Tumor Susceptibility of *Rassf1a* Knockout Mice

Stella Tommasi,[1] Reinhard Dammann,[3] Zhongqiu Zhang,[4] Yian Wang,[4] Limin Liu,[1] Walter M. Tsark,[1] Sharon P. Wilczynski,[2] Jie Li,[4] Ming You,[4] and Gerd P. Pfeifer[1]

[1]Division of Biology and [2]Department of Anatomic Pathology, City of Hope National Medical Center, Duarte, California; [3]Medizinische Fakultät, Martin-Luther-Universität Halle-Wittenberg, Halle/Saale, Germany; and [4]Department of Surgery, Washington University School of Medicine, St. Louis, Missouri

Abstract

The human Ras association domain family 1 (*RASSF1*) gene is located at 3p21.3 in an area that is believed to harbor at least one important tumor suppressor gene. The two major isoforms of *RASSF1*, *RASSF1A* and *RASSF1C*, are distinguished by alternative NH_2-terminal exons and the two transcripts initiate in two separate CpG islands. *RASSF1A* is one of the most frequently inactivated genes described thus far in human solid tumors. Inactivation of *RASSF1A* most commonly involves methylation of the promoter and CpG island associated with the *RASSF1A* isoform. In contrast, *RASSF1C* is almost never inactivated in tumors. Here, we have derived *Rassf1a* knockout mice in which exon 1-α of the *Rassf1* gene was deleted, leading to specific loss of *Rassf1a* but not *Rassf1c* transcripts. *Rassf1a*-targeted mice were viable and fertile. *Rassf1a*$^{-/-}$ mice were prone to spontaneous tumorigenesis in advanced age (18–20 months). Whereas only two tumors developed in 48 wild-type mice, six tumors were found in 35 *Rassf1a*$^{+/-}$ mice ($P < 0.05$) and thirteen tumors were found in 41 *Rassf1a*$^{-/-}$ mice ($P < 0.001$). The tumors in *Rassf1a*-targeted mice included lung adenomas, lymphomas, and one breast adenocarcinoma. *Rassf1a*$^{-/-}$ and wild-type mice were treated with two chemical carcinogens, benzo(*a*)pyrene and urethane, to induce skin tumors and lung tumors, respectively. *Rassf1a*$^{-/-}$ and *Rassf1a*$^{+/-}$ mice showed increased tumor multiplicity and tumor size relative to control animals. The data are consistent with the tumor-suppressive role of *Rassf1a*, which may explain its frequent epigenetic inactivation in human tumors. (Cancer Res 2005; 65(1): 92-8)

Introduction

The Ras association domain family 1 (*RASSF1*) gene (1) is located at 3p21.3 within an area of common heterozygous and homozygous deletions, which occur frequently in a variety of human solid tumors (2–4). The *RASSF1* locus encodes two major transcripts and several tissue-specific splice variants. The two major transcripts initiate in two CpG islands separated by about 3.5 kb. They have different NH_2-terminal exons and are referred to as *RASSF1A* and *RASSF1C* (1, 5). Both RASSF1A and RASSF1C proteins contain a Ras association domain (6). RASSF1A also contains a diacylglycerol-phorbol ester binding domain near its NH_2 terminus (1). Only one of the isoforms of the *RASSF1* gene, *RASSF1A*, is inactivated by DNA methylation of its promoter CpG island at a high frequency in many human solid tumors, as observed originally in lung cancers (1, 5, 7). *RASSF1A* is probably the most frequently methylated gene described thus far in human cancer (8, 9). *RASSF1A* methylation occurs in a broad spectrum of carcinomas and other solid tumors (1, 5, 7, 9–29). In several types of tumors, *RASSF1A* is inactivated at a high frequency. For example, methylation of *RASSF1A* is found in 80% of small cell lung cancers (5, 16), over 60% of breast tumors (5, 10, 30), over 70% of prostate cancers (12, 13, 31), and in 90% of hepatocellular carcinomas (15, 29, 32). In contrast, inactivation of *RASSF1C* in human tumors has rarely been reported.

The biological function of RASSF1A is unknown. Transfection of *RASSF1A* reduces the growth of human cancer cells *in vitro* and *in vivo* supporting a role for *RASSF1* as a tumor suppressor gene (1, 5, 11, 12). RASSF1A's closest homologue is the RAS effector protein NORE1A (33). RASSF1A and NORE1A form heterodimers suggesting that RASSF1A may function in signal transduction pathways involving RAS-like small GTPase proteins. Recent data indicate that RASSF1A itself binds to RAS only weakly and that binding to activated RAS may require heterodimerization of RASSF1A and NORE1 (34). There is evidence for an association of both NORE1 and RASSF1A with the proapoptotic kinase MST1. This interaction is involved in cellular processes leading to apoptosis (35). Other investigators have described the role of RASSF1A in the suppression of cyclin D accumulation and cell cycle progression (36). Recently, we presented evidence that RASSF1A is a microtubule-binding protein that can stabilize microtubules, and that overexpression of RASSF1A causes metaphase arrest (37). RASSF1A localizes to the mitotic apparatus and controls mitotic progression by modulating microtubule dynamics and/or by interacting with components of the anaphase-promoting complex (37–39).

Animal models for studying the function of *RASSF1A* have not yet been described. Here, we have created a mouse knockout for *Rassf1a*. In a model which closely mimics the situation in human tumors, the *Rassf1a* isoform was specifically inactivated in the mouse. We studied the tumor susceptibility of *Rassf1a* knockout mice, both for spontaneous tumor formation and for chemical carcinogen-induced tumor formation.

Materials and Methods

Generation of Gene-Targeted Mice. The *Rassf1a* knockout vector was made by high-fidelity PCR with the proofreading-capable Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) and 129/SvImJ mouse ES cell DNA as the template. The *PgK-neo* cassette was flanked by 3.9 kb of genomic sequence upstream of *Rassf1a* exon 1-α and by 2.5 kb of sequence downstream of exon 1-α. The 5′ insert was generated with primers UMBLU1 (5′-GGGGCCTGCGCAGCTTCCAGCT-3′) and LM10A1 (5′-TGGGCGGGGCACGGCACAA-3′), cloned into the pCR2.1 vector and verified by sequencing. The insert was released by *Eco*RI digestion and cloned into the *Eco*RI sites of the pKSneolox-cloning vector. The primers UM11AA

Note: S. Tommasi, R. Dammann, Z. Zhang, and Y. Wang contributed equally to this work.

