Supporting Information

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SI Methods

Materials. All cell culture reagents were obtained from Invitrogen. Primers were made by MWG and restriction endonucleases were obtained from New England Biolabs. The *Escherichia coli* strains TOP10 and Stbl3 (Invitrogen) were used for transformation and plasmid amplification.

Plasmid Constructions. To generate the indicated loxP-flanked conditional stop elements (see supporting information (SI) Fig. S1*A*), a *PGK-neo* resistance cassette kindly provided by P. Soriano (1), four head to tail cloned *SV40* or *BGH* polyadenylation sites (pA) excised from pGL3-basic (Promega) or pcDNA3.1 (Invitrogen), respectively, a RNA polymerase pause site (RNA-PPS) generously provided by N. Proudfoot (2), and a linker containing stop codons in all reading frames were cloned into pBluescript SK+ (Stratagene). Subsequently, loxP sites were inserted 5′ and 3′ of each stop element to generate loxP-flanked stop cassettes termed loxP-stop-loxP (LSL). The different LSL elements were subcloned into pCMV-fLuc-pA, a plasmid containing the human cytomegalovirus (*CMV*) major immediate early promoter/enhancer from pIRES (Clontech), a *firefly luciferase* (*fLuc*) expression cassette from pGL3-basic, and a rabbit *β-globin* polyadenylation site from pTriEx-2 Neo (Novagen) (Fig. S1*A*). To generate a LSL-silenced TVA expression plasmid termed pRosa26-LSL-ATG-Tva (see Fig. S2*A*), the LSL cassette from PGK-neo 4xSV40-pA RNA-PPS was removed by restriction endonuclease digestion and inserted upstream of the splice acceptor (SA) of pRosa26-SA-Tva-IRES-lacZnls. This plasmid contains the murine *Rosa26* promoter from pBroad3 (Invitrogen), a splice acceptor kindly provided by P. Soriano (1), the quail *TVA* coding sequence (800 bp) kindly provided by P. Bates (3), an internal ribosome entry site from the encephalomyocarditis virus (pIRES, Clontech), a *lacZ* cassette with a nuclear localization signal (lacZnls), and the human *β-globin* polyadenylation site, both from pBroad3. To generate the expression plasmid pRosa26-ATG-LSL-Tva, in which the LSL element disrupts the ORF of the *TVA* transgene, we inserted the LSL cassette just after the ATG start codon of *TVA* (Fig. S2*A*). To generate an antibody against mutated TVA by genetic immunization, the LSL element was removed from pRosa26-ATG-LSL-Tva by incubation with Cre-recombinase (Novagen) and the ATG-loxP-Tva cassette was subcloned into pcDNA3 resulting in pcDNA3-ATG-loxP-Tva. To generate RCASBP(A) vectors for stable RNAi, oligos for *firefly luciferase* (*fLuc*), murine *TP53,* and control shRNA were annealed and cloned into pEntr-H1-TO (Invitrogen) as recommended by the manufacturer. Subsequently, the shRNA expression cassettes were transferred into a Gateway compatible RCASBP(A) vector by recombineering (Gateway system, Invitrogen). The *fLuc* and *TP53* oligo sequences were the same as described in ref. 4. The target sequence of the control shRNA oligo was 5′-GCAAAGACCATTACGAAGGATCTAT-3′. RCASBP(A)-Kras^{G12D} and RCASBP(A)-AP plasmids were kindly provided by S. Orsulic (5). RCASBP(A)-EGFP was generated by subcloning of the coding sequence of *EGFP* from pEGFP (Clontech) into the blunted ClaI restriction site of RCASBP(A) (a gift from S. Hughes, National Cancer Institute, Frederick, MD). The Cre-recombinase expression plasmid pIC-Cre was generously provided by K. Rajewski (6). pCMV-fLuc-IRES-TVA was generated by subcloning of the *fLuc* coding sequence into multiple cloning site (MCS) A and subsequent ligation of the TVA cDNA into MCS B of pIRES. Integrity of all cloned sequences was confirmed by automated DNA sequencing (GATC) by using an ABI Prism 377 DNA sequencer (Applied Biosystems).

Construction of the Targeting Vector and Generation of the *LSL-R26$^{Tva\text{-}lacZ}$* Mouse Line. Targeting the *Rosa26* locus by a knockin strategy was performed on the basis of plasmid pROSA26–1, kindly provided by P. Soriano (1). To generate the targeting vector, the SA-ATG-LSL-TVA-IRES-LacZnls element from pRosa26-ATG-LSL-Tva (see Fig. S2*A*) was inserted into the blunted XbaI site of pROSA26–1 (Fig. S3*A*). The linearized targeting vector was electroporated into 129S6 embryonic stem (ES) cells. ES cells were selected in 200 μg/ml G418, and appropriately targeted clones were identified by PCR as described (1). Correct recombination was verified by Southern blot analysis with ^{32}P-labeled 5′ and 3′ external probes and EcoRV and BglI digested genomic DNA, respectively (see Fig. S3*B*). An internal probe for the neomycin resistance gene confirmed single copy insertion. ≈20% of the analyzed ES cell clones showed correct homologous recombination, and two clones were injected into C57BL/6J blastocysts (Polygene). Germ-line transmission was achieved in 2/2 clones harboring the targeted allele. A three-primer PCR strategy was used to genotype animals as described (1).

