Use of avian retroviral vectors to introduce transcriptional regulators into mammalian cells for analyses of tumor maintenance

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A key issue in cancer biology is whether genetic lesions involved in tumor initiation or progression are required for tumor maintenance. This question can be addressed with mouse models that conditionally express oncogenic transgenes, i.e., under the control of tetracycline (tet)-dependent transcriptional regulators. We have developed a system for studying tumor maintenance by using avian retroviral [i.e., replication-competent avian leukosis virus long terminal repeat with splice acceptor (RCAS)] vectors to deliver the reverse tet transcriptional transactivator (rtTA) gene to somatic mammalian cells. rtTA can regulate any transgene in which the protein coding sequence is preceded by a tet-operator (tet-o); RCAS viruses infect only cells engineered to express ectopically the avian retroviral receptor, TVA. One vector, RCAS-*rtTA-IRES-GFP*, also encodes GFP to identify infected cells. Infection of cells from β-actin TVA transgenic mice with this vector permits efficient regulation of tet-responsive transgenes. Sarcomas arise when p53-deficient murine embryonic fibroblasts carrying β-actin TVA and tet-o-*K-ras4b*^{G12D} transgenes are infected with RCAS-*rtTA-IRES-GFP* and introduced into nude mice treated with the tet analog, doxycycline (dox); when dox is withdrawn, *K-ras4b*^{G12D} levels fall, cells undergo apoptosis, and tumors regress. Regression can be prevented by means of a genetic complementation assay in which tumors are superinfected before dox withdrawal with other RCAS viruses, such as those carrying an active allele of *K-ras*. Many TVA and tet-regulated transgenic mice have been generated; thus, this method for somatic cell-specific and temporally controlled gene expression may have broad applications for the study of oncogenesis and tumor maintenance, as well as other cell functions and development.

Cancers are thought to arise from a multistage process during which tumor cells progressively acquire activating mutations in oncogenes and inactivating mutations in tumor suppressor genes (1). In most cases, it is not known whether a genetic lesion that is necessary for the initial development or progression of a specific tumor is also required for the maintenance of that tumor's survival. Moreover, the mechanisms by which oncogenes sustain tumor phenotypes are not well understood.

Recently, the development of mouse models that permit conditional expression of a specific gene of interest has led to a more systematic study of tumor maintenance *in vivo* (reviewed in refs. 2 and 3). Most of the inducible methods rely on the control of gene expression by tetracycline (tet) (4). Three elements are required: a tet analog [such as doxycycline (dox)], which is exogenously administered; a tet transcriptional transactivator; and a tet-responsive gene of interest regulated by a tet operator. In the tet-on system, which involves the reverse tetracycline transcriptional transactivator (rtTA), a tet-regulated gene is expressed only in bitransgenic mice administered dox.

We have developed a system for studying tumor maintenance by using avian retroviral [i.e., replication-competent avian leukosis virus long terminal repeat with splice acceptor (RCAS)] vectors to deliver the rtTA gene to somatic mammalian cells, which harbor tet-regulated transgenes and express the gene product of *tv-a* (TVA), which encodes the receptor for subgroup A avian leukosis virus-derived retroviruses. Ectopic expression of TVA by cells confers susceptibility to infection; RCAS-based viruses do not infect normal mammalian cells. The advantages and disadvantages of the TVA avian retroviral system have been reviewed (5, 6) and are well documented (7, 8). We infected murine embryonic fibroblasts (MEFs) derived from wild-type and/or p53-deficient β-actin TVA transgenic animals carrying tet operator (tet-o)-*lacZ* or tet-o-*K-ras4b*^{G12D} transgenes to demonstrate that this approach can be used to achieve dox-dependent gene expression of tet-regulated transgenes *in vitro* and *in vivo*, and tumor progression and regression *in vivo*. We also show that tumor regression can be prevented through a genetic complementation assay in which tumors are superinfected before dox withdrawal with other RCAS viruses; we establish proof of principle with RCAS carrying a constitutively active allele of *K-ras*.

Materials and Methods

Mouse Strains. β-actin TVA (9), tet-o-*K-ras4b*^{G12D} (10), and p53$^{-/-}$ (11) mice have been described. Athymic nude mice (*nu/nu*; National Cancer Institute) were maintained either on standard mouse or dox-impregnated food pellets (Harlan-Teklad, Madison, WI). Tet-o-*lacZ* mice were generated on an FVB/N background by using standard techniques with pBI-G (CLONTECH), which expresses the reporter enzyme β-galactosidase and a dominant-negative form of p53 (dnp53) simultaneously under the control of a single tet-responsive element. (The dnp53 element has yet to be fully characterized). All mice were housed in accordance with institutional guidelines. Genotyping was performed by PCR with DNA extracted from tails or embryonic cells. PCR primers for tet-o-*lacZ* mice were: revmp1.2 5′-GCCTGCGACGGCGGCATCTGC-3′; and rt p53.1 5′-TCCGCGGGCGTAAACGCTTCG-3′.