Requests for reprints: Gerd P. Pfeifer, Beckman Research Institute, City of Hope National Medical Center, 1450 East Duarte Road, Duarte, CA 91010. Phone: 626-301-8853; Fax: 626-930-5366; E-mail: gpfeifer@coh.org.

(5′-GCGGCAGTGGAGGGCTGGTCA-3′) and LM121 (5′-TGTGAGAGGTGCGCGTGCTCC-3′) were utilized to clone the 3′ insert into pCR2.1. The orientation and integrity of the fragment was confirmed by sequencing and it was cloned into the *Xho*I and *Kpn*I sites of pKSneolox. The resultant construct replaces exon 1-α of *Rassf1a* with the *PgK-neo* cassette. Gene targeting was done in the ES cell line 129S3/SvImJ. The gene-targeting vector was linearized with *Kpn*I and introduced into ES cells by electroporation. G418-resistant ES cell colonies were transferred to 96-well tissue culture plates for expansion and analysis. To identify homologous recombinants, Southern blot analyses using diagnostic restriction enzyme digests with probes that are external to the regions of homology included in the targeting vector were done. A 402-bp probe downstream of the 3′ insert of the *Rassf1a* knockout was generated using primers U33 (5′-GGCGAGGCTGAAACACCTTCCT-3′) and LM8109 (5′-CCCTGGCTCAACCGGTCTGTGA-3′). Genomic DNA of the ES cell colonies was digested with *Hin*dIII and separated on 0.8% TAE agarose gels, blotted, hybridized with the single-stranded labeled PCR probe, and then visualized. In all colonies with an apparent knockout allele, correct targeting was verified by Southern blots using *Nde*I digestion and external and internal hybridization probes.

Once the correctly targeted ES cell clones had been identified and confirmed by Southern blot analysis, they were used for injection into C57BL/6 blastocysts and generation of mouse chimeras. Male chimeras were mated to C57BL/6 females. After germ line transmission and breeding to homozygosity, the absence or presence of *Rassf1a* and *Rassf1c* transcripts, respectively, was confirmed by reverse transcription-PCR.

Reverse Transcription-PCR Analysis. Total RNA was isolated from early passage primary mouse embryo fibroblasts using the Trizol reagent (Invitrogen, Carlsbad, CA). Mouse embryo fibroblasts were derived from 13.5 days postcoitum embryos and genotyped as described below. For reverse transcription-PCR analysis, 200 ng of RNA was pre-associated with a lower primer from exon 4 of the mouse *Rassf1* gene (5′-GATGAAGCCTGTGTAGGAGCCATCCT). After the reverse transcription reaction, either an upper primer from exon 1-α (5′-GTCGTGGCCACCGTTTCCAG) or an upper primer specific for exon 2-γ (5′-CTGAAACACCTTCCTTCGAAATGACCT) was used in the PCR reaction to measure *Rassf1a* and *Rassf1c* mRNA levels, respectively. PCR conditions were 95°C for 30 seconds, 61°C for 30 seconds, and 68°C for 40 seconds (35 cycles), followed by a 10 minutes extension at 68°C. Glyceraldehyde-3-phosphate dehydrogenase primers were used in the same reverse transcription reactions as a control for mRNA integrity (25).

Animals and Genotyping. Animals were housed in plastic cages with hardwood bedding and dust covers, in a high-efficiency particulate air–filtered, environmentally controlled room (24 ± 1°C, 12/12 hours light/dark cycle). Animals were given Rodent Lab Chow #5001 (Purina, St. Louis, MO) and water *ad libitum*. The mice used in the study were genotyped by PCR analysis for identification of the *Rassf1a* genotype. The primer sequences were as follows: UMIOAI, 5′-TTGTGCCGTGCCCCGCCCA; LMIIAA, 5′-TGACCAGCCCTCCACTGCCGC; and Neo48U, 5′-GGGCCAGCTCATTCCTCCCAC. The multiplex PCR produces a 520-bp band for the wild-type allele and a 380-bp band for the knockout allele and both for heterozygous mice (see example in Fig. 1*C*).

Spontaneous Tumorigenesis Studies. At the age of 6 weeks, animals were assigned to three different groups (wild-type, *Rassf1a* heterozygous, and *Rassf1a* homozygous knockout, according to the genotype) and were on a 129SvJ × C57BL/6 background (roughly 50% each). At 18 to 20 months of age, the mice were euthanized by carbon dioxide asphyxiation and subjected to complete necroscopy of all major organs. Any abnormal tissues found were fixed in 10% neutral-buffered formalin followed by 70% ethanol and paraffin embedding. Tissue sections (5 μm) were stained with H&E for histopathologic evaluation. Immunohistochemistry was done using rat antibodies specific for the B cell marker CD45R/B220 (BD Biosciences, San Diego, CA) and the T cell marker CD3 (DakoCytomation, Carpinteria, CA) followed by secondary antibody, an avidin-biotin amplification system and peroxidase staining (Vector Laboratories, Burlingame, CA).

Skin Tumorigenesis Studies. A standard skin carcinogenesis study was done. Eight-week-old mice on a mixed 129 × C57BL/6 background were assigned to three groups according to the *Rassf1a* genotypes. All mice had their backs shaved weekly. Forty-eight hours prior to initial treatment, the dorsal skin of mice was shaved. All mice were treated topically twice weekly for 10 weeks with benzo(*a*)pyrene (100 nmol/mouse; Sigma Chemical, Co., St. Louis, MO) dissolved in 200 μl acetone. Skin tumors were monitored and recorded thrice a week for the duration of the studies. This bioassay was terminated at 7 months after the final benzo(*a*)pyrene exposure. For each mouse, the skin tumors were enumerated and three diameters were measured; only tumors with a diameter of > 2 mm were included. Part of the dorsal normal skin tissue and tumors were isolated and placed in individual tubes and were immediately frozen in liquid nitrogen. The rest of the tumors were fixed in 10% neutral-buffered formalin overnight, followed by 70% ethanol and paraffin embedding. Tissue sections (5 μm) were stained with H&E for histopathologic analysis. A gross necropsy was also done. The total tumor volumes of skin tumors were calculated by $V = (4/3)\pi r^3$ or $V = 2r\pi h$ (r, radius) dependent on the tumor shape.