Mouse Embryonic Fibroblasts (MEFs). All experiments were performed with early passage MEFs (p2–3) prepared from day 12–14 embryos. *LSL-R26$^{Tva\text{-}lacZ/+}$* mice were crossed with wild-type animals to generate MEFs with loxP sites flanking the transcriptional stop cassette that disrupts the TVA transgene in the Rosa26 locus. To generate MEFs with expression of TVA and lacZnls due to Cre-mediated deletion of the LSL element, *LSL-R26$^{Tva\text{-}lacZ/+}$* mice were crossed to the general deleter strain *Prm-Cre*. Mice and MEFs were on a mixed 129S6;C57BL/6 background. MEFs were grown in DMEM supplemented with 10% FCS and infected with high-titer (10^8 U/ml) RCASBP(A)-EGFP retroviral stocks. Three days after infection, EGFP expression was visualized by using an Axiovert 200M fluorescent microscope equipped with an AxioCam MRm charge-coupled device camera system (Carl Zeiss). High-resolution images (1,388 × 1,040 pixels) were captured and analyzed using AxioVision 4.3 software (Carl Zeiss).

***In Vivo* Bioluminescence and e*x Vivo* Fluorescence Imaging.** Mice were anesthetized with medetomidine, midazolam, and fentanyl, injected with D-luciferin (Synchem) at 150 mg/kg i.p. and imaged after 10 min by using an *in vivo* imaging system (Hamamatsu) as described (7). To assess presence of EGFP expression, mice were killed and subjected to fluorescence stereomicroscopy (Stemi 11, Carl Zeiss). Emitted fluorescence was collected on a Zeiss color charge-coupled device camera system (AxioCam MRc). High-resolution images were captured and analyzed by using AxioVision 4.3 software (Carl Zeiss).

Cell Culture, Transient and Stable Transgene Expression, and Reporter Gene Assays. HEK293, TD-2, and MiaPaCa-2 cells were cultured and transiently or stably transfected as described (7, 8). Alkaline phosphatase (AP) activity was detected as described by Holland *et al.* (9) with minor modifications. In biref, RCASBP(A)-AP-infected cells were fixed with ice-cold 4% paraformaldehyde for 5 min, and washed three times with PBS; endogenous AP was heat-inactivated in AP buffer (100 mM Tris, pH 9.5; 100 mM NaCl; 50 mM $MgCl_2$) at 65°C for 30 min. AP staining solution

(BM purple, Roche) was then added and left to develop overnight in the dark. Luciferase assays were performed essentially as described (10) by using a luminometer (EG&G Berthold) and a Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized in all samples for total protein content. Data are presented as relative light units of firefly luciferase per μg of protein.

β-Galactosidase Staining. Cells, tissues, and embryos were fixed in 4% paraformaldehyde at 4°C and washed three times in lacZ wash buffer (2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40 in PBS) for 30 min. β-Galactosidase activity was detected using lacZ staining buffer [35 mM potassium ferrocyanide, 35 mM potassium ferricyanide, 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) in lacZ wash buffer] at 37°C overnight. Stereoscopic pictures were taken by using a Zeiss Stemi 11 microscope. For histological analysis, organs were cryosectioned after fixation, postfixed in 0.2% glutaraldehyde in PBS, and stained as described above. Counterstaining was done with eosin or nuclear fast red.

Generation of Polyclonal TVA Antibody, Histochemistry, and Immunohistochemistry. For cryosections, tissues were fixed for 2 h at 4°C in 4% paraformaldehyde, cryopreserved in graded solutions of sucrose (15% 4 h; 30% overnight), embedded in Tissue-Tek (Miles), snap-frozen, and serially sectioned (10 μm thick) on a cryostat. EGFP expression was visualized by fluorescence microscopy (Axiovert 200 M) after counterstaining with DAPI (Vector Laboratories) to identify nuclei. For histopathological analysis, specimens were fixed in 10% buffered formalin, embedded in paraffin, and sectioned (3 μm thick). Cryosections or dewaxed paraffin-embedded tissue sections were stained with H&E. For TVA immunohistochemistry, cryosections were stained with a rabbit polyclonal TVA antiserum (1:100) obtained by genetic immunization (Genovac GmbH) using the expression plasmid pcDNA3-ATG-loxP-Tva that encodes for the N-terminal mutated TVA receptor (see Fig. S2*A*). For CK-19 immunodetection, formalin-fixed paraffin-embedded tissue sections were dewaxed and placed in a microwave (10 min., 600 watt) to recover antigens before incubation with the primary antibody (1:100, TROMAIII, Developmental Studies Hybridoma Bank, Iowa City, IA). Primary antibodies were followed by secondary antibodies conjugated to biotin (Vector Laboratories). Peroxidase-conjugated streptavidin was used with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) as chromogen for detection as described (7). Hematoxylin was used for nuclear and eosin for cytoplasmatic counterstaining.

