Loss of p53 in craf-induced Transgenic Lung Adenoma Leads to Tumor Acceleration and Phenotypic Switch\textsuperscript{1,2}

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ABSTRACT

One of the most frequent malignancies in humans is lung adenocarcinoma. To develop novel diagnostic and therapeutic approaches for the management of this disease, animal models are required. We have used transgenic mice with lung-targeted expression of the CRAf kinase to evaluate genes altered frequently in human lung adenocarcinoma for their effect on tumor progression. Here we report that loss of p53 dramatically accelerates tumor development and induces a phenotypic switch in the target cell from cuboid to a nonciliated columnar morphology. Coexpression of lung epithelial cell markers surfactant protein C and Clara cell antigen suggests that tumor cell dedifferentiation could be involved in this process. The effect of p53 is specific, because loss of one of its target genes, p21\textsuperscript{CIP1/WAF1}, did not have this effect on cell phenotype although tumor latency was also reduced significantly. Neither loss of p53 nor p21 stimulated acquisition of the metastasis program beyond the stage of bronchiolar extension. This mouse model for pulmonary adenoma and adenocarcinoma should be very helpful for a better understanding of pathogenesis and treatment of this most deadly human cancer.

INTRODUCTION

The lung exposes an enormous surface area (80–120 m\textsuperscript{2} in humans) to the environment to efficiently load the blood with oxygen and vent out CO\textsubscript{2}. Thus, the epithelial cells lining the surface of the proximal and distal lung are exposed continuously to air pollutants such as cigarette smoke. Not surprisingly, these epithelial cells are at risk of oncogenic transformation. In fact, lung cancer is the leading cause of cancer death in the world today (1, 2). There are at least five distinct epithelial cell types in the lung that all derive from a common progenitor in the foregut endoderm. In contrast with our detailed understanding of cell lineage relationships in the hematopoietic system, the number of lineages and the relationship of cell types within a lineage of lung epithelial cells are poorly understood (3). The most frequent lung cancer in humans is adenocarcinoma, and the cell of origin of this cancer type is the subject of intense investigation (1). Although the histology of the most frequent natural lung tumors in mice and humans may differ (3), the amenability of the mouse to detailed genetic and biochemical analysis makes it a very useful and important tool for investigating mechanisms of the human disease.

Earlier molecular genetic studies on the potential involvement of cancer genes in lung tumors of mice and humans have highlighted close to a dozen regulators of growth factor signal transduction and cell cycle progression, and frequently included ras and p53 (4). Although craf has not been detected in genetically altered form in human lung cancer, Raf protein serine/threonine kinases are effectors of Ras signaling and, therefore, are likely involved at least in those 30% of lung cancers in which mutated ras is detected (5, 6). Moreover CRAf protein has been found to be overexpressed in a large fraction of human lung cancers (7). The basis for increased CRAf protein content in lung tumor cells presumably is translational rather than transcriptional up-regulation (7), a phenomenon that we have also observed to occur in cultures of rodent fibroblast cell lines expressing oncogenic Ras (8). In contrast to the situation with craf, frequent mutations in braf have been reported in human melanoma and a number of other solid human tumors (9, 10), making the availability of an animal model for Raf kinase-driven tumorigenesis highly desirable. We reported recently that lung targeted expression of normal CRAf kinase in transgenic mice induced development of adenomas at high frequency (11). Oncogenically activated CRAf (CRAf BXB) caused the same histological type of adenoma to occur more rapidly in 100% of mice (11).

A remarkable feature of the Raf lung tumors was the lack of tumor progression as judged from extensive histological examination. There are multiple Raf signaling pathways that cell culture experiments suggest are jointly required for Raf transformation. These include the mitogenic cascade (Raf-mitogen-activated protein/ERK\textsuperscript{2} kinase-ERK), activation of nuclear factor xB by Raf (12), and cooperation of Raf with Bcl-2 in suppression of apoptosis (13–16). We reported recently that bcl-2 is required for efficient lung tumor development in mice (17). CRAf is today a validated target for treatment of a variety of solid tumors (18). Two types of Raf inhibitors have thus far been introduced in clinical trials to the treatment of human solid tumors, antisense oligonucleotides that specifically target craf and a low molecular weight kinase inhibitor (18). The antisense oligos have also been tested with human tumor cell lines in culture, where they were shown to induce apoptotic cell death (19) suggesting that the survival activity of CRAf (20) was a major factor in cell transformation. We speculate that Raf-induced cell survival activity may reduce DNA damage and perhaps thereby also tumor progression (21). Cell culture experiments with a hormone-regulated version of CRAf kinase also demonstrated that high intensity Raf signaling up-regulates the cyclin-dependent kinase inhibitor p21 in part through p53, and thereby induces cell cycle arrest (22, 23), a second pathway that might potentially contribute to genomic stability and inhibition of tumor progression.