Viral Constructs and Virus Production. For RCAS-*rtTA*, an ≈1-kb fragment containing rtTA was excised from pcDNA6c-rtTA with *Eco*RI, blunted with DNA polymerase I, large (Klenow) fragment, and inserted into RCAS-X, which was digested with *Not*I (blunted). For RCAS-*rtTA-IRES-GFP*, the *Eco*RI rtTA fragment was inserted into the *Eco*RI site of the cloning vector pIRES2-EGFP (enhanced GFP) (CLONTECH). Subsequently, *p*-rtTA-IRES2-EGFP was digested with *Xho*I, blunted, and digested with *Not*I; the resulting 2.3-kb rtTA-IRES-GFP fragment was inserted into RCAS-X, digested with *Cla*I (blunted)

Abbreviations: tet, tetracycline; tet-o, tet operator; rtTA, reverse tet transcriptional transactivator; dox, doxycycline; RCAS, replication-competent avian leukosis virus long terminal repeat with splice acceptor; TVA, gene product of *tv-a*, which encodes the receptor for subgroup A avian leukosis virus-derived retroviruses; MEF, murine embryonic fibroblast; IRES, internal ribosomal entry site; HA, hemagglutinin.

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and *Not*I. For RCAS-*rtTA-IRES-PURO*, the *Eco*RI rtTA fragment was inserted into the *Eco*RI site of the cloning vector, pQCXIP (CLONTECH). A 2.5-kb *Not*I–*Cla*I fragment was then excised and inserted into RCAS-Y, which was digested with the same enzymes. RCAS-*GFP*, RCAS-*AP*, RCAS-*K-ras4b*^{G12D}, and RCAS-*K-ras4b*^{G12D} hemagglutinin (HA) have been described (8).

To produce retroviruses carrying the tet-transactivator, DF-1 chicken fibroblasts (12, 13) were transfected, cultured, and processed as published (8). Viral titers for RCAS-*rtTA-IRES-GFP* were measured by end point dilution assays on uninfected DF-1 cells by using either frozen concentrated virus or fresh viral supernatants. Infections at various dilutions were scored as positive if infected cells expressed GFP by fluorescent microscopy (Leica).

Cell Culture. Day 12–14 MEFs were derived by standard protocols from the progeny of β-actin TVA mice crossed to either tet-o-*lacZ* or tet-o-*K-ras4b*^{G12D} mice; the latter were bred on a wild-type or p53-deficient background. MEFs were cultured in DF-1 media for no more than 2 weeks in total; for infections, new viral supernatant was added to medium twice a day for 4–5 days. Dox (Sigma-Aldrich) was added to appropriate cultures at a concentration of 1 μg/ml. RCAS-rtTA-infected MEFs were examined for GFP expression before adding dox either *in vitro* or *in vivo* by fluorescent microscopy and/or fluorescence activated cell sorting; the latter was performed by using FACSCalibur and/or FACS Vantage SE systems (Becton-Dickinson).

Tumorigenicity Assays. To induce MEF tumors, the flanks of nude mice maintained on normal or dox-containing diets were injected s.c. with cell populations containing at least 1×10^6 GFP-positive cells resuspended in 200 μl of PBS at each site of injection (one to two sites per mouse). For RCAS-*K-ras4b*^{G12D}-infected cells, $>1 \times 10^6$ total cells were injected per site. MEFs were scored as tumors if a visible nodule ($\geq$5 mm in diameter) appeared at the site of injection; tumors were measured by calipers in two dimensions (length and width) at regular intervals. Tumor volume was calculated by determining the average radius and with the equation, $(4/3)\pi r^3$. On average, tumors formed in $\approx$8 weeks. Mice injected with negative control MEFs were observed for up to 21 weeks.

To observe for tumor regression, mice with measurable tumors had dox removed from their diet. Tumors were then measured at regular intervals. At the time of harvest, skin-exposed tumors were analyzed for GFP expression by imaging with a high-intensity light source and appropriate filter (Intralux 6000-1, Volpi Manufacturing, Auburn, NY). Tumor fragments were snap-frozen in liquid nitrogen for DNA, RNA, or protein extraction. Other fragments were fixed in 10% formalin for histological and immunohistochemical studies. Tumor pieces were also finely minced, washed in PBS, and plated in culture medium for isolation of tumor cells.

For genetic complementation assays, existing MEF tumors were injected with DF-1 producer cells or concentrated virus. Dox was withdrawn 3 days after injection, and tumors were observed for regression as above.

RT-PCR. Total RNA from cells or frozen tissue was isolated by using Trizol (Invitrogen). Samples were treated with RNase-free DNase (Invitrogen), and RT-PCR was performed by using Superscript One-Step RT-PCR (Invitrogen) for 35 cycles with an annealing temperature of 58°C and 1 μg of total RNA per sample. Primers for the tet-regulated *K-ras4b*^{G12D} transgene were: K-ras4b forward (DT12) 5′-GGGAATAAGTGTGAT TTGCCT-3′ and mp-1 reverse (revmp917) 5′-GCTATTCTG TGCATCTAGTATT-3′. β-actin primers were obtained from Promega. PCR products were resolved on 2% agarose gels and were visualized with ethidium bromide.