PCR, RT-PCR, and Quantitative Real-Time RT-PCR. Genomic DNA was isolated from mouse tissues and tumors as described (10). Total RNA was extracted from cells, tissues and tumors with the RNeasy kit and treated with DNAseI (Qiagen). Five micrograms of total RNA was reverse transcribed by using random hexamers and the TaqMan Reverse Transcription Kit (Applied Biosystems) as described (11). PCR was performed with specific primer pairs spanning codon 12 of RCASBP(A)-$Kras^{G12D}$ (forward: 5′-ATGACTGAGTATAAGCTTGTGGTGGT-3′; reverse: 5′-ATGGCAAATACACAAAGAAAGCC-3′) for 40 cycles (annealing 58°C, 30 sec; extension 72°C, 60 sec; denaturation 94°C, 30 sec) by using a proofreading polymerase (PfuUltra, Stratagene). For detection of RCASBP(A) proviral DNA, envA-specific primers were used (forward: 5′-ACCGGGGGATGCGTAGGCTTCA-3′; reverse: 5′-CCGCAACACCCACTGGCATTACC-3′) as described (12) (annealing 65°C, 30 sec; extension 72°C, 60 sec; denaturation 94°C, 30 sec; 35 cycles). Amplification products were subjected to DNA sequence analysis (GATC). Quantitative real-time PCR was performed as described (13) on an ABI 7700 Sequence Detection System by using standard curves and SYBR Green kit (Applied Biosystems) with the following primers: TVA forward: 5′-CTCTGCCAGCCAGGAATCAC-3′, TVA reverse: 5′-CATCTCACCAGCTCACAGCAA-3′. For quantification of murine TP53 mRNA we used the QuantiTect primer assay Mm_Trp53_1_SG (Qiagen). All reactions were performed in triplicate. As endogenous reference, the globally expressed housekeeping gene cyclophilin was quantified as described (7).

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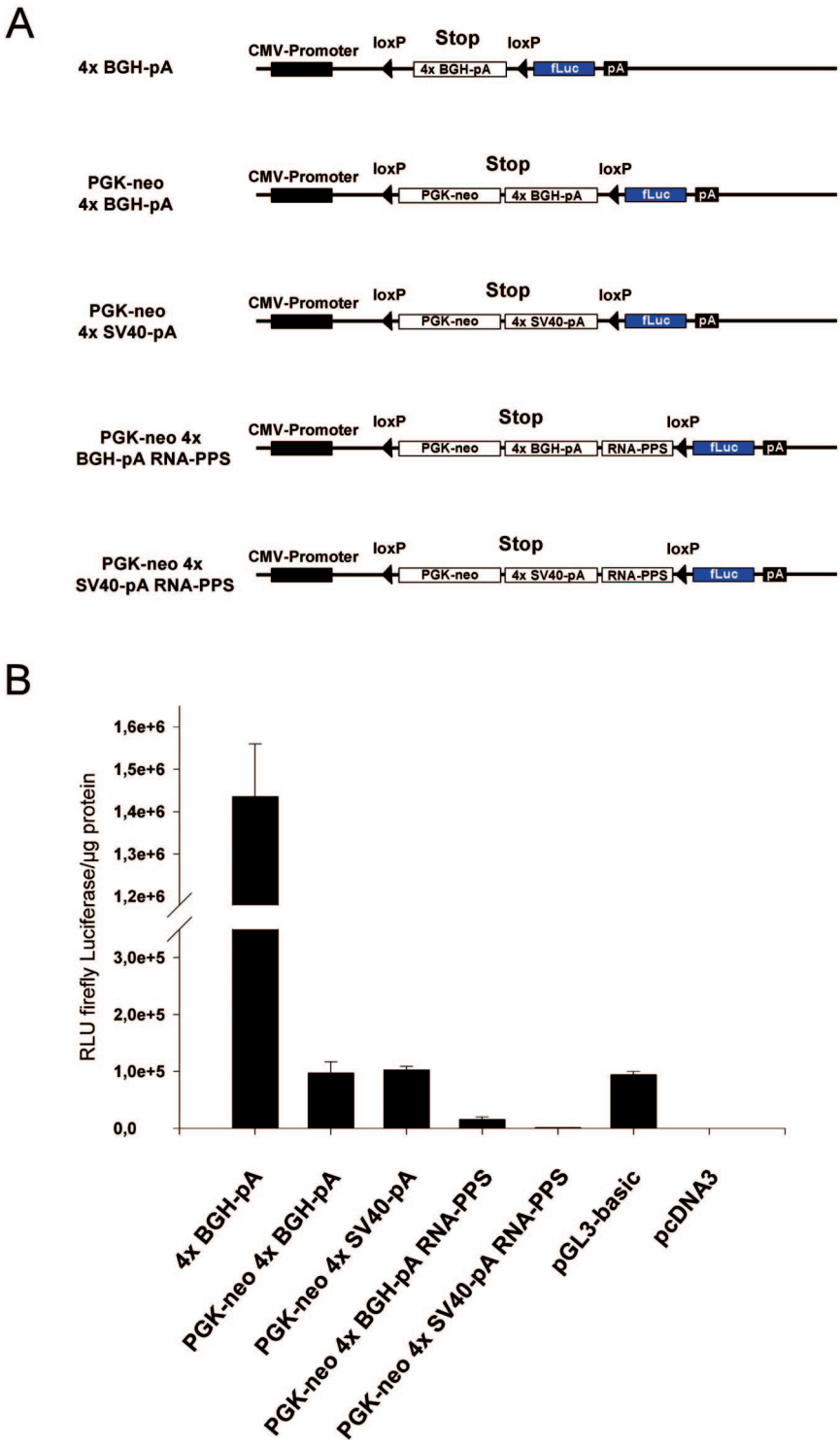