To evaluate any connection between Raf and p53-p21 signaling pathways in the development of CRAf-induced lung tumor development in mice we crossed lung-targeted craf transgenics (11) with p53 (24) or p21 knockout mice (25), and examined the effect on tumor growth by histological and immunohistochemical analysis. Loss of p53 in SP-C-craf BXB mice not only shortened tumor latency and induced a high degree of nuclear atypia but caused a phenotypic switch from cuboid to nonciliated columnar epithelial tumor cells that may indicate acquisition of progenitor cell properties. These findings

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\textsuperscript{4} The abbreviations used are: ERK, extracellular signal-regulated kinase; RT-PCR, reverse transcription-PCR; RT, reverse transcription; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; TTF, thyroid transcriptional factor; PCNA, proliferating cell nuclear antigen; SP-C, surfactant protein C.
Histopathological Evaluation. Mice were subjected to complete autopsy including both gross and microscopic examination. Organs were fixed in 3.7% formaldehyde in PBS, embedded in paraffin, sectioned at 6 μm, and stained with H&E. For morphometric studies the 3–4 pieces of the lungs from 1.5 months old SP-C-craf BXB and SP-C-craf BXB/p53−/− or p53−/− mice were sectioned serially in their entirety. Every 10th section was stained with H&E and evaluated by light microscopy for the presence of neoplasia. The average of foci for 1-mm² area of the lung in single- and double-transgenic mice was counted by recording the number of individual foci on each section in several frames. Diameter of tumors was determined with an ocular micrometer. Statistical analysis was performed by Student’s t test using a P < 0.05. Percentage of papillary tumors were determined on both genetic backgrounds. Analysis was done by two histology readers (T. P. and L. M. F.). Every point on the plots represents the mean of values obtained from both readers. The difference between two independent calculations was <5%. Analysis of nuclear atypia distinguished among grade I (slight atypia), grade II (moderate atypia), and grade III (severe atypia), as described by Suzuki et al. (26). Grade I denotes nuclei that are uniform in size and equal to or only slightly larger than those of type II alveolar epithelial cells. Grade II denotes nuclei that were uniform in size and up to twice the size of those of type II alveolar epithelial cells, and grade III grade denotes increased nuclear:cytoplasmic ratio, prominence of nucleoli, and the presence of giant tumor cells. To compare the tumor growth as a function of the time lungs were isolated and weighed, and a linear regression assay was done to calculate the regression coefficients. For weight determination, only lungs free of lymphoma metastasis were used.

Immunohistochemistry and TUNEL Assays. For the immunohistochemical detection of CRaf protein and SP-C, paraffin-embedded 6-μm thick sections were deparaffinized, dehydrated, and microwaved for 6 min in 10 mM sodium citrate buffer (pH 5.5). Subsequently, the slides were incubated for 6 min in peroxidase blocking solution (3% H₂O₂ in PBS). After antigen retrieval, slides were rinsed in distilled water, incubated in 20% sucrose PBS at 4°C for 30 min and placed in blocking buffer (2.5% goat serum in PBS) for 40 min. Subsequently, the slides were incubated in the presence of an antihuman CRaf SP63 rabbit polyclonal antiserum (11) diluted 1:500 in blocking buffer or antiserum for detection of SP-C rabbit polyclonal antiserum (a gift from Jeffrey A. Whitsett, The Children’s Hospital Research Foundation, Cincinnati, OH; Ref. 27) diluted 1:1000 at 4°C overnight. Antigen-antibody complexes were detected with the avidin-biotin complex immunoperoxidase system (Vectastain ABC kit; Vector). The sections were then counterstained with hematoxylin. To identify expression of TTF-1, Clara cell antigen (CC10), or PCNA, the deparaffinized tissue sections (5 μm) were rehydrated and microwaved twice for 10 min in 10 mM sodium citrate buffer (pH 5.5) and incubated with primary antibodies: mouse antirat (mouse) TTF-1 monoclonal antibodies (Dako) diluted 1:100, mouse antigoat antibodies (Dako) diluted 1:100 in 50 mM Tris-buffer (pH 7.4); polyclonal antiserum (PharMingen) diluted in 1:100 in 50 mM Tris-buffer (pH 7.4) overnight at room temperature. Subsequently, the slides were rinsed with 50 mM Tris-buffer (pH 7.4), incubated with biotinylated secondary rabbit anti-antisera antibodies (Dako) 1:50 or rabbit anti-goat antibodies (Dako) diluted 1:50 in 50 mM Tris buffer (pH 7.4) for 30 min at room temperature, rinsed with Tris-buffer, incubated with streptavidin-biotinylated alkaline phosphatase-complex (Strept AB Complex; Dako) for 30 min at room temperature, followed by Fast-red reaction for 20 min at room temperature and counterstaining with hematoxylin. All of the immunohistochemical reactions were carried out in parallel with reactions lacking primary antibodies as the negative controls. TUNEL assay was performed as described previously (17). Apoptotic and PCNA indices were determined by counting 10 randomly chosen fields per 3–4 sections and determining the percentage of apoptotic or proliferating cells per 2000 cells at ×400. Statistical analyses for PCNA and TUNEL labeling were performed by Student’s t test, and differences were considered significant when P < 0.05.