Lung Tumorigenesis Studies. Eight-week-old mice on a mixed 129 × C57BL background were assigned to three groups according to the *Rassf1a* genotypes. Animals were given two i.p. injections of urethane (1 mg/g body weight; Sigma) in 0.2 mL PBS 1 week apart. Seven months after treatment with carcinogens, animals were euthanized by carbon dioxide asphyxiation. For each mouse, portions of the tumors plus some normal lung tissue were frozen in liquid nitrogen. The remaining tissue and tumors were fixed in Tellyesniczky's solution overnight, followed by 70% ethanol treatment. Each lung was examined with the aid of a dissecting microscope to obtain the tumor count and size. Tumor volumes were determined by measuring the three-dimensional size of each tumor and by using the average of the three measurements as the diameter. Only tumors with a diameter of > 1 mm were included. The radius (diameter/2) was determined, and the total tumor volume was calculated by: volume = $(4/3)\pi r^3$.

***Rassf1a* Methylation Analysis.** The genomic DNA from mouse skin and lung tumors of *Rassf1a*$^{+/-}$ mice was isolated using the Trizol solution (Invitrogen) according to the manufacturer's protocols. The methylation status of the *Rassf1a* promoter region was determined by chemical modification of genomic DNA with sodium bisulfite and methylation-specific PCR. Bisulfite treatment converts cytosine bases to uracil bases but has no effect on 5-methylcytosine bases. The bisulfite-treated DNA was used as a template for the methylation-specific PCR reaction. Primers for the unmethylated DNA-specific reaction were: F, 5′-GGTGTTGAAGTTGTGGTTTG-3′; R, 5′-TATTATACCCAAAACAATACAC-3′. Primers for the methylated DNA-specific reaction were: F, 5′-TTTTGCGGTTTCGTTCGTTC-3′; R, 5′-CCCGAAACGTACTACTATAAC-3′. The reactions were incubated at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, for 35 cycles. The PCR product obtained from methylated DNA was 213 bp, and the fragment obtained from unmethylated DNA was 204 bp. The amplified fragments were confirmed by DNA sequencing. DNA from normal lung was used as a control for unmethylated *Rassf1a*, and normal lung DNA treated with SssI DNA methyltransferase was used as a control for methylated *Rassf1a*. Water was used as a negative control, 25 μl of each PCR reaction was loaded onto a 6% nondenaturing polyacrylamide gel, which was stained with ethidium bromide, and photographed under UV light.

Results

Two major transcripts, termed *Rassf1a* and *Rassf1c* originate at the human and mouse genomic *Rassf1* loci and the organization of the exons is highly conserved between mouse and human. They code for two different proteins, RASSF1A and RASSF1C that differ in their NH_2-terminal sequence but have common COOH-terminal sequences (1, 5). Although *RASSF1C* is almost never inactivated in human tumors, loss of *RASSF1A* expression is very commonly observed in a variety of human solid tumors and is accompanied by methylation of the CpG island associated with the transcription start site of *RASSF1A*.

In order to study the function of *Rassf1a* in the mouse, we have carried out gene targeting experiments in which exon 1-α of the

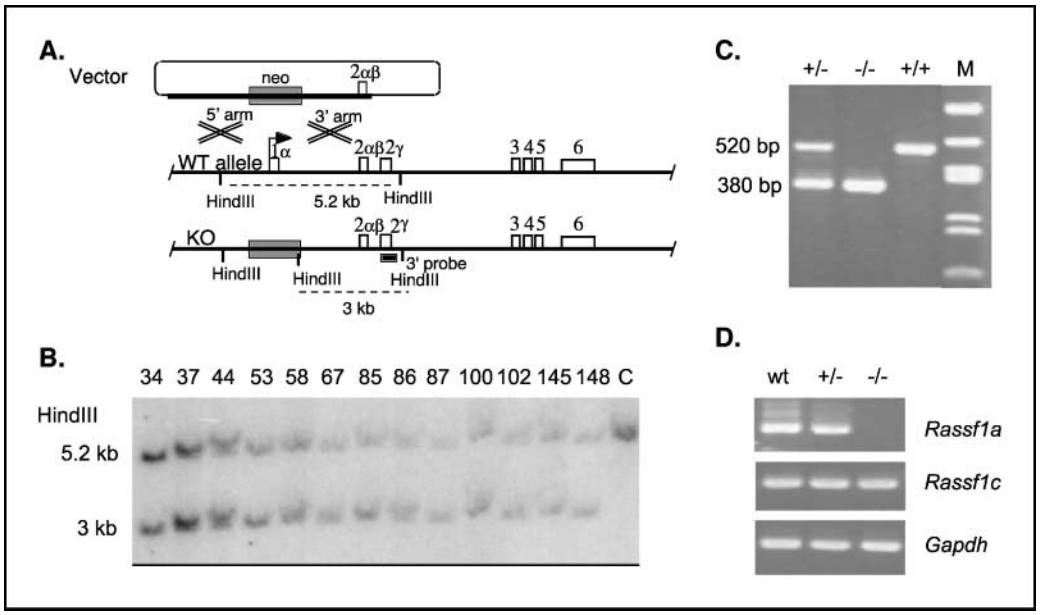

Figure 1. Generation of *Rassf1a* knockout mice. *A,* strategy for generating a mouse knockout of *Rassf1a*. The wild-type (*wt*) allele represents the organization of the exons (*open boxes*) of the human and mouse genes, which is conserved. For the mouse knockout of *Rassf1a*, the entire exon 1-α was deleted. The necessary homologies for the replacement vector are provided by the 5′ and 3′ arms, respectively. *B,* Southern blot analysis of ES cell clones targeted with the vector for the knockout of *Rassf1a*. After correct targeting, the expected size of the *Hin*dIII fragment is 3 kb. This band is present in the 13 targeted clones shown. "*C*" is a non-targeted clone. *C,* example of PCR genotyping of *Rassf1a* gene-targeted mice. *D,* reverse transcription-PCR analysis of expression of *Rassf1a* and *Rassf1c* in fibroblasts derived from the *Rassf1a*-targeted mice. Expression of glyceraldehyde-3-phosphate dehydrogenase was analyzed as a control.