Fig. S1. Generation and functional characterization of loxP-flanked transcriptional stop elements. (*A*) Schematic representation of the indicated loxP-stop-loxP cassettes inserted 5′ of the firefly luciferase (fLuc) coding sequence of pCMV-fLuc-pA. BGH, bovine growth hormone; CMV, cytomegalovirus; pA, polyadenylation site; RNA-PPS, RNA polymerase pause site. (*B*) Suppression of firefly luciferase expression by different stop cassettes. Equal amounts of the indicated plasmids were transfected in TD-2 cells. As negative control, we used the promoterless fLuc plasmid pGL3-basic and ''empty'' pcDNA3. Luciferase activity was assayed 48 h after transfection and normalized for total cellular protein content of lysed TD-2 cells. Data are expressed as means ± SD of three independent experiments in triplicate.

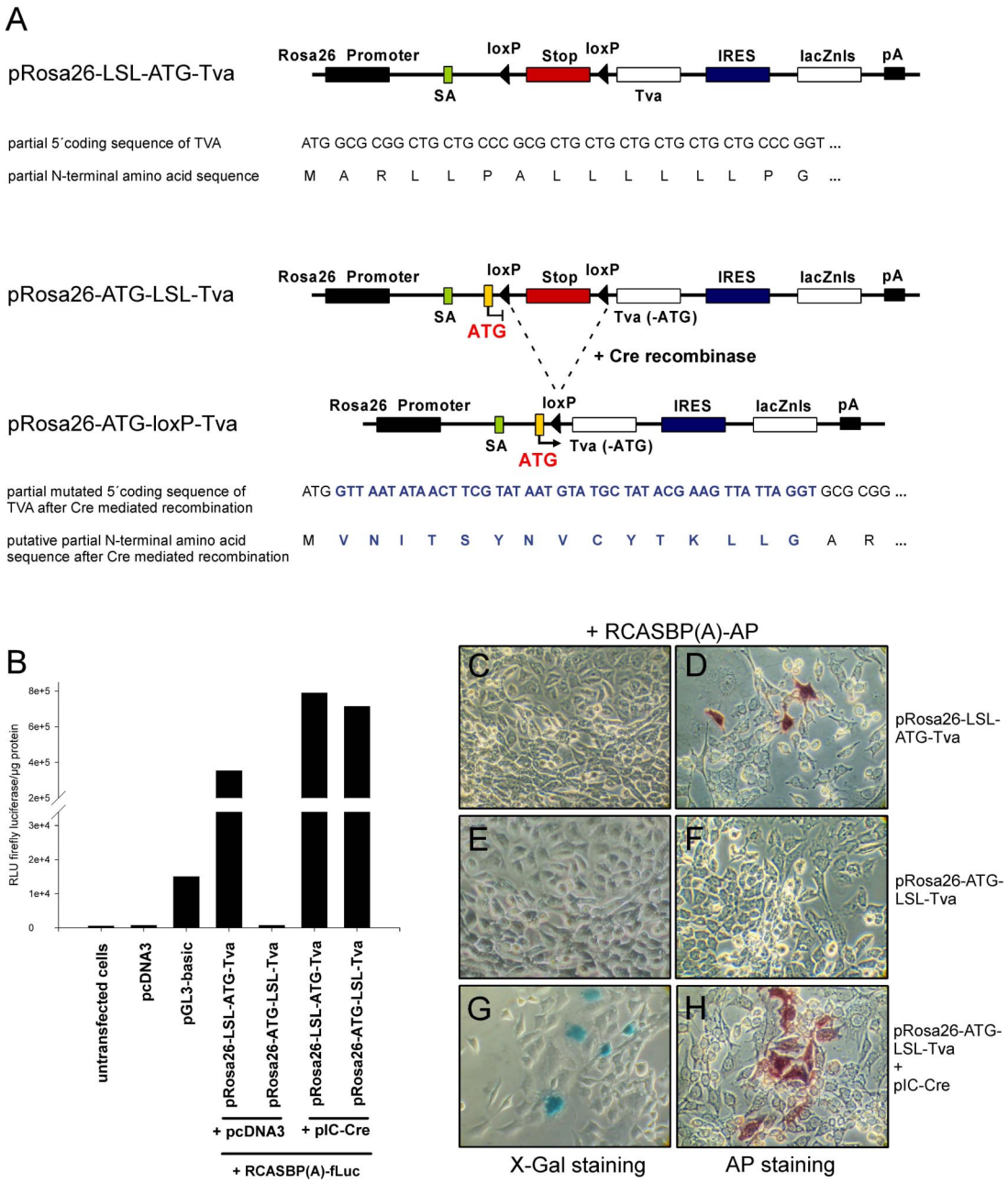

Fig. S2. Disruption of the TVA ORF by a loxP-stop-loxP (LSL) cassette renders transiently transfected TD-2 cells resistant to RCASBP(A)-mediated retroviral infection. (*A*) Schematic representation and partial sequence of LSL-silenced TVA-IRES-lacZnls expression plasmids under the control of the *Rosa26* promoter. (*Top*) pRosa26-LSL-ATG-Tva plasmid that encodes for wild-type TVA receptor after Cre-mediated recombination (partial 5′ coding and N-terminal amino acid sequence of wild-type TVA below). (*Middle*) pRosa26-ATG-LSL-Tva plasmid. The LSL cassette disrupts the ORF of TVA. (*Bottom*) pRosa26-ATG-loxP-Tva plasmid. After Cre-mediated recombination of pRosa26-ATG-LSL-Tva a N-terminally mutated TVA receptor is expressed (partial 