Western Blot Analysis. Western blot analysis was performed as described previously (17). p21(CIP1/WAF1) protein was detected by rabbit antismooth muscle α- actin polyclonal antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200. Donkey antirabbit immunoglobulins linked to horseradish peroxidase (Amersham) diluted 1:1000 were used as secondary antibodies. The binding of the antibodies was detected by ECL Western blotting detection reagents (Amersham). CCl₄ mouse myoblast cell line (28) highly expressing p21(CIP1/WAF1) was used as positive control.

MATERIALS AND METHODS

Generation and Genotyping of Bitransgenic Mice. All of the mice were housed in pathogen-free conditions and handled in accordance with federal animal protection laws, and reviewed by a local ethics committee. Animals were monitored daily for signs of respiratory distress by inspection of their mobility and sacrificed before signs of extensive respiratory distress. Transgenic mouse line SP-C-craf BXB-23 (B6xD2 background; Ref. 11) was crossed with p53-deficient mice on two genetic backgrounds [C57Bl/6-Tmp5tm1(24) purchased from Jackson Laboratories and 129/Sv-Tmp5tm1(24) purchased from Centre National De La Recherche Scientifique, Orleans, France]. To produce animals hemizygous for the transgene and heterozygous for targeted mutation at the tumor suppressor locus, the SP-C-craf BXB mice were mated with p53−/− mice. The resultant mice were subsequently backcrossed with p53−/− mice to yield SP-C-craf BXB/p53−/− and SP-C-craf BXB/p53−/−−/− animals. The breeding was done with both strains of mice. Accordingly, the resulting SP-C-craf BXB/p53−/− and SP-C-craf BXB/p53−/−−/− mice had either 92.5% B6/7.5% D2 or 75% 129/Sv/25% B6 backgrounds. Genotyping of animals was performed by PCR using mouse tail DNA. SP-C-craf BXB transgene, wild-type, and targeted alleles of p53 were detected by PCR as described previously (17, 24). Loss of p53 was analyzed by quantitative PCR. The primers described above for genotyping of p53 were used with the following modifications: 40 ng of purified DNA were amplified for 32 cycles. To validate that the PCR was quantitative, the amount of wt p53 allele was reduced by serial 2-fold dilution in control reactions starting from 20 ng to 1.25 ng in the presence of a constant amount (20 ng) of the targeted allele. The gel photograph was digitized (as an 8-bit grayscale tiff-file) and additionally analyzed with the use of the Scion “Image” software to determine the intensity of the individual bands. The calibration curve of the serial dilution was monotonous and used to derive the p53 levels in the lung specimens. p21 CIP1/WAF1-deficient mice (129/Sv background; Ref. 25) were a gift from Philip Leder (Harvard Medical School, Boston, MA). The breeding scheme for production of SP-C-craf BXB/p21CIP1/WAF1−/− mice was the same as for SP-C-craf BXB/p53−/− mice. A 300-bp fragment of the wild-type allele of p21 CIP1/WAF1 was detected using primers located in exon 2 of the p21 CIP1/WAF1 gene: forward primer 5′-AGTGTGCGTGTTCCTTGCTGTC-3′ and reverse primer 5′-ACGCGCAAGCCACTGGTCTTCC-3′. The following PCR protocol was used: 95°C for 5 min followed by 35 cycles; 30 s at 95°C, 30 s at 72°C, and 1 min at 72°C, followed by 6-min extension at 72°C. The targeted allele was identified by amplification of a 208-bp fragment using primers corresponding to the neo cassette of knockout vector: 5′-ACCTGACAGCAATATGAGTCTC-3′ and 5′-GTCTGTAGCTGCTACCCG-3′ using the following PCR protocol: 95°C for 5 min followed by 35 cycles; 30 s at 95°C, 30 s at 68°C, and 1 min at 72°C, followed by 6-min extension at 72°C.