Immunohistochemistry. Formalin-fixed/paraffin-embedded 5-μm-thick sections were hematoxylin/eosin-stained for morphological evaluation. Immunohistochemistry was performed with polyclonal rabbit anti-Ki-67 (Vector Laboratories, 1:1,000) and anti-HA (Y-11, Santa Cruz Biotechnology, 1:100) antibodies. TUNEL (14) was performed as per established lab protocols, with a 1:5 dilution of standard TdT enzyme (Roche Applied Science). Substrates were visualized by using appropriate ABC kits (Vector Laboratories).

β-Galactosidase Visualization. MEFs were washed in PBS and fixed with 0.2% glutaraldehyde and 2% formaldehyde (Sigma-Aldrich) in PBS for 5 min. After another wash with PBS, MEFS were incubated at 37°C for >1 h in X-gal mix: 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, 1 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside in PBS. Cells were washed again in PBS and then examined for blue nuclei by light microscopy (Leica).

Southern Blotting. DNA was extracted from DF-1 cells producing either RCAS-*GFP* or RCAS-*rtTA-IRES-GFP* retroviruses and was double digested with *Eco*RV and *Not*I restriction enzymes. Membranes were hybridized to either P^{32}-dATP-labeled rtTA (*Eco*RI fragment) or IRES-GFP (*Bam*HI/*Not*I) probes.

Results

Generation of RCAS Vectors Encoding rtTA. RCAS retroviral vectors produce replication-competent viruses that use the viral long terminal repeat to drive expression of a gene of interest. To produce RCAS retroviruses capable of delivering a tet-on transactivator, we used an $\approx$1-kb *rtTA* cDNA fragment to generate three separate RCAS vectors: RCAS-*rtTA*, RCAS-*rtTA-IRES-GFP*, and RCAS-*rtTA-IRES-PURO* (Fig. 1). The first vector encodes the *rtTA* gene alone. In the second vector, the *rtTA* gene was placed in tandem with *IRES-GFP*, which contains an internal ribosome entry site (IRES) and a cDNA encoding an enhanced version of GFP, so that *rtTA*-infected cells could be followed more readily by assessing for expression of GFP. In the third vector, the *rtTA* gene was placed in tandem with *IRES-PURO*, so that *rtTA*-infected cells could be selected *in vitro* by killing uninfected cells with the antibiotic, puromycin. To initiate retrovirus production, DF-1 chicken fibroblast producer cells were transfected with the various RCAS-*rtTA*-encoding plasmids. RCAS-*rtTA-IRES-GFP* was most fully characterized, because of the convenience of tracking infected cells with green fluorescence. After spread of the virus within the culture media, nearly all cells expressed GFP by fluorescent microscopy, and cells expressed *rtTA* mRNA by the RT-PCR method (data not shown). Viral titers (1×10^6 ml) were comparable to that of other RCAS viruses, such as RCAS-*GFP*.

Infection with RCAS-*rtTA-IRES-GFP* Confers the Ability to Regulate Expression of Tet-Responsive Genes by Dox *in Vitro*. To test the ability of RCAS-*rtTA-IRES-GFP* to introduce functional rtTA into TVA-positive cells, we infected MEFs derived from the bitransgenic progeny of β-actin TVA transgenic mice (in which essentially all cells express TVA; ref. 9) crossed to mice harboring a tet-responsive reporter gene, *lacZ*, which encodes β-galactosidase (Fig. 1*B*). MEFs were selected for analysis because they are easily isolated, proliferate quickly, and are amenable to retroviral infection *in vitro*. Three separate embryos were analyzed.

After infection with RCAS-*rtTA-IRES-GFP*, $\approx$60% of infected cells expressed GFP as assessed by flow cytometry (Fig. 2*A Right*). No GFP was detectable in uninfected cells (Fig. 2*A*