Rassf1 gene was deleted (Fig. 1*A*). This targeting strategy should selectively eliminate the *Rassf1a* transcript but should leave expression of the *Rassf1c* isoform unaltered, a situation that is commonly found in human tumors. Several recombinant ES cell clones, in which exon 1-α was targeted, were identified (Fig. 1*B*). Two ES cell clones (#102 and #145) were used for blastocyst injection and generation of gene-targeted mice (Fig. 1*B* and *C*). Reverse transcription-PCR analysis confirmed that the *Rassf1a* transcript is missing and that *Rassf1c* is still expressed (Fig. 1*D*). *Rassf1a* homozygous knockout mice were viable and fertile, and no overt phenotype was immediately apparent. All experiments described here were subsequently carried out with the clone #145 mice. Mice were genotyped and assigned to three different groups according to genotype (wild-type, heterozygous, and homozygous *Rassf1a* knockout). They were kept and observed over extended periods of time. One $Rassf1a^{-/-}$ mouse died at 40 days of age and a splenic lymphoma was found. When the mice reached 18 to 20 months of age, they were euthanized and subjected to complete necroscopy. Only two lymphomas were found in a total of 48 wild-type mice (Tables 1 and 2). Six of thirty-five *Rassf1a* heterozygous mice developed tumors (two lymphomas and four lung adenomas). However, a total of 13 tumors were found in 41 $Rassf1a^{-/-}$ mice. The differences between wild-type and *Rassf1a* knockout mice were statistically significant (Table 1), in particular for the homozygous mice ($P < 0.001$, χ^2 test).

The types of tumors found in the $Rassf1a^{-/-}$ mice were hematologic tumors, lung tumors, one adenocarcinoma of the breast, and one rectal squamous papilloma. All tumors were subjected to histopathologic analysis. Examples are shown for the breast adenocarcinoma, two lung tumors, and one lymphoma (Fig. 2). Two homozygous *Rassf1a* knockout animals developed both lymphomas and adenomas of the lung. Most tumors in the *Rassf1a* knockout mice were morphologically similar to spontaneously observed tumors (40). The breast tumor measured 1.2 cm in diameter and was composed of multiple small glands and microcysts composed of relatively uniform cuboidal cells with vesicular nuclei and mild to moderate pleomorphism. The epithelial component was embedded in an abundant myxomatous stroma with spindled cells (Fig. 2*A* and *B*). Histologically similar tumors have been classified in the mouse as breast adenocarcinoma type C. The lung tumors were well circumscribed but nonencapsulated and composed of closely packed acini of cuboidal and low columnar epithelial cells (Fig. 2*C* and *D*). Some tumors showed papillary formations and others had more nuclear pleomorphism with rare intranuclear inclusions (Fig. 2*F*). A few animals had both the epithelial tumor as well as lymphoma (Fig. 2*E*). In general, the hematopoietic tumors extensively involved both solid organs such as the lung and spleen as well as soft tissues. Morphologically, they were composed of small atypical lymphoid cells with plasmacytic differentiation. Immunologic characterization indicated that five of the six lymphomas analyzed, including the tumors in $Rassf1a^{-/-}$ mice, were high-grade B cell lymphomas.

We then conducted standard skin and lung carcinogenesis experiments to characterize the susceptibility of *Rassf1a* gene-targeted mice to tumor formation. In the skin carcinogenesis studies, benzo(*a*)pyrene was applied to the shaved dorsal skin of mice beginning at 8 weeks of age. Tumor formation was evaluated 7 months after the treatments (Fig. 3). Tumor size was significantly increased in *Rassf1a* knockout mice (Fig. 3*A* and *B*). However, tumor multiplicity was increased only in heterozygous *Rassf1a* knockout mice. Importantly, all tumors found in $Rassf1a^{+/+}$ mice were skin papillomas. However, approximately 50% of the skin

Table 1. Tumor susceptibility of *Rassf1a* knockout mice

Genotype	Tumors/animals	
Wild-type	2/48 (4.2%)	
$Rassf1a^{+/-}$	6/35 (17.1%)	$P = 0.05$*
$Rassf1a^{-/-}$	13/41 (31.7%)	$P < 0.001$*

*χ^2 test.

Table 2. Tumor types in *Rassf1a* knockout mice

Wild-type	
Lymphoma	2
Rassf1a$^{+/-}$	
Lymphoma	1
Lung adenoma	4
Sarcoma	1
Rassf1a$^{-/-}$	
Lymphoma	4
Leukemia	1
Lung adenoma	5
Breast adenocarcinoma	1
Lung papillary tumor	1
Rectal papilloma	1

tumors found in either *Rassf1a*$^{+/-}$ or *Rassf1a*$^{-/-}$ mice were skin squamous cell carcinomas.

In the lung tumorigenesis study, mice at 8 weeks of age were injected with urethane, a well-established lung carcinogen. Lungs were dissected at 7 months after the treatments. Both tumor load and tumor multiplicity were increased in *Rassf1a* gene-targeted mice relative to wild-type mice but the difference between heterozygous and homozygous mice was not significant (Fig. 3). All lung tumors found in either *Rassf1a*$^{+/+}$, *Rassf1a*$^{+/-}$, or *Rassf1a*$^{-/-}$ mice were lung adenomas.

Because the multiplicity of skin tumors was highest in the heterozygous animals and the most common mechanism for inactivation of *Rassf1a* is by methylation silencing, we examined if the remaining allele of *Rassf1a* had become methylated in the tumors. We investigated a total of 17 benzo(*a*)pyrene-induced skin tumors from wild-type and *Rassf1a* heterozygous mice as well as nine lung tumors found in *Rassf1a*$^{+/-}$ mice. No methylation was found in the