5′ nucleotide sequence and predicted N-terminal amino acid sequence of mutated TVA below; nucleotides and amino acids encoded by the product of Cre-recombination in bold and blue). (*B*) TD-2 cells were transiently cotransfected with the indicated LSL-silenced TVA-IRES-lacZnls expression plasmids and a Cre-recombinase expression vector (pIC-Cre) or empty pcDNA3 as control. Six hours later, cells were infected with 10^7 units/ml RCASBP(A)-firefly luciferase (fLuc) and assayed for fLuc activity after 72 h. Firefly luciferase activity expressed as relative light units (RLUs) was normalized for total cellular protein content of TD-2 cells. Untransfected cells and cells transfected with empty pcDNA3 or the promoterless fLuc vector pGL3-basic were used as controls. (*C–H*) The LSL-silenced TVA-IRES-lacZnls expression plasmids pRosa26-LSL-ATG-Tva (*C* and *D*) and pRosa26-ATG-LSL-Tva (*E* and *F*) were transiently transfected into TD-2 cells. Six hours later, cells were infected with 10^7 units/ml RCASBP(A)-alkaline phosphatase (RCASBP(A)-AP). After 72 h cells were stained for X-Gal (*C* and *E*) or alkaline phosphatase (*D* and *F*) activity as described in *Material and Methods*. (*G* and *H*) Cotransfection of pRosa26-ATG-LSL-Tva and the Cre-recombinase expression plasmid pIC-Cre resulted in recombination of the LSL element with subsequent expression of nuclear lacZ as shown by X-Gal staining (*G*). Furthermore, recombination of pRosa26-ATG-LSL-Tva plasmid renders transfected cells susceptible to infection with RCASBP(A)-AP as shown by alkaline phosphatase staining (*H*).