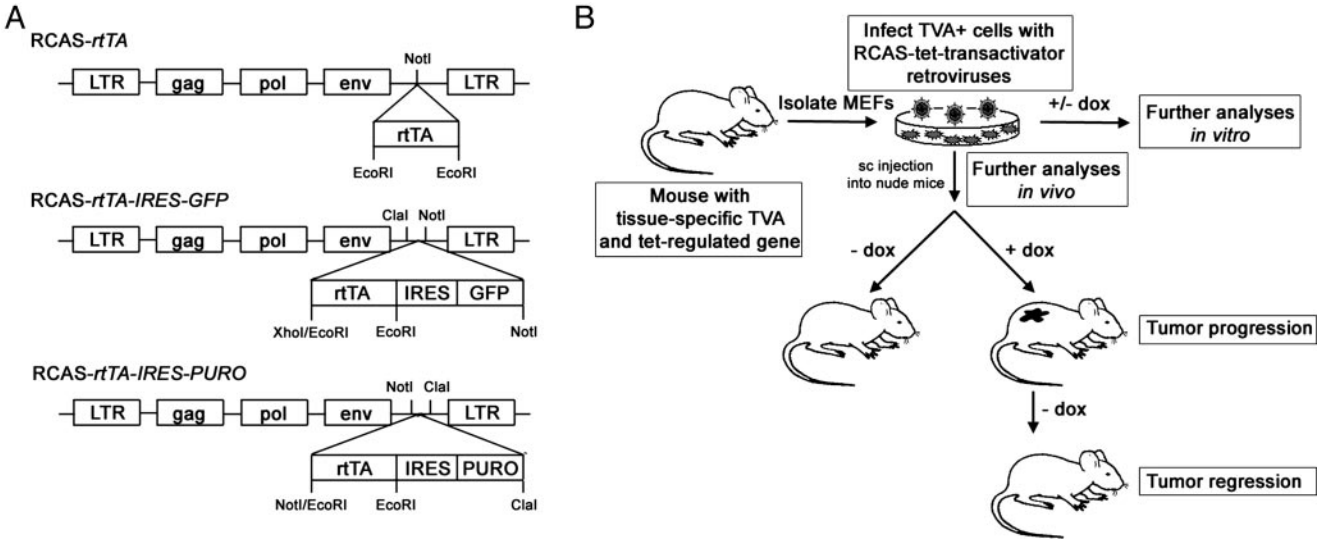

Fig. 1. Use of avian retroviral vectors to introduce transcriptional regulators into mammalian cells. (*A*) Constructs of RCAS-*rtTA*, RCAS-*rtTA-IRES-GFP*, and RCAS-*rtTA-IRES-PURO*. LTR, long terminal repeat; gag/pol/env, viral genes; rtTA, reverse tet transcriptional transactivator; IRES, internal ribosomal entry site; GFP; and PURO, puromycin. (*B*) Experimental strategy to characterize rtTA-encoding vectors. MEFs are harvested from mice harboring a tet-regulated gene and expressing the TVA receptor in nearly all cells (β-actin TVA). Cells are infected with RCAS-tet-transactivator retroviruses *in vitro* and then studied either *in vitro* or *in vivo*, through injection into nude mice. Expression of the tet-regulated gene is controlled by the exogenous administration of the tet analog, dox.

Left). *LacZ*-positive (blue) nuclei were observed in MEFs cultured in the presence of dox; the enzyme was coexpressed with GFP (Fig. 2 *B* and *C Right Lower*). No blue nuclei were detected in rtTA-infected MEFs cultured in the absence of dox (Fig. 2*C Top*), or in either uninfected MEFs or MEFs infected with RCAS-*GFP*, cultured with dox (Fig. 2*C Left* and *Lower Center*). Dox-regulated expression of lacZ was also observed after infection of MEFs with RCAS-*rtTA* and RCAS-*rtTA-IRES-PURO* (data not shown; see *Discussion*).

In unsorted cultures of MEFs infected with RCAS-*rtTA-IRES-GFP*, not all GFP-positive cells appeared to express β-galactosidase. To investigate this possibility further, GFP-positive MEFs were sorted by flow cytometry and stained for the reporter. Approximately one-third of GFP-positive cells contained blue nuclei, raising the possibility that cells were infected with retroviral mutants in which the GFP coding region was intact, but the rtTA region was deleted. To assess this prospect, Southern blots were performed on DNA extracted from DF-1 producer cells infected with RCAS-*rtTA-IRES-GFP*, by using probes encompassing either the *rtTA* or *IRES-GFP* fragments from the *rtTA-IRES-GFP* construct. By densitometry, only $\approx$5% had substantive deletions of the *rtTA* fragment (data not shown). This finding suggests that β-galactosidase staining was not detected in all GFP-positive cells, probably because in some infected MEFs either (*i*) rtTA levels were too low to activate transcription of tet-o-*lacZ*, and/or (*ii*) β-galactosidase was expressed, but below the level of detection in our assay.

Next, we used RCAS-*rtTA-IRES-GFP* to infect MEFs derived from the bitransgenic progeny of β-actin TVA and tet-o-*K-ras4b*^{G12D} mice (10), which were bred on a wild-type or p53-deficient background. MEFs infected with RCAS-*GFP* and cultured with dox did not express mRNA for tet-o-*K-ras4b*^{G12D}, as assessed by RT-PCR (Fig. 2*D*). By contrast, expression of the mutant K-ras mRNA was detected in RCAS-*rtTA-IRES-GFP*-infected cells grown in the presence of dox. Similar results were obtained in wild-type and p53-deficient backgrounds (data not shown). By 25 h after removal of drug, low levels of expression were still found (data not shown), but by 39 h after dox withdrawal, mutant K-ras mRNA was no longer detected (Fig. 2*D*). Taken together, these results demonstrate that RCAS-*rtTA-IRES-GFP* delivers functional tet-transactivators to TVA-positive cells, and allows efficient control of tet-regulated genes by dox *in vitro*.