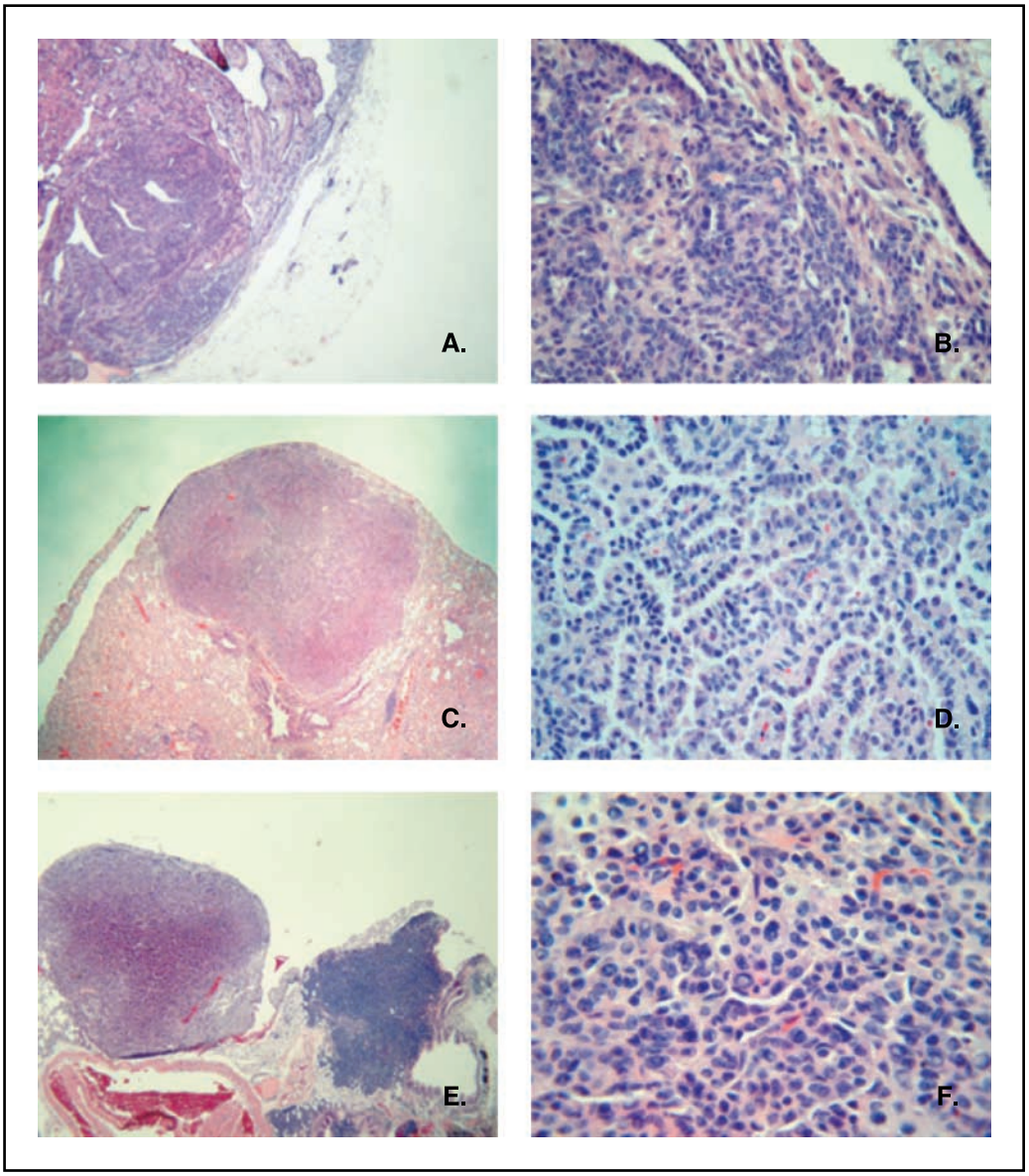

Figure 2. Histologic analysis of tumors found in *Rassf1a*$^{-/-}$ mice. *A,* breast adenocarcinoma; the tumor is well circumscribed and benign breast tissue is seen at the periphery (4× magnification). *B,* breast adenocarcinoma; the breast tumor is composed of rather uniform cells, forming small glands and cysts in a loose myxomatous matrix (40× magnification). *C,* lung adenoma; the tumor is well circumscribed and nonencapsulated (4× magnification). *D,* lung adenoma; the tumor is composed of closely packed acini of cuboidal to low columnar cells (40× magnification). *E,* a lung adenoma and multiple nodules of lymphoma in the same lung (4× magnification). *F,* some lung tumors were more cellular with intranuclear invaginations and moderate pleomorphism (60× magnification). Tissues were paraffin-embedded and stained with H&E stain.