RNA Preparation and RT-PCR Analysis. Total RNA was prepared from lung tissues using the TRIzol LS Reagent and treated with amplification grade DNase I (Life Technologies, Inc.). For semiquantitative PCR of RNA, cDNA was prepared by RT of 5 μg of each RNA sample using Moloney murine leukemia virus-RT (Life Technologies, Inc.). The PCR amplifications were performed in a 50-μl reaction volume containing 5 μl of each RT reaction mixture. Primers for p53 detection were 5′-TCTGGACAGC-CCAAGTCTG-3′ (sense) and 5′-GGAGTCCTCCAGTGATGTA-3′ (antisense). Primers for p73 detection were 5′-AGATGGTCAAGATGCAGCCC-3′ (sense) and 5′-TGATGAGTACGACAGTGGCCG-3′ (antisense). Primers for β-actin detection were 5′-GTGTCGACACAGACCTGTTGAG-3′ (sense) and 5′-GCAATGCTGCTATCAGTGGG-3′ (antisense). The PCR conditions for amplification were as follows: 95°C denaturation for 2 min followed by 95°C for 30 s, 56°C for 30 s, 72°C for 1 min for 33 cycles using p53 and p73, and 25 cycles using β-actin primers followed by 6-min extension at 72°C. Cycle curve studies confirmed that for the amounts of cDNA being amplified, the reactions had not reached the plateau of the amplification curve with either primer pair. PCR control reaction without RT yielded no detectable fragments with either primer pair. RT-PCR product for p53 was transferred to Hybond-N membrane and detected subsequently by Southern Blot analysis using the same fragment as a probe. DNA sequencing of the PCR products verified the identity of the p53 and p73 sequences.
RESULTS

Loss of p53 Accelerates Lung Tumor Growth and Shortens the Life Span of SP-C-craf BXB Transgenic Mice. Lung-targeted expression of a constitutively active form of CRaf kinase, CRaf BXB, leads to rapid, multifocal induction of adenoma that are the cause of death after more than a year (Fig. 1A). We have used these transgenic mice to model human lung carcinogenesis by introducing additional genetic changes that occur frequently in human tumors. To examine the effect that loss of one or both alleles of p53 has on CRaf BXB induced adenoma formation, the SP-C-craf BXB mice were mated with p53 negative mice that had been generated by targeted deletion (24). The breeding scheme was designed so that all of the transgenic mice were maintained in a hemizygous state for the SP-C-craf BXB transgene to avoid possible gene dosage effects and to allow examination of effects of p53 both on B6 and 129/Sv backgrounds.

Removal of one allele of p53 shortened the life span of the transgenic mice by approximately one-third (Fig. 1A, solid squares versus open circles). Loss of the second allele occurred in the tumors as a function of time as indicated (Supplemental Fig. 1D). In the absence of both alleles, the survival curve became coincident with that of p53-negative mice that die predominantly from lymphoma (Fig. 1A, open triangles versus open squares). Monitoring animal health revealed that respiratory distress that was pronounced in 7–8 month-old SP-C-craf BXB mice was apparent already at 5–6 months in p53+/− and at 3–3.5 months in double-negative mice on either B6 or 129/Sv backgrounds.

Histological examination of lungs from bitransgenic and control mice showed that the latency of formation of small foci of cuboidal adenoma cells was reduced 2-fold (from 4 weeks to 2 weeks) or 4-fold (to 1 week) when SP-C-craf BXB mice were bred into a background deficient in one versus two copies of p53 (data not shown). The number of foci per mm² at 1.5 months of age was increased on p53 removal in a dose-dependent fashion suggesting that oncogenic Raf and loss of p53 may cooperate in the initiation of adenomatous