Dox-Dependent Tumor Progression *in Vivo*. To determine whether we could use our TVA-based approach to achieve tumor progression, we took advantage of the fact that ras-transformed MEFs lacking the tumor suppressor gene, p53, form tumors in nude mice (15). Thus, MEFs were harvested from five individual p53-deficient embryos containing both the β-actin TVA and tet-o-*K-ras4b*^{G12D} transgenes, infected with various RCAS vectors, and assessed in a nude tumorigenicity assay (Fig. 1*B*). As expected, infection of the above MEFs with RCAS-*K-ras4b*^{G12D}, which results in the constitutive expression of activated K-ras, led to tumor formation in the absence of dox (5 of 5 sites injected; Table 1). More importantly, in the presence of dox, tumors developed in 30 of 34 sites injected with RCAS-*rtTA-IRES-GFP*-infected MEFs. No tumors were observed in mice who were either fed a normal diet and injected with similarly infected MEFs (0/9), or in mice who were on dox and injected with RCAS-*GFP*-infected MEFs (0/5). Taken together, these results demonstrate that dox-dependent tumor progression was attained.

Tumors that developed because of inducible activated K-ras were compared with those that arose from constitutively expressed activated K-ras. The inducible K-ras tumors expressed GFP as assessed by bioluminescence imaging, whereas tumors that arose because of constitutively expressed K-ras tumors did not (data not shown). Cultures of tumor cells also expressed GFP by immunofluorescence and FACS analyses (data not shown). On a molecular level, only the inducible K-ras tumors expressed the tet-o-*K-ras4b*^{G12D} transgene as assessed by RT-PCR (data not shown). However, tumors that arose from both the dox-regulated and constitutively expressed *K-ras* oncogenes were histologically indistinguishable. Both were highly cellular sarcomas; they contained spindled and epithelioid cells, atypical and pleomorphic nuclei, and numerous mitotic figures (Fig. 3*A*). Moderate numbers of inflammatory cells were distributed between the neoplastic cells, with a regionally varying density.

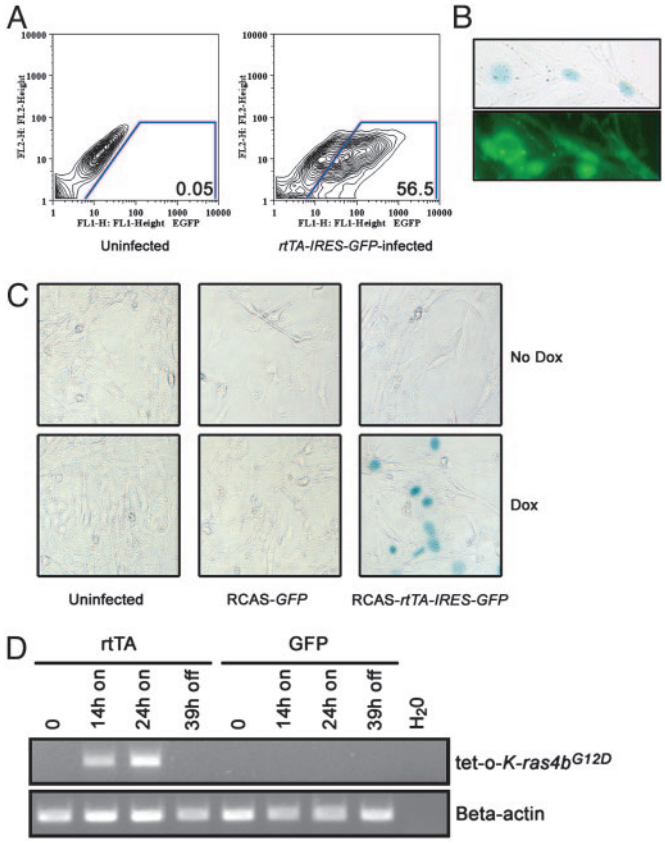

Fig. 2. Dox-dependent expression of tet-regulated genes in infected TVA-positive MEFs *in vitro*. (*A*) Flow cytometry contour plots of MEFs freshly harvested and analyzed for expression of GFP (FL-1 channel). Percentages of GFP-positive cells are indicated by the numbers in the bottom right corner of each gated panel. Nearly 60% of cells infected with RCAS-*rtTA-IRES-GFP* express GFP (*Right*), whereas uninfected cells do not (*Left*). [Data are plotted in two dimensions to enable distinction between autofluorescence only (FL-2 channel), and autofluorescence plus fluorescence because of GFP (FL-1 channel).] (*B*) After induction with dox, GFP (cytoplasmic; *Lower*; magnification: ×400) and a tet-regulated reporter gene, *lacZ* (fused to a nuclear localization signal), are coexpressed (blue nuclei; *Upper*; magnification: ×400). (*C*) MEFs harboring a tet-regulated *lacZ* gene express β-galactosidase only after infection with RCAS-*rtTA-IRES-GFP* (*Right*), and only when cultured in the presence of dox (*Right Lower*). Uninfected cells (*Left*) and cells infected with RCAS-*GFP* (*Center*) do not express β-galactosidase, either in the absence or presence of dox (magnification: ×200). (*D*). MEFs harboring tet-o-*K-ras4b*^{G12D} express the transgene after dox is added to the culture media, as assessed by RT-PCR. The ras transgene is no longer expressed after dox is removed. Cells infected with RCAS-*GFP* do not express the *K-ras* transgene, even in the presence of dox. M, marker; 0 (time 0), no dox; 14 h on, 14 h on dox; 24 h on, 24 h on dox; and 39 h off, 39 h off dox after 24-h exposure to dox.