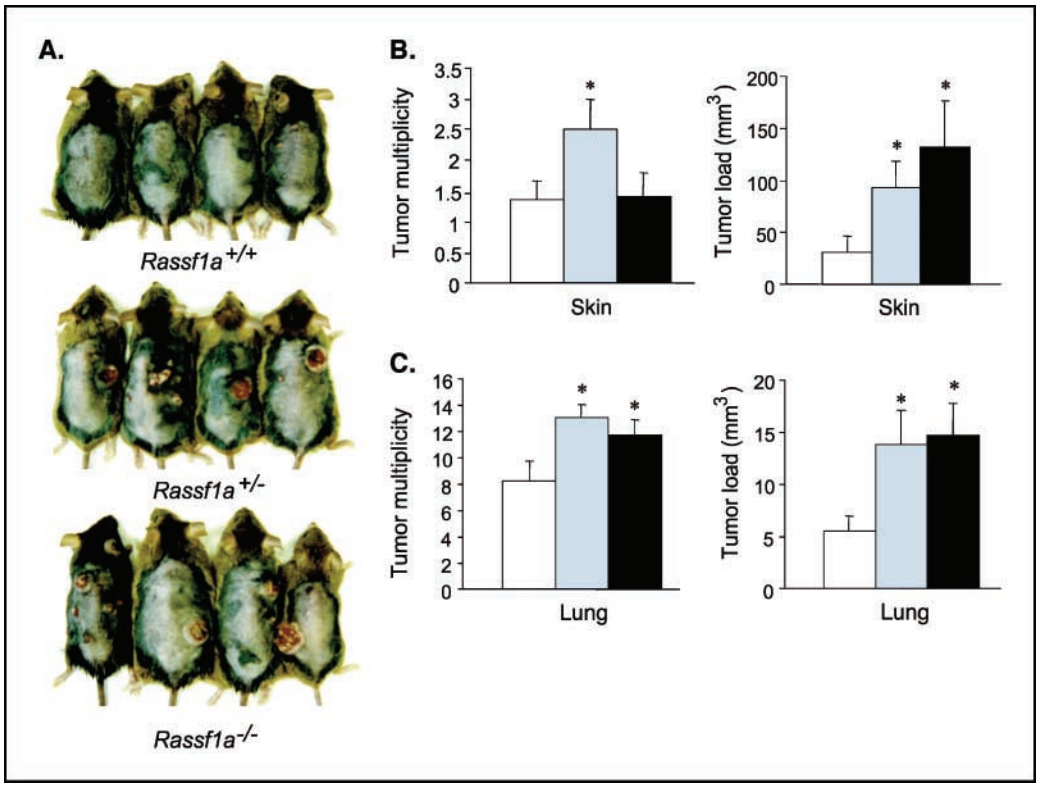

Figure 3. *Rassf1a* gene-targeted mice are susceptible to chemical carcinogen-induced tumorigenesis. *A,* skin carcinogenesis; cohorts of 8-week-old mice with different genotypes (*Rassf1a*$^{+/+}$, *Rassf1a*$^{+/-}$, *Rassf1a*$^{-/-}$) were exposed twice weekly for 10 weeks to the chemical carcinogen benzo(*a*)pyrene on the dorsal skin. Photographs were taken at 7 months after the last exposure. *B* and *C,* quantitation of the skin and lung carcinogenesis data. Skin tumors (*B*), were induced with benzo(*a*)pyrene and lung tumors (*C*), were induced by injection of urethane. Tumor load and tumor multiplicity were determined 7 months after the exposures. *White columns,* *Rassf1a*$^{+/+}$ mice; *gray columns,* *Rassf1a*$^{+/-}$ mice; and *black columns,* *Rassf1a*$^{-/-}$ mice. The number of animals for the skin carcinogenesis study was (*Rassf1a*$^{+/+}$, $n = 14$; *Rassf1a*$^{+/-}$, $n = 23$; *Rassf1a*$^{-/-}$, $n = 19$). The number of animals for the lung tumorigenesis study was (*Rassf1a*$^{+/+}$, $n = 8$; *Rassf1a*$^{+/-}$, $n = 26$; *Rassf1a*$^{-/-}$, $n = 21$). *Bars,* SE; **, $P < 0.05$, *t* test.

tumors from wild-type mice but 3 out of 10 skin tumors arising in heterozygous animals showed the presence of methylated *Rassf1a* alleles (Fig. 4, samples 13, 17, and 18); Table 3). Because these mice have only one copy of *Rassf1a*, the presence of a band derived from the unmethylated allele is expected to arise from normal cell contamination or from partial methylation present within the tumor. No methylation was found in the lung tumors from heterozygous animals.

Discussion

Methylation of the *RASSF1A* gene is one of the most common epigenetic aberrations described thus far in human tumors (1, 5, 7–32, 41–44). The common epigenetic silencing and its location within an area of overlapping homozygous deletions at 3p21.3 (4) suggest that *RASSF1A* may be a tumor suppressor gene. Here we present evidence that mice, which lack the *Rassf1a* isoform, are susceptible to late stage spontaneous tumorigenesis and to formation of lung and skin tumors induced by chemical carcinogens. Although inactivation of *RASSF1A* in human non-melanoma skin tumors has not yet been investigated, there are several reports documenting *RASSF1A* methylation in human lung tumors (1, 5, 7, 42–44). A breast adenocarcinoma was found in one *Rassf1a*$^{-/-}$ mouse and epigenetic inactivation of *RASSF1A* in human breast cancers is a common event and occurs in over 60% of the tumors (5, 10, 30).

Heterozygous *Rassf1a* knockout mice were significantly tumor-prone, both for spontaneous tumor formation and for the chemically induced tumors. This may suggest that the *Rassf1a* gene has the characteristics of conferring haploinsufficiency when only one allele is lost. There are now many examples demonstrating that loss of a single gene copy is sufficient to promote tumor formation (45, 46). Another possibility is that the single remaining *Rassf1a* allele in heterozygous mice is particularly

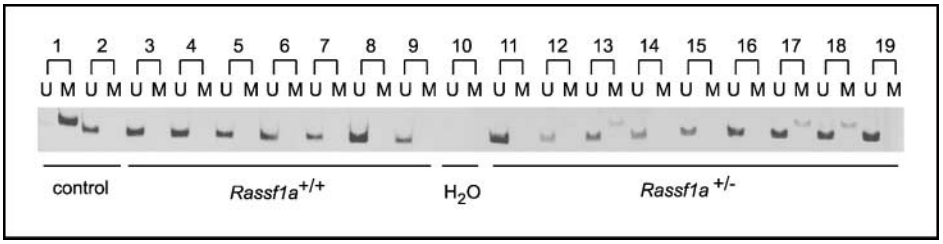

Figure 4. Analysis of *Rassf1a* methylation in skin squamous cell carcinomas induced by benzo(*a*)pyrene. Tumors from wild-type (*Rassf1a*$^{+/+}$) and *Rassf1a* heterozygous (*Rassf1a*$^{+/-}$) mice were analyzed using methylation-specific PCR. Normal lung DNA treated with SssI DNA methyltransferase was used as a control for methylated *Rassf1a* (*lanes 1* and *2*) and untreated DNA from normal lung was used as a control for unmethylated *Rassf1a* (*lanes 3* and *4*). *U,* PCR product derived from unmethylated *Rassf1a* alleles (204 bp); *M,* PCR product derived from methylated *Rassf1a* alleles (213 bp).

Table 3. Methylation-specific PCR analysis of *Rassf1a* in carcinogen-induced tumors in *Rassf1a* wild-type and heterozygous mice

Genotype	No.	Carcinogen	Tumors	*Rassf1a* methylation
Rassf1a$^{+/+}$	7	benzo(*a*)pyrene	skin squamous cell carcinoma	0
Rassf1a$^{+/-}$	10	benzo(*a*)pyrene	skin squamous cell carcinoma	3 (30%)
Rassf1a$^{+/-}$	9	urethane	lung adenoma	0

prone to inactivation. We have tested this possibility using carcinogen-induced skin and lung tumors. For the skin tumors, tumor multiplicity was particularly high in heterozygous mice (Fig. 3). The data for skin tumors indicate that methylation was more common in tumors from heterozygous animals than in wild-type animals, although not all tumors from heterozygous mice showed methylation (Fig. 4). However, no methylation was found in lung tumors from heterozygous mice. The data from lung tumors and most skin tumors support the hypothesis of haploinsufficiency. However, the observation that *Rassf1a* promoter methylation is seen in a subset of skin tumors from heterozygous animals indicates that some skin tumors may not fit this hypothesis. We are pursuing immunohistochemistry studies on tumor tissue from heterozygous mice to show Rassf1a expression. However, we have not found or developed an antibody that works, and this makes this approach unfeasible at present. Further, we believe that studies on *Rassf1a* mutations in tumors from *Rassf1a*$^{+/-}$ mice are not likely to reveal any changes because mutational inactivation of *RASSF1A* in human tumors is exceedingly rare. On the other hand, loss of heterozygosity at 3p21.3 is a common and early event in the progression of several human cancers and is observed, for example, in breast and lung cancers (2, 4, 47, 48). Methylation of *RASSF1A* has been observed in early lesions of the lung, breast, and thyroid gland (14, 49, 50).