Fig. 1. Acceleration of tumor growth in SP-C-craf BXB transgenic mice on removal of p53. A, survival curves of SP-C-craf BXB/p53+/− mice. B, mean number of foci/1 mm². Difference of SP-C-craf BXB/p53−/− versus SP-C-craf BXB/p53+/− and SP-C-craf BXB is statistically significant (P < 0.01). C, mean ratio of tumor area:lung area (in %) in lung sections from 1.5-month-old SP-C-craf BXB/p53+/− mice. Difference of SP-C-craf BXB/p53+/− versus SP-C-craf BXB/p53−/− and SP-C-craf BXB is statistically significant (P < 0.019). The number of mice in each group is indicated. D, weight increase of lungs from SP-C-craf BXB/p53+/− mice. All of the groups are statistically different (P < 0.01).
transformation (Fig. 1B). This cooperation may be even more pronounced in terms of acceleration of growth, as the increase in focus area more than doubled when both alleles of p53 were missing (Fig. 1C). This relationship is also evident from the gross morphometric evaluation (Fig. 1D) when the slope increase from double-negative is compared with that of single-negative mice. Only lungs free of lymphoma metastasis were used. Lymphomas occur at high frequency (65%; n = 37) in p53-negative mice that can metastasize to the lung (5 mice in 37 analyzed lungs). The apparently nonlinear increase in tumor growth may indicate that alleviation of p21 up-regulation by high intensity Raf signaling (23) may require deletion of all of the p53 molecules in SP-C-craf BXB mice. In fact, RT-PCR analysis of p53 transcript levels showed age-dependent up-regulation specifically of p53 (not p73) expression in CRaf BXB lung tumors suggesting that loss of one copy of p53 may be compensated by increased gene expression under these conditions (data not shown). p21 protein levels are reduced but still readily detectable in p53-negative SP-C-craf BXB tumor cells examined at 5 months of age (data not shown).

**Phenotypic Switch from Cuboidal- to Columnar-Cell Tumors on Loss of p53.** p53 deletion not only affects the rate of lung tumor growth in SP-C-craf BXB mice but also the tumor cell phenotype, an observation that was made with animals either on the B6 or 129/Sv background (Supplemental Fig. 1, A and B). This change is illustrated in Fig. 2. Fig. 2B shows the homogeneous (solid) appearance of Raf-only tumors in SP-C-craf BXB transgenics at 6 months of age. As described previously (11), these tumors consist of cuboid epithelia comparable with type II pneumocytes (grade I, Fig. 2D). Sometimes an intra-alveolar micropapillary growth pattern was seen (Fig. 2E; Ref. 11). In the absence of p53, papillary tumors became the predominant form with increasing age (Fig. 2C). Undetectable below the age of 2.5 months, they constituted approximately one-half to two-thirds of the tumor size at the time of sacrifice. Typically, these regions of the tumor were papillary in appearance, but no true fibrovascular cores were apparent (Fig. 2F; Fig. 3F; Fig. 4F). There are also areas of mixed tumor phenotypes having small cuboidal, large cuboidal, and columnar cell areas (data not shown) suggesting that the large cuboidal cells may represent a transition form on the path to the large columnar cells in a cell lineage. Extensive serial sectioning did not provide any evidence of a bronchiolar origin of the papillary tumors.

**Evaluation of Tumor Progression by Comparison of Nuclear Morphology.** A hallmark of the SP-C-craf BXB lung adenomas is their lack of nuclear atypia and tumor cell heterogeneity (11). This stability of tumor phenotype is remarkable in light of the enormous tumor mass that accumulates from only a few initial foci per lung by continuous concentric growth in the case of CRaf wild-type tumors (11). One possible explanation may be that a CRaf-driven increase in cell survival is more critical than an increased rate of proliferation for CRaf-induced lung tumor development. Raf-induced suppression of apoptosis may involve mechanisms such as reduction of oxygen-free radicals that would also lower DNA damage and, thus, attenuate mutation-driven tumor progression. Deletion of p53 should overcome this block and promote progression perhaps all of the way to a metastatic phenotype. In Fig. 3, nuclear morphology is compared between early (2.5 months) and late (6 months) cuboid cell SP-C-craf BXB tumors, as well as between early (2.5 months) versus late (6 months) p53 negative cuboid cell and columnar cell adenomas. Comparison of Fig. 3, A and D, shows little or no increase in the very low level of nuclear atypia, whereas the p53-negative cuboid cell (Fig. 3, B and E) or columnar cell tumors (Fig. 3, C and F) have a significantly

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**Fig. 2. Phenotypic switch of lung tumor cells in p53-negative SP-C-craf BXB mice at 6 months of age.** Histological sections of lungs from wild-type (A) and SP-C-craf BXB mice showing large numbers of adenomas (B). The lung of a SP-C-craf BXB/p53<sup>−/−</sup> mouse with solid cuboidal (bottom) and papillary (top) tumors (C). Solid (D) and mini-papillary (E) tumors from a SP-C-craf BXB mouse containing cuboidal cells. F, a papillary tumor from SP-C-craf BXB/p53<sup>−/−</sup> mouse containing large columnar cells. H&E staining. The scale bars are shown in the right corner. Bar, 1 mm (A–C) and 30 μm (D–F).
higher level of atypical nuclei already at 2.5 months (Supplemental Fig. 1C). Another histological feature that is consistent with tumor progression is the occurrence of bronchial extension (Fig. 3, G and H) that we observed in 4 of 25 SP-C-craf BXB/p53−/− mice. However, even in these areas there is no cell dispersal or a transition to spindle-shaped cells detectable that might indicate emergence of a full invasive, metastatic carcinoma. Moreover, we did not detect evidence for metastasis by a search for epithelial cells expressing SP-C in mediastinal lymph nodes, liver, kidney, and all of the other major organs (data not shown).