There was no histologic evidence of specific lines of mesenchymal differentiation.

Analyses of Tumor Maintenance. To determine whether we could study the role of activated K-ras in the maintenance of the induced sarcomas, we withdrew dox from the diet of the tumor-bearing nude mice. Fifteen of 16 tumors regressed when measured >2 days after drug withdrawal (Fig. 3 *B* and *C*). In the one tumor that did not regress, its dimensions (length vs. width) changed at day 4 after drug withdrawal, but the overall tumor volume remained the same. Because the mouse bearing this single tumor was killed 4 days after withdrawal of dox, the tumor could not be further evaluated for regression.

RT-PCR demonstrated loss of expression of the tet-o-*K-ras4b*^{G12D} transgene within the tumors (Fig. 4*A*). Regressing tumors underwent proliferative arrest, as assessed by Ki-67 staining (Fig. 4*B*). Such tumors also displayed multiple characteristics of programmed cell death, including pyknotic nuclei,

Table 1. Summary of nude mouse tumorigenicity assay

Infecting retrovirus	Dox status	Tumors/sites of injection
RCAS-*K-ras4b*^{G12D}	Absent	5/5
RCAS-*rtTA-IRES-GFP*	Absent	0/9
RCAS-*GFP* alone	Present	0/5
RCAS-*rtTA-IRES-GFP*	Present	30/34

p53-deficient MEFs derived from bitransgenic beta-actin TVA and tet-o-*K-ras4b*^{G12D} mice were infected *in vitro* with RCAS-*K-ras4b*^{G12D}, RCAS-*rtTA-IRES-GFP*, or RCAS-*GFP*. MEFs were then injected subcutaneously into the flanks of athymic nude mice fed either a normal diet or a diet containing dox, and the mice were observed for tumor development at each site of injection.

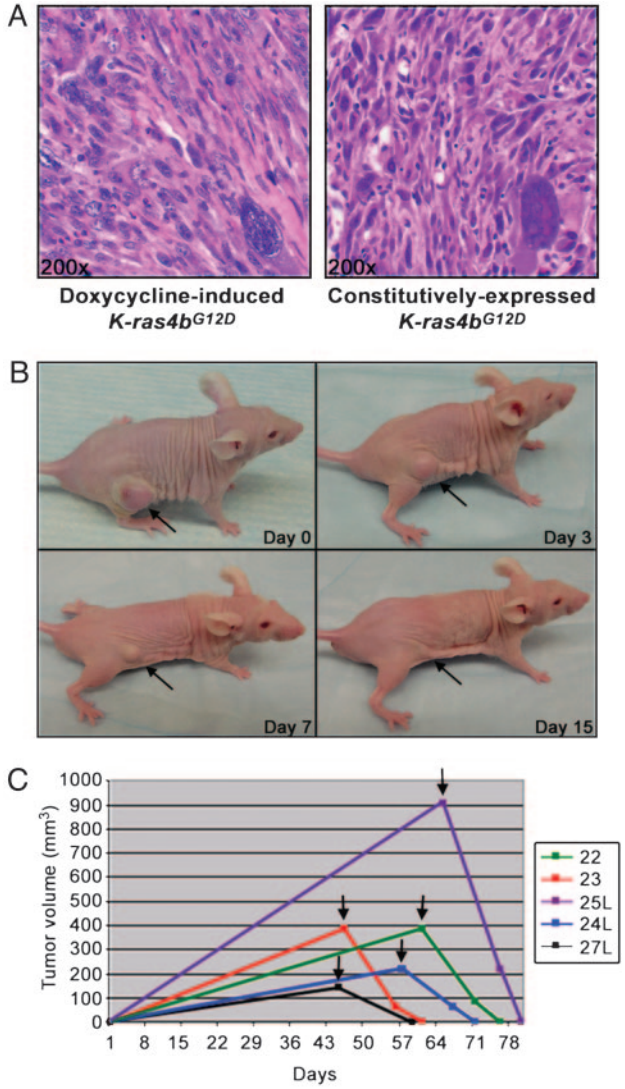

Fig. 3. Dox-dependent expression of tet-regulated *K-ras* oncogene in infected TVA-positive MEFs *in vivo*. (*A*) Comparison of tumors that develop in nude mice from inducible *K-ras4b*^{G12D} vs. constitutively expressed *K-ras4b*^{G12D}. By hematoxylin/eosin staining, both tumors were highly cellular sarcomas. (*B*) Serial photographs of a single MEF tumor that regresses after removal of dox from the mouse's diet. Day 0 indicates the first day of dox withdrawal. (*C*) Graph of the tumor volume of five representative individual MEF tumors that progressed in the presence of dox, and fully regressed after dox was withdrawn (the time of which is indicated by an arrow).