In summary, we provide the first genetic model to support the role of *RASSF1A* as a tumor suppressor gene. It will be interesting to determine if loss of *RASSF1A* in conjunction with the inactivation or overexpression of other genes will produce a more severe phenotype. For example, the closest homologue of RASSF1A, NORE1A, which is about 55% identical to RASSF1A, has been reported to be inactivated in human tumors as well (51, 52). Because RASSF1A has been proposed to be a negative regulator of activated Ras (53, 54), experiments in which loss of RASSF1A function is combined with expression of activated k-Ras may be informative. Finally, because RASSF1A stabilizes microtubules and plays a role in mitotic control (37, 38), the simultaneous inactivation of *RASSF1A* and mitotic checkpoint control genes, such as *MAD2*, may dramatically increase tumor susceptibility.

Acknowledgments

Received 8/5/2004; revised 10/9/2004; accepted 11/3/2004.

Grant support: NIH grants (CA88873 to G.P. Pfeifer, and CA093643 to M. You) and by grant BMBF-FKZ01ZZ0104 to R. Dammann.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Donna Isbell for technical help with the mouse colony.

References

1. Dammann R, Li C, Yoon JH, Chin PL, Bates S, Pfeifer GP. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. Nat Genet 2000;25:315–9.
2. Kok K, Naylor SL, Buys CH. Deletions of the short arm of chromosome 3 in solid tumors and the search for suppressor genes. Adv Cancer Res 1997;71:27–92.
3. Sekido Y, Ahmadian M, Wistuba II, et al. Cloning of a breast cancer homozygous deletion junction narrows the region of search for a 3p21.3 tumor suppressor gene. Oncogene 1998;16:3151–7.
4. Lerman MI, Minna JD. The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: identification and evaluation of the resident candidate tumor suppressor genes. The International Lung Cancer Chromosome 3p21.3 Tumor Suppressor Gene Consortium. Cancer Res 2000;60:6116–33.
5. Burbee DG, Forgacs E, Zochbauer-Muller S, et al. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. J Natl Cancer Inst 2001;93:691–9.
6. Ponting CP, Benjamin DR. A novel family of Ras-binding domains. Trends Biochem Sci 1996;21:422–5.
7. Agathanggelou A, Honorio S, Macartney DP, et al. Methylation associated inactivation of RASSF1A from region 3p21.3 in lung, breast and ovarian tumours. Oncogene 2001;20:1509–18.
8. Pfeifer GP, Yoon JH, Liu L, Tommasi S, Wilczynski SP, Dammann R. Methylation of the RASSF1A gene in human cancers. Biol Chem 2002;383:907–14.
9. Dammann R, Schagdarsurengin U, Strunnikova M, et al. Epigenetic inactivation of the Ras-association domain family 1 (RASSF1A) gene and its function in human carcinogenesis. Histol Histopathol 2003;18: 665–77.
10. Dammann R, Yang G, Pfeifer GP. Hypermethylation of the CpG island of Ras association domain family 1A (RASSF1A), a putative tumor suppressor gene from the 3p21.3 locus, occurs in a large percentage of human breast cancers. Cancer Res 2001;61:3105–9.
11. Dreijerink K, Braga E, Kuzmin I, et al. The candidate tumor suppressor gene, RASSF1A, from human chromosome 3p21.3 is involved in kidney tumorigenesis. Proc Natl Acad Sci U S A 2001;98:7504–9.
12. Kuzmin I, Gillespie JW, Protopopov A, et al. The RASSF1A tumor suppressor gene is inactivated in prostate tumors and suppresses growth of prostate carcinoma cells. Cancer Res 2002;62:3498–502.
13. Liu L, Yoon JH, Dammann R, Pfeifer GP. Frequent hypermethylation of the RASSF1A gene in prostate cancer. Oncogene 2002;21:6835–40.
14. Schagdarsurengin U, Gimm O, Hoang-Vu C, Dralle H, Pfeifer GP, Dammann R. Frequent epigenetic silencing of the CpG island promoter of RASSF1A in thyroid carcinoma. Cancer Res 2002;62:3698–701.
15. Schagdarsurengin U, Wilkens L, Steinemann D, et al. Frequent epigenetic inactivation of the RASSF1A gene in hepatocellular carcinoma. Oncogene 2003;22: 1866–71.
16. Dammann R, Takahashi T, Pfeifer GP. The CpG island of the novel tumor suppressor gene RASSF1A is intensely methylated in primary small cell lung carcinomas. Oncogene 2001;20:3563–7.
17. Morrissey C, Martinez A, Zatyka M, et al. Epigenetic inactivation of the RASSF1A 3p21.3 tumor suppressor gene in both clear cell and papillary renal cell carcinoma. Cancer Res 2001;61:7277–81.
18. Lo KW, Kwong J, Hui AB, et al. High frequency of promoter hypermethylation of RASSF1A in nasopharyngeal carcinoma. Cancer Res 2001;61:3877–81.
19. Byun DS, Lee MG, Chae KS, Ryu BG, Chi SG. Frequent epigenetic inactivation of RASSF1A by aberrant promoter hypermethylation in human gastric adenocarcinoma. Cancer Res 2001;61:7034–8.
20. Honorio S, Agathanggelou A, Wernert N, Rothe M, Maher ER, Latif F. Frequent epigenetic inactivation of the RASSF1A tumour suppressor gene in testicular tumours and distinct methylation profiles of seminoma and nonseminoma testicular germ cell tumours. Oncogene 2003;22:461–6.
21. Chan MW, Chan LW, Tang NL, et al. Frequent hypermethylation of promoter region of RASSF1A in tumor tissues and voided urine of urinary bladder cancer patients. Int J Cancer 2003;104:611–6.
22. Lee MG, Kim HY, Byun DS, et al. Frequent epigenetic