Expression of Raf Transgene and Lung Cell-specific Markers in Lung Tumors from Bitransgenic Mice. What might be the mechanism by which p53 loss induces the emergence of nonciliated columnar epithelial cells in the solid cuboid cell SP-C-craf BXB lung tumors? It seems unlikely that induction of genomic instability is the trigger, because the phenotypic switch from cuboid to columnar appears too orderly, more reminiscent of a developmental switch than a random event. Unfortunately, the knowledge of lineage relationships between different epithelial lung cell types and of molecules that might mediate their interconversion is very limited. Therefore, it is difficult to make an educated guess as to candidate p53 target genes that upon relief of suppression by p53 would bring about the switch in programs from the cuboidal to nonciliated columnar epithelial cell type.

In an attempt to get closer to identifying candidate target genes, the tumor cells were analyzed by immunohistochemistry for expression of cell type-specific markers. The exogenous SP-C promoter is known to be activated not only in type II pneumocytes but also in other bronchio-alveolar epithelial cells including Clara cells. In contrast, the endogenous SP-C promoter is active in distal columnar precursor cells during embryonic development but in the adult is restricted to type II pneumocytes (29, 30). Therefore, CRaf protein expression above the level of endogenous CRaf was determined. Fig. 4, A and B, clearly establishes that increased expression of CRaf indicative of transgene expression is not restricted to the cuboidal but includes the nonciliated columnar cells in the p53−/− papillary tumors. Thus, the papillary tumor is not an entirely new entity initiated and maintained by new cancer genes activated via genomic mutations as a result of p53 loss in the cuboidal cells.

Consistent with SP-C-craf BXB expression in both tumor types,
SP-C expression was also detected in cuboid as well as in columnar cell tumors (Fig. 4, C and D). Next we examined expression of TTF-1, a homeodomain transcription factor known to be involved in lung development and SP-C expression. TTF-1 nuclear staining is detectable in the columnar cells of papillary tumors as well as in the solid cuboidal cell CRaf BXB tumor. Finally, expression of CC10 was determined (Fig. 4, G and H). CC10 is a marker for Clara cells that are the most prevalent nonciliated columnar epithelial cell in the lung. During development CC10 is found in columnar epithelial cells of the primordial lung buds (30). There is significant cytoplasmic expression of CC10 in the p53-negative columnar cell tumor but not in the cuboidal adenomas. From this we conclude that there are shared and distinct marker proteins in both tumor cell types consistent with the emergence of one from the other and suggestive of CC10 being a direct or indirect target of p53. It is unclear whether expression of the Clara cell marker in the nonciliated columnar cells constitutes ectopic expression, i.e., a scrambled phenotype that is an artifact of tumor development or whether these cells have re-emerged as a result of a dedifferentiation process and resemble undifferentiated cells of the distal murine lung bud (31).

Survival versus Growth Induction by Loss of p53. The accelerated tumor growth that we observed on deletion of p53 in SP-C-craf BXB transgenic mice might be caused by an increase in cell survival, an increase of proliferation, or a combination of both. To distinguish among these possibilities tissue sections were examined for apoptotic cells by TUNEL assay and the fraction of proliferating cells by staining for PCNA (Fig. 5). Papillary tumors in p53-negative mice showed the highest proportion of apoptotic cells; all of the others were indistinguishable (Fig. 4D, 4E, and 5A, B, and E). Papillary tumors also had the largest fraction of PCNA-positive cells. We conclude that the preponderance of the papillary tumor component in SP-C-craf BXB/p53+/mice close to the time of death (4–7 months) is the result of high proliferative activity. The cuboidal cell adenoma that also has a high degree of nuclear atypia (Fig. 3, B and E) appears to be driven both by a high survival rate and stimulation of proliferation above the level of cuboidal cell tumors in the presence of p53 (Fig. 5, C, E, and F).