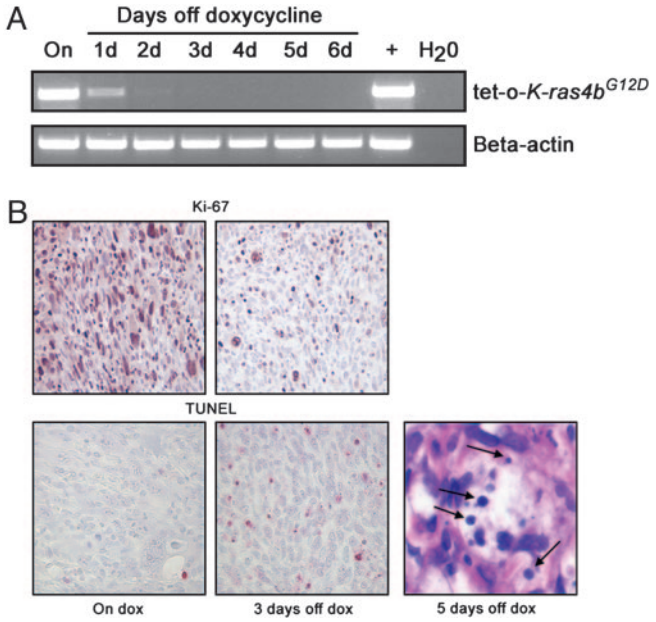

Fig. 4. Regressing MEF tumors undergo proliferative arrest and apoptosis. (*A*) RT-PCR analysis of MEF tumors demonstrating that expression of the tet-regulated *K-ras* transgene diminishes to undetectable levels after mice are withdrawn from dox. (*B*) Regressing tumors undergo proliferative arrest (Ki-67, *Upper*; magnification: ×200) and apoptosis (TUNEL staining, *Left* and *Lower Center*; magnification: ×200). (*Right Lower*) The appearance of apoptotic bodies is shown (arrows; magnification: ×400). A total of 19 tumors were examined by TUNEL staining, 8 tumors were never withdrawn from dox.

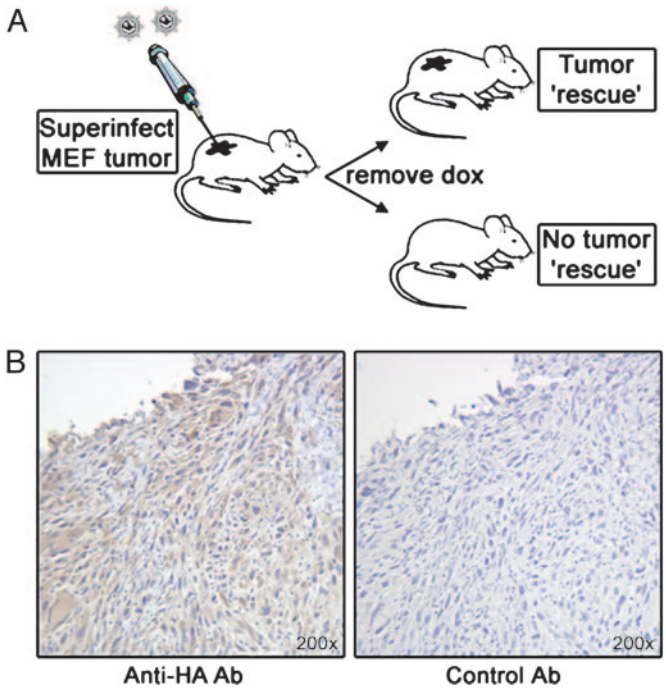

Fig. 5. Genetic complementation assay. (*A*) Experimental design. MEF tumors, comprised of cells derived from p53-deficient embryos carrying β-actin TVA and tet-o-*K-ras4b*^{G12D} transgenes, are induced in nude mice on dox as outlined in Fig. 1*B*. Once tumors form, they are superinfected with other RCAS-based retroviruses encoding cDNAs of interest. Mice are subsequently withdrawn from dox to determine whether the newly introduced gene can rescue tumors from death. (*B*) Representative tumor superinfected with RCAS-*K-ras4b*^{G12D}-HA, which encodes a HA epitope-tagged, constitutively expressed mutant K-ras; these tumors persist after dox withdrawal and express HA. (*Left*) Anti-HA antibody. (*Right*) Control antibody. Tumors reinfected with RCAS-*AP*, which encodes alkaline phosphatase, completely regress (data not shown).

apoptotic bodies, nuclear condensation, blebbing, and increased numbers of TUNEL-positive cells (Fig. 4*B*). These characteristics were rarely noted in tumors before dox withdrawal. Detailed examination of hematoxylin/eosin- and TUNEL-stained slides of regressing tumors revealed no specific clustering of apoptotic cells around tumor microvasculature and no obvious decrease in microvascularity.

Interestingly, two of two tumors that regressed completely on separate mice after withdrawal of the inducer for 3 weeks recurred rapidly at the same site after dox was readministered. After withdrawal from dox again, these tumors appeared dox-independent, in that they no longer regressed (data not shown). Further studies are needed to determine the cause of regrowth and dox-independence.