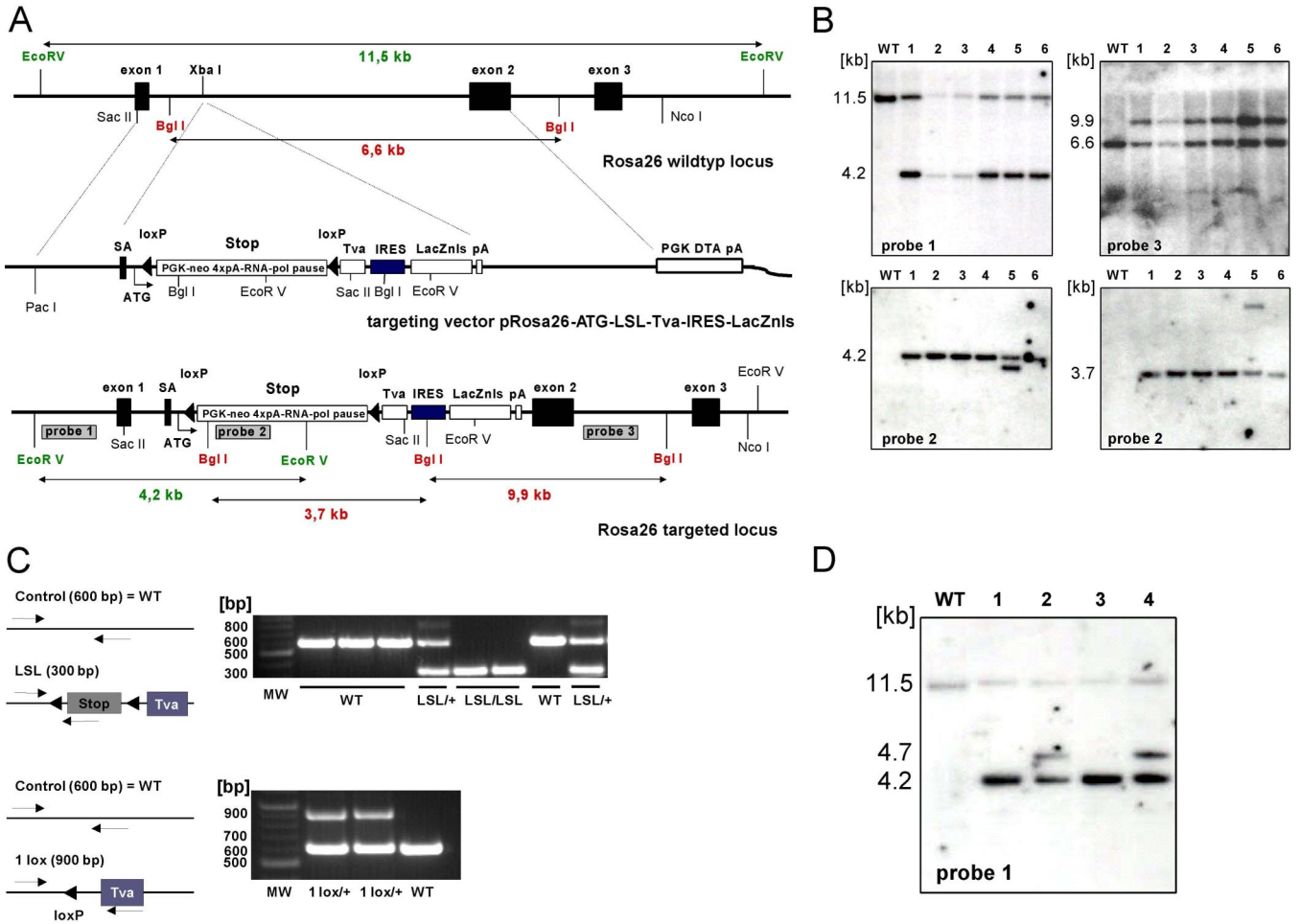

Fig. S3. Targeted integration of a silenced Tva-IRES-LacZnls cassette into the *Rosa26* locus. (*A*) From top to bottom, diagrams of *Rosa26* wild-type locus, the targeting vector pRosa26-ATG-LSL-TVA-IRES-lacZnls, and the targeted *Rosa26* locus. Restriction sites, sizes of DNA fragments, and the location of the 5′ (probe 1), neo (probe 2), and 3′ (probe 3) probes, are indicated. Black arrowheads flanking the transcriptional stop cassette represent loxP sites. (*B*) Southern blot analysis of DNA from wild-type (WT) and targeted embryonic stem cells (1–6) after digestion with EcoRV (*Left*) and BglI (*Right*). The expected bands for the wild-type and targeted alleles are indicated [probe 1: WT, 11.5 kb; mutant, 4.2 kb. probe 2: WT, no band; mutant, 4.2 kb (EcoRV) and 3.7 kb (BglI). probe 3: WT, 6.6 kb; mutant, 9.9 kb]. (*C Top*) PCR analysis of DNA from wild-type (WT), heterozygous (LSL/+) and homozygous (LSL/LSL) *LSL-R26*$^{Tva\text{-}lacZ}$ mice with retained stop cassette. (*Bottom*) PCR analysis of DNA from wild-type (WT) and heterozygous (1 lox/+) *LSL-R26*$^{Tva\text{-}lacZ/+}$*;Prm-Cre* mice with deleted stop cassette. Sizes of wild-type and mutant PCR products are indicated. (*D*) Southern blot analysis of DNA isolated from wild-type (WT) and heterozygous *LSL-R26*$^{Tva\text{-}lacZ/+}$*;Ptf1a/p48*$^{Cre/+}$ (lanes 1–4) mice. DNA was isolated from the pancreas (WT, lanes 2 and 4) and the liver (lanes 1 and 3). After digestion with EcoRV and hybridization with probe 1, selective deletion of the stop cassette in the pancreas was shown by an additional 4.7-kb band corresponding to the deleted allele.

