SP-C cells (arrowheads). Similar results obtained from three animals of each genotype. (*E*) Limiting dilution analysis of primary colonies from *Bmi1*$^{+/+}$ or *Bmi1*$^{-/-}$ BASCs infected with empty shRNA or p16/p19^{ARF} shRNA viruses. The percentage of wells without colony formation for the corresponding number of plated cells is shown with the formula from regression analysis. Data from two independent experiments (mean ±SD) were used. (*F*) Limiting dilution analysis of secondary colonies derived from passaged primary colonies from (*D*). Mean ±SD. Figure legend same as in (*D*).