Tumor Rescue by Genetic Complementation. Infected TVA-positive mammalian cells remain susceptible to repeated rounds of infection with multiple RCAS vectors (16–18). To determine whether we could take advantage of this feature to rescue tumors from cell death and regression after withdrawal of dox (Fig. 5*A*), we superinfected existing MEF tumors with RCAS-*K-ras4b*^{G12D}-HA, which encodes a HA epitope-tagged, constitutively expressed mutant K-ras. Mice were then withdrawn from dox to initiate tumor regression. Four of four tumors injected with RCAS-*K-ras4b*^{G12D}-HA persisted after dox withdrawal. Rescued tumors expressed HA (Fig. 5*B*) and did not express the mutant *K-ras* transgene, as assessed by RT-PCR (data not shown). By contrast, four of four tumors injected with RCAS-AP, which encodes alkaline phosphatase, completely regressed. Experiments involving superinfection with RCAS vectors carrying other cDNAs of interest will be of future interest.

Discussion

The studies presented here demonstrate that avian leukosis-derived retroviruses can be used to deliver fully functional tet transactivators to TVA-positive mammalian cells. This RCAS-TVA-Tet approach permits tight conditional expression of tet-regulated transgenes in cell culture and/or in live animals. Other viruses such as Moloney murine leukemia virus (Mo-MuLV) and adenovirus have been used to introduce tet-transactivators to cells with tet-regulated transgenes (19, 20). However, RCAS-based retroviruses have three advantages for such delivery. First, tissue-specificity can be achieved, because infection is limited to tissues that express the TVA receptor. Second, the lack of viral protein production by RCAS-based retroviruses prevents cell-to-cell spreading of infection and decreases the probability of an immune response by the host (as is seen especially with adenoviruses; ref. 21). Third, TVA-positive mammalian cells can be repeatedly reinfected with multiple RCAS vectors carrying different genes (refs. 16–18 and Fig. 5); thus, various genes can be introduced sequentially into the same cells expressing an inducible transgene. The last advantage permits genetic complementation studies as described above.

We have initially used murine embryonic fibroblasts from β-actin TVA mice to demonstrate the utility of the RCAS-TVA-Tet method *in vitro* and *in vivo*. We have consequently developed a fibrosarcoma tumor model in which to study mouse tumor progression and regression in the context of activated K-ras. Studies thus far of regressing MEF tumors demonstrate that, as in other conditional tumor models (2, 3, 10), MEF tumors depend on the sustained expression of the inciting oncogene (i.e., *K-ras4b*^{G12D}) for tumor maintenance. Of note, the fact that MEF tumors regress in athymic nude mice, which lack conventional αβ T cells (22), is consistent with the view that the adaptive

immune system is not critical for the process of tumor elimination (23).

Others using tet-regulated systems have observed low levels of background gene expression in the noninduced state whenever rtTA is present at relatively high intracellular concentrations (24). As shown above, by using RCAS-*rtTA-IRES-GFP*, we have not observed any inappropriate tet-transgene expression, either *in vitro* or *in vivo*, with MEFs derived from tet-o-*lacZ* or tet-o-*K-ras4b*^{G12D} mice. Similarly, in initial experiments with RCAS-*rtTA*, we have not observed any leaky tet-regulated transgene expression (data not shown). However, in preliminary studies with RCAS-*rtTA-IRES-PURO*, using *lacZ*-reporter MEFs, we have detected some β-galactosidase expression in the absence of dox *in vitro*. The number of *lacZ*-positive cells was still increased when cells were cultured with dox, indicating dox-dependent transcriptional regulation, and leakiness was eliminated by diluting the viral stock of *rtTA-IRES-PURO* 10-fold before use (data not shown). These results highlight the importance of determining optimal multiplicities of infection to achieve tight conditional gene regulation. The efficiency of tet-regulation will also need to be tested for cell types other than MEFs.

Multiple strains of transgenic mice expressing TVA in various tissues and carrying tet-regulated genes have been generated (see Tables 2 and 3, which are published as supporting information on the PNAS web site, www.pnas.org). Thus, the RCAS-TVA-Tet system could be generally applied to study in different organs the role of a variety of genes in tumor progression and maintenance. This approach can also be used to restrict the number of target cells that express a tet-regulated transgene, which, in turn, may help limit the number of tumor foci that develop, and thus perhaps aid in the generation of mouse tumor models that more faithfully mimic human cancer. In cases where the cell type to be studied is sparse (e.g., melanoblasts; ref. 23), a more conventional tet-regulated approach may be sufficient to generate limited numbers of tumors. However, in cases where the cell type to be studied is abundant (e.g., type II lung epithelial cells; ref. 10), use of avian retroviral vectors to introduce rtTA into bitransgenic animals harboring tet-regulated genes and expressing TVA within a specific tissue may be more useful.

In addition to facilitating the study of tumor progression and maintenance, the RCAS-TVA-Tet method could be useful for studying cell migration and/or development, temporally controlling a gene of interest by administration and withdrawal of dox at different times. One could then follow infected cells during and after timed expression of a gene of interest, either by assessing GFP expression (through the use of RCAS-*rtTA-IRES-GFP*), or by detecting evidence of retroviral insertion within infected cells. These manipulations would add a further layer of complexity to the already flexible RCAS-TVA mouse model approach.

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