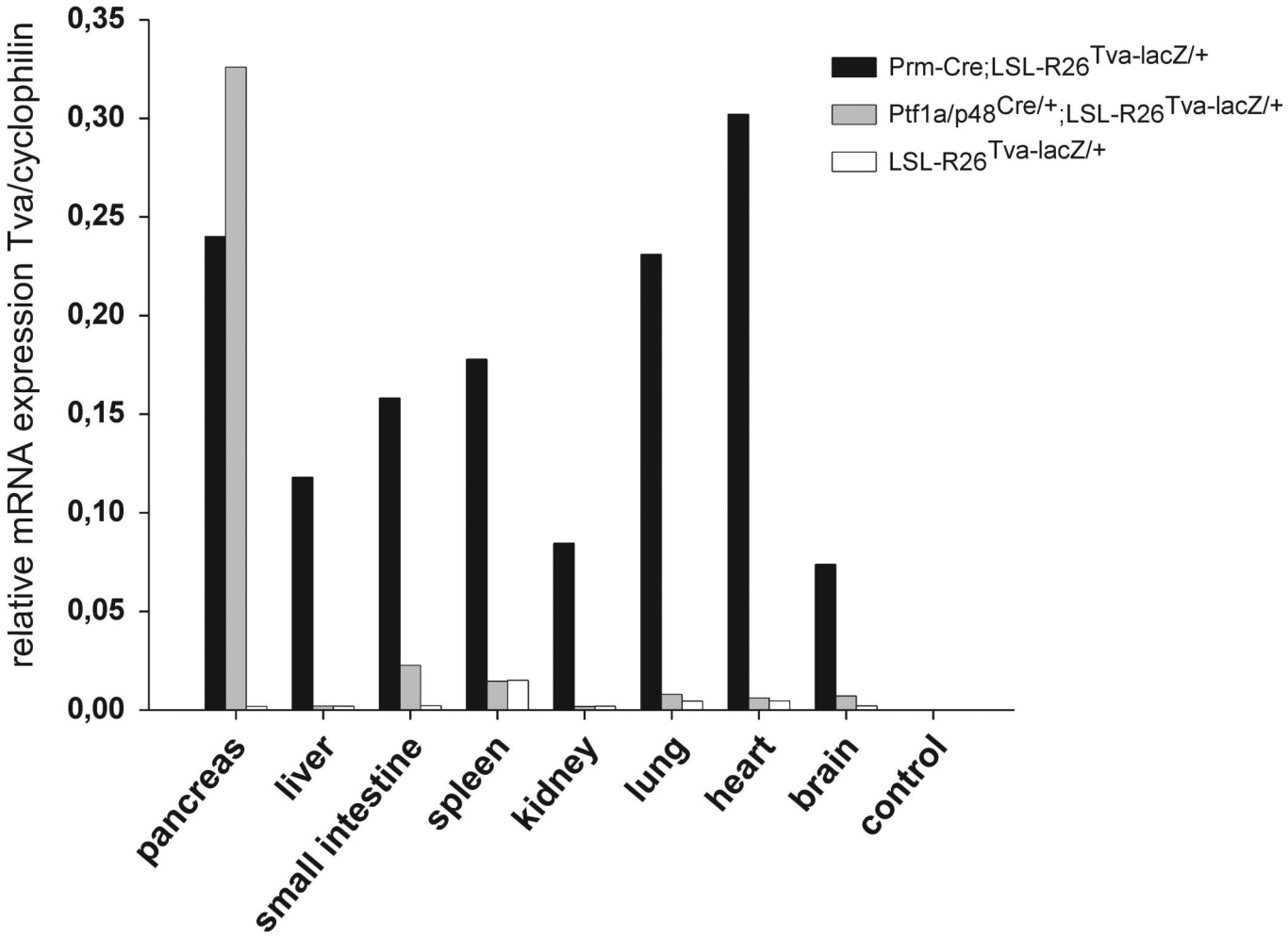

Fig. S4. Quantitative analysis of TVA mRNA expression by real time RT-PCR in the indicated tissues of *Prm-Cre;LSL-R26*$^{Tva\text{-}lacZ/+}$, *Ptf1a/p48*$^{Cre/+}$*;LSL-R26*$^{Tva\text{-}lacZ/+}$, and *LSL-R26*$^{Tva\text{-}lacZ/+}$ mice. Relative amounts of TVA transcripts were calculated by using standard curves and normalized against the globally expressed cyclophilin housekeeping gene measured in the same RNA preparation.

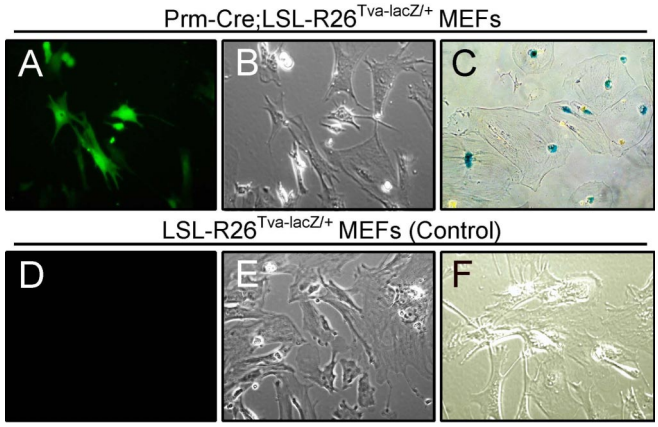

Fig. S5. Ectopic expression of TVA renders murine cells susceptible to retroviral RCASBP(A)-mediated somatic gene transfer *in vitro*. (*A–F*) Murine embryonic fibroblasts (MEFs) isolated from *Prm-Cre;LSL-R26*$^{Tva\text{-}lacZ/+}$ (*A–C*) and control *LSL-R26*$^{Tva\text{-}lacZ/+}$ (*D–F*) animals were infected with RCASBP(A)-EGFP viruses in culture. Fluorescent (*A* and *D*) and corresponding phase contrast (*B* and *E*) images show EGFP expression only in cells that have undergone Cre-mediated recombination (*A*) but not in cells with intact stop cassette (*D*). Identical exposure time was used for all images. X-Gal staining confirms recombination of MEFs isolated from *LSL-R26*$^{Tva\text{-}lacZ/+}$*;Prm-Cre* mice (*C*), whereas MEFs from *LSL-R26*$^{Tva\text{-}lacZ/+}$ mice are nuclear lacZ negative (*F*).