inactivation of RASSF1A in human bladder carcinoma. Cancer Res 2001;61:6688–92.
23. Lusher ME, Lindsey JC, Latif F, Pearson AD, Ellison DW, Clifford SC. Biallelic epigenetic inactivation of the RASSF1A tumor suppressor gene in medulloblastoma development. Cancer Res 2002;62:5906–11.
24. Maruyama R, Toyooka S, Toyooka KO, et al. Aberrant promoter methylation profile of bladder cancer and its relationship to clinicopathological features. Cancer Res 2001;61:8659–63.
25. Spugnardi M, Tommasi S, Dammann R, Pfeifer GP, Hoon DS. Epigenetic inactivation of RAS association domain family protein 1 (RASSF1A) in malignant cutaneous melanoma. Cancer Res 2003;63:1639–43.
26. Toyooka S, Carbone M, Toyooka KO, et al. Progressive aberrant methylation of the RASSF1A gene in simian virus 40 infected human mesothelial cells. Oncogene 2002;21:4340–4.
27. van Engeland M, Roemen GM, Brink M, et al. K-*ras* mutations and RASSF1A promoter methylation in colorectal cancer. Oncogene 2002;21:3792–5.
28. Wagner KJ, Cooper WN, Grundy RG, et al. Frequent RASSF1A tumour suppressor gene promoter methylation in Wilms' tumour and colorectal cancer. Oncogene 2002;21:7277–82.
29. Zhang YJ, Ahsan H, Chen Y, et al. High frequency of promoter hypermethylation of RASSF1A and p16 and its relationship to aflatoxin B1-DNA adduct levels in human hepatocellular carcinoma. Mol Carcinog 2002;35:85–92.
30. Yan PS, Shi H, Rahmatpanah F, et al. Differential distribution of DNA methylation within the RASSF1A CpG island in breast cancer. Cancer Res 2003;63: 6178–86.
31. Maruyama R, Toyooka S, Toyooka KO, et al. Aberrant promoter methylation profile of prostate cancers and its relationship to clinicopathological features. Clin Cancer Res 2002;8:514–9.
32. Zhong S, Yeo W, Tang MW, Wong N, Lai PB, Johnson PJ. Intensive hypermethylation of the CpG island of Ras association domain family 1A in hepatitis B virus-associated hepatocellular carcinomas. Clin Cancer Res 2003;9:3376–82.
33. Vavvas D, Li X, Avruch J, Zhang XF. Identification of Nore1 as a potential Ras effector. J Biol Chem 1998;273:5439–42.
34. Ortiz-Vega S, Khokhlatchev A, Nedwidek M, et al. The putative tumor suppressor RASSF1A homodimerizes and heterodimerizes with the Ras-GTP binding protein Nore1. Oncogene 2002;21:1381–90.
35. Khokhlatchev A, Rabizadeh S, Xavier R, et al. Identification of a novel Ras-regulated proapoptotic pathway. Curr Biol 2002;12:253–65.
36. Shivakumar L, Minna J, Sakamaki T, Pestell R, White MA. The RASSF1A tumor suppressor blocks cell cycle progression and inhibits cyclin D1 accumulation. Mol Cell Biol 2002;22:4309–18.
37. Liu L, Tommasi S, Lee DH, Dammann R, Pfeifer GP. Control of microtubule stability by the RASSF1A tumor suppressor. Oncogene 2003;22:8125–36.
38. Song MS, Song SJ, Ayad NG, et al. The tumour suppressor RASSF1A regulates mitosis by inhibiting the APC-Cdc20 complex. Nat Cell Biol 2004;6:129–37.
39. Jackson PK. Linking tumor suppression, DNA damage and the anaphase-promoting complex. Trends Cell Biol 2004;14:331–4.
40. Turusov VS. Pathology of tumors in laboratory animals, volume II: tumors of the mouse. Lyon: WHO IARC; 1979.
41. Harada K, Toyooka S, Maitra A, et al. Aberrant promoter methylation and silencing of the RASSF1A gene in pediatric tumors and cell lines. Oncogene 2002; 21:4345–9.
42. Endoh H, Yatabe Y, Shimizu S, et al. RASSF1A gene inactivation in non-small cell lung cancer and its clinical implication. Int J Cancer 2003;106:45–51.
43. Tomizawa Y, Kohno T, Kondo H, et al. Clinicopathological significance of epigenetic inactivation of RASSF1A at 3p21.3 in stage I lung adenocarcinoma. Clin Cancer Res 2002;8:2362–8.
44. Li J, Zhang Z, Dai Z, et al. RASSF1A promoter methylation and Kras2 mutations in non small cell lung cancer. Neoplasia 2003;5:362–6.
45. Santarosa M, Ashworth A. Haploinsufficiency for tumour suppressor genes: when you don't need to go all the way. Biochim Biophys Acta 2004;1654:105–22.
46. Cook WD, McCaw BJ. Accommodating haploinsufficient tumor suppressor genes in Knudson's model. Oncogene 2000;19:3434–8.
47. Wistuba II, Behrens C, Milchgrub S, et al. Sequential molecular abnormalities are involved in the multistage development of squamous cell lung carcinoma. Oncogene 1999;18:643–50.
48. Maitra A, Wistuba II, Washington C, et al. High-resolution chromosome 3p allelotyping of breast carcinomas and precursor lesions demonstrates frequent loss of heterozygosity and a discontinuous pattern of allele loss. Am J Pathol 2001;159:119–30.
49. Honorio S, Agathanggelou A, Schuermann M, et al. Detection of RASSF1A aberrant promoter hypermethylation in sputum from chronic smokers and ductal carcinoma *in situ* from breast cancer patients. Oncogene 2003;22:147–50.
50. Xing M, Cohen Y, Mambo E, et al. Early occurrence of RASSF1A hypermethylation and its mutual exclusion with BRAF mutation in thyroid tumorigenesis. Cancer Res 2004;64:1664–8.
51. Hesson L, Dallol A, Minna JD, Maher ER, Latif F. NORE1A, a homologue of RASSF1A tumour suppressor gene is inactivated in human cancers. Oncogene 2003; 22:947–54.
52. Chen J, Lui WO, Vos MD, et al. The t(1;3) breakpoint-spanning genes LSAMP and NORE1 are involved in clear cell renal cell carcinomas. Cancer Cell 2003;4:405–13.
53. Vos MD, Ellis CA, Bell A, Birrer MJ, Clark GJ. Ras uses the novel tumor suppressor RASSF1 as an effector to mediate apoptosis. J Biol Chem 2000;275:35669–72.
54. Zhang Z, Wang Y, Vikis HG, et al. Wildtype Kras2 can inhibit lung carcinogenesis in mice. Nat Genet 2001; 29:25–33.

Tumor Susceptibility of *Rassf1a* Knockout Mice

Stella Tommasi, Reinhard Dammann, Zhongqiu Zhang, et al.

Cancer Res 2005;65:92-98.