Does Loss of p53 Mediate the Phenotypic Switch via p21? The increased proportion of proliferating columnar cells in papillary tumors is evident from higher levels of PCNA immunostaining (Fig. 5). This is consistent with p53 action through the cell cycle inhibitor p21. To determine whether loss of p21 might have similar or perhaps even more pronounced effects on the S phase fraction of tumor cells than deletion of p53, because other members of the p53 family of proteins are still present in the bitransgenic mice, the SP-C-craf BXB mice were bred with p21–/− deficient mice that had a 129/Sv back-
ground. As can be seen from the survival curves (Fig. 6A), there is a dose-dependent shortening of life span on removal of one versus two alleles of p21, but this effect is not quite as strong as that seen on deletion of p53 (Fig. 1A). The level of PCNA staining also shows a somewhat lower increase than has been observed in SP-C-craf BXB/p53−/− mice, and the same holds true for the gross morphometric parameter lung weight. No differences were observed in the fraction of apoptotic cells between p21-negative and wild-type tumors (data not shown). We conclude that p21 is not the only p53 effector involved in the cooperation between CRaf BXB and loss of p53. Moreover, p21 is apparently not the p53 target that mediates phenotypic stability, because the phenotypic switch from cuboid to nonciliated columnar cells was absent in the SP-C-craf BXB/p21−/− mice. This finding makes it less likely that switch induction is a function of growth rate and is consistent with a model where a target gene that is up-regulated in the absence of p53, such as perhaps CC10 (Fig. 4), would be responsible.

**DISCUSSION**

The present study demonstrates that development of lung tumors by targeted expression of CRaf BXB in type II pneumocytes occurs with
shorter latency when either of two tumor suppressor loci, p53 or p21<sup>CIP1/WAF1</sup>, which are frequently altered in human non-small cell lung carcinoma, is deleted. Absence of p53 not only accelerates disease development but specifically promotes a phenotypic switch in transformed type II pneumocytes to nonciliated columnar cells. Moreover, loss of p53 induces signs of tumor progression such as nuclear atypia and bronchiolar extension, although a full metastatic phenotype has not been observed.

**Acceleration of Tumor Development.** We have described previously a mouse model for induction of multifocal adenomas by activated or wild-type CRaf, which is overexpressed frequently on the protein level in human tumors. The latency of tumor development parallels CRaf kinase activity toward mitogen-activated protein/ERK kinase as a substrate, and tumors occur without the need for introduction of additional genetic changes (11). These findings were surprising on two accounts: the deviation from the general believe that multiple genetic insults are required for tumor formation (32, 33) and the observation that CRaf signaling induces cell cycle arrest at high signal intensity (23). The last observation suggested that tumor induction might have involved secondary changes such as K-ras mutation that was known to occur in the majority of chemically induced lung cancers in rodents (34) or p53 inactivation, as the latter might cancel the cell-cycle inhibitory p21 up-regulation typical of high-intensity CRaf signaling. Because neither of these changes were detected in our earlier study we decided to examine the impact of introducing those mutations that are observed frequently in human lung adenocarcinoma by breeding <i>craf</i> transgenic with p53 or p21<sup>CIP1/WAF1</sup> knockout mice. As the histology of CRaf adenomas was stable over 18 months, changes induced by loss of p53 or p21 were expected to be readily identifiable. In fact the number of initial adenomatous foci dramatically increased in the absence of p53 (Fig. 1B) consistent with relief of suppression of CRaf BXB driven proliferation mediated by high levels of p21 (23). Such a mechanism is supported by determination of the fraction of proliferating cells by PCNA staining that shows a parallel, ∼3-fold increase (Fig. 5F). In addition to the increased number of tumor foci at 1.5 months of age (Fig. 1B), small foci could be detected much earlier in bitransgenic mice (1 week versus 2–4 weeks). Reduction of p21 levels was presumably not the only mechanism by which p53 deletion accelerated disease as suggested by two observations: (a) the fact that p21 removal was not as powerful an accelerator as that of p53; and (b) the pronounced nuclear atypia in the absence of p53 (Fig. 3). Nuclear atypia indicated that additional genetic changes might have contributed to the relief of suppression of CRaf BXB oncogenesis at early ages. Other changes that are known to cooperate with <i>raf</i> oncogenes include activation of c-myc (35, 36), and small G-proteins of the Ras and Rho families (37, 38). A broad screen for genomic and gene expression alterations is currently underway.

Surprisingly, a metastatic phenotype was not observed in either bitransgenic SP-C-<i>craf</i> BXB tumor model. In the absence of p53 there was extension of bronchioles, but more noteworthy was the absence of widespread and frequent metastasis. The shortened life span of double-negative p53 mice that die between 150 and 200 days of age mainly because of metastasizing lymphoma induced by loss of p53 may be one explanation, although the acceleration in the death curve seen on removal of just one p53 allele (Fig. 1A) should have left plenty of time for activation of one of the many metastasis genes that have been described (39). Future experiments will address the question whether CRaf BXB in type II pneumocytes cells does in fact actively suppress genetic instability in a p53/p21-independent manner or whether triggering of the metastatic phenotype in Raf-transformed pneumocytes requires a specific gene alteration that rarely occurs even in the absence of p53.

The other dominant phenotype of SP-C-<i>craf</i> BXB/p53<sup>−/−</sup> tumors besides adenoma initiation and growth acceleration is the emergence of a histologically new cell type, nonciliated columnar cells, that also shows pronounced nuclear atypia increasing with age.
Phenotypic Switch from Cuboid to Columnar Epithelial Cells.
The hallmark of CRaf BXB expanded type II pneumocytes in our transgenic model is their retention of cell type identity over a period of up to 18 month or the time of death of the mice. This correlation between Rafl oncogenesis and an orderly differentiated phenotype is reminiscent of the histogenesis of Rafl tumors induced with retrovirus carrying the v-rafi oncogene (36, 40, 41). On i.p. injection, newborn mice typically die of splenomegaly leading to ruptured spleens because of erythroid hyperplasia (41, 42). In addition to the erythroid lineage, other cell lineages were affected, but the most striking observation was an amplification of histologically normal looking erythroid lineage cells that eventually killed the animal predominantly because of the accumulated tissue mass (43, 44). This pattern was altered dramatically when v-rafl was combined with a second oncogene v-myc (41, 45–47). In this combination the pattern of tumor formation was altered in that: (a) target cells typical for myc oncogenesis were added to those typical for v-rafl; (b) both sets of target cells were transformed with shortened latency; (c) new cell types were targeted for transformation that had not been seen with either oncogene alone; and finally, (d) in addition to the widened spectrum of tumors, an instability of the transformed cell phenotype was observed for the first time that was examined in some detail by us and others in the myeloid-B-lymphoid lineage (48–50). Whereas v-rafl alone induced pre-B lymphomas in this lineage, addition of v-myc stimulated rapid transition of the B-cell lineage to mature B cells, which, however, had a tendency to shut down expression of the rearranged immunoglobulin genes and display properties of mature macrophages (50, 51). We interpreted this observations at that time as a lineage switch that involved retrodifferentiation of mature B cells to a branch point in their ontogenetic tree where a bipotential progenitor could feed both into the B and myeloid lineage followed by differentiation in the latter. Although several more recent observations have delineated potential mechanisms (52, 53) the issue is not fully resolved.

The phenotypic switch of type II pneumocytes to noniliated columnar cells that express SP-C, TTF-1, and CC10 (Fig. 5) on loss of p53 may also be because of a specific effect on phenotype programing within the lineages of lung epithelial cells. Epithelial plasticity has been proposed (54) to explain apparent transdifferentiation of transformed Clara cells to other pulmonary epithelial cells under promoter influence of the cellular environment. In addition, calcium-driven ectopic v-Ha-ras expression induces murine tumors that dedifferentiate into a phenotype with both endocrine and nonendocrine features (55). Our findings link a specific genetic alteration, p53 loss, to the induction of plasticity and should facilitate molecular dissection of this phenomenon. The parallel between loss of p53 in the current study and addition of v-myc in our earlier work on lineage switch in the B-myeloid cell lineages suggests that the same underlying mechanism may be at work. Myc transformation may force selection of cells that are mutant in either Ink4a/Arf or p53 (56). We are currently exploring this possibility by breeding SP-C-Craf BXB with SP-C-c-myc mice. We also identified recently a distal effector kinase of Raf, 3pk, that includes elements of the cell membrane apparatus among its substrates. The potential involvement of this and related enzymes in the lung epithelial cell switch will be a topic of future investigation.

It is not clear whether the noniliated columnar epithelial cells observed in the bitransgenic animals correspond to a cell type that is a normal constituent of lung epithelial cell lineage(s) or whether it displays a scrambled phenotype. Future experiments with progenitor cells that differentiate in culture and determination of gene expression profiles at all of the stages of differentiation should help to settle this question. An additional limitation of the current work is the use of the SP-C promoter that presumably restricts the range of switched phenotypes to those cells that allow expression of the SP-C-craf BXB transgene (30).

In summary, we have dissected a useful mouse model of human lung adenocarcinoma for conditions of tumor progression and generation of tumor heterogeneity via phenotypic switch. These mice should be valuable for evaluation of a variety of novel cancer gene-based drugs in preclinical trials. Moreover, our findings should facilitate studies on the regulation of cell memory to differentiated states and its disturbance in the process of malignant transformation.

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REFERENCES
Loss of $p53$ in $craf$-induced Transgenic Lung Adenoma Leads to Tumor Acceleration and Phenotypic Switch