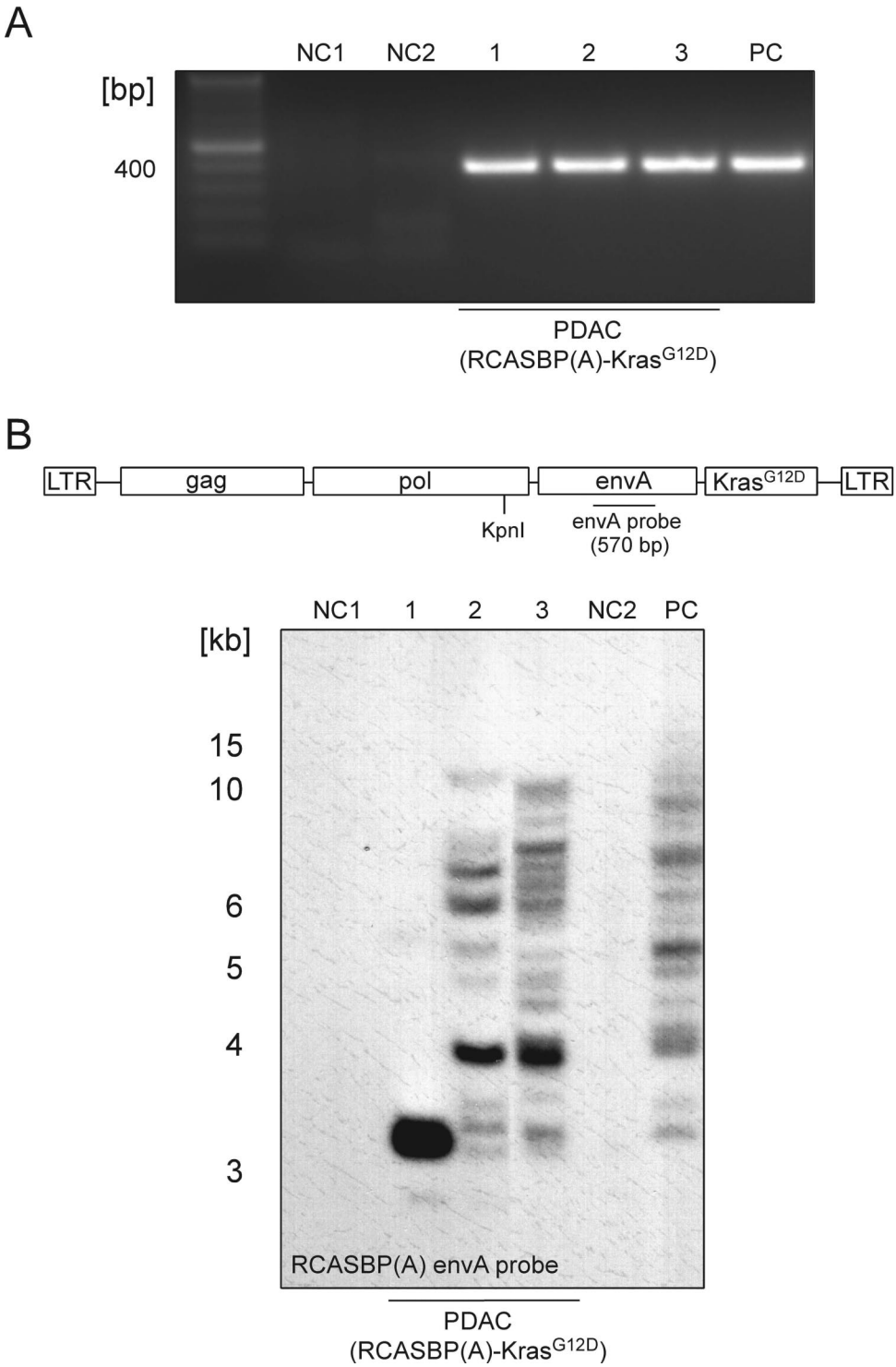

Fig. S6. PCR (*A*) and Southern blot (*B*) analysis of provirus integrations in PDAC of *Ptf1a/p48$^{Cre/+}$;LSL-R26$^{Tva\text{-}lacZ/+}$* mice infected with RCASBP(A)-Kras^{G12D}. (*A*) DNA from normal pancreas of uninfected *Ptf1a/p48$^{Cre/+}$;LSL-R26$^{Tva\text{-}lacZ/+}$* mouse (NC1) and PDAC from a *Ptf1a/p48$^{Cre/+}$;LSL-R26$^{Tva\text{-}lacZ/+}$;LSL-Kras$^{G12D/+}$;LSL-TP53$^{R172H/+}$* (NC2) animal were used as negative controls. DNA from three RCASBP(A)-Kras^{G12D}-induced PDAC (1–3) were subjected to PCR analysis to demonstrate integration of RCASBP(A)-Kras^{G12D} provirus in the genomic DNA. As positive control (PC), TVA expressing MiaPaCa2$^{fLuc\text{-}IRES\text{-}TVA}$ cells transduced with RCASBP(A)-Kras^{G12D} were used. (*B*) Restriction mapping of PDAC by Southern blot analysis. (*Upper*) Schematic representation of RCASBP(A)-Kras^{G12D} provirus. KpnI restriction site and localization of envA hybridization probe generated by digestion of RCASBP(A) vector with NdeI and AflII is indicated. (*Lower*) Southern blot analysis of KpnI-digested DNA from normal pancreas of an uninfected *Ptf1a/p48$^{Cre/+}$;LSL-R26$^{Tva\text{-}lacZ/+}$* animal (NC1), three RCASBP(A)-Kras^{G12D}-induced PDAC (1–3), PDAC from an uninfected *Ptf1a/p48$^{Cre/+}$;LSL-R26$^{Tva\text{-}lacZ/+}$;LSL-Kras$^{G12D/+}$;LSL-TP53$^{R172H/+}$* animal (NC2), and TVA-positive MiaPaCa2$^{fLuc\text{-}IRES\text{-}TVA}$ cells transduced with RCASBP(A)-Kras^{G12D} (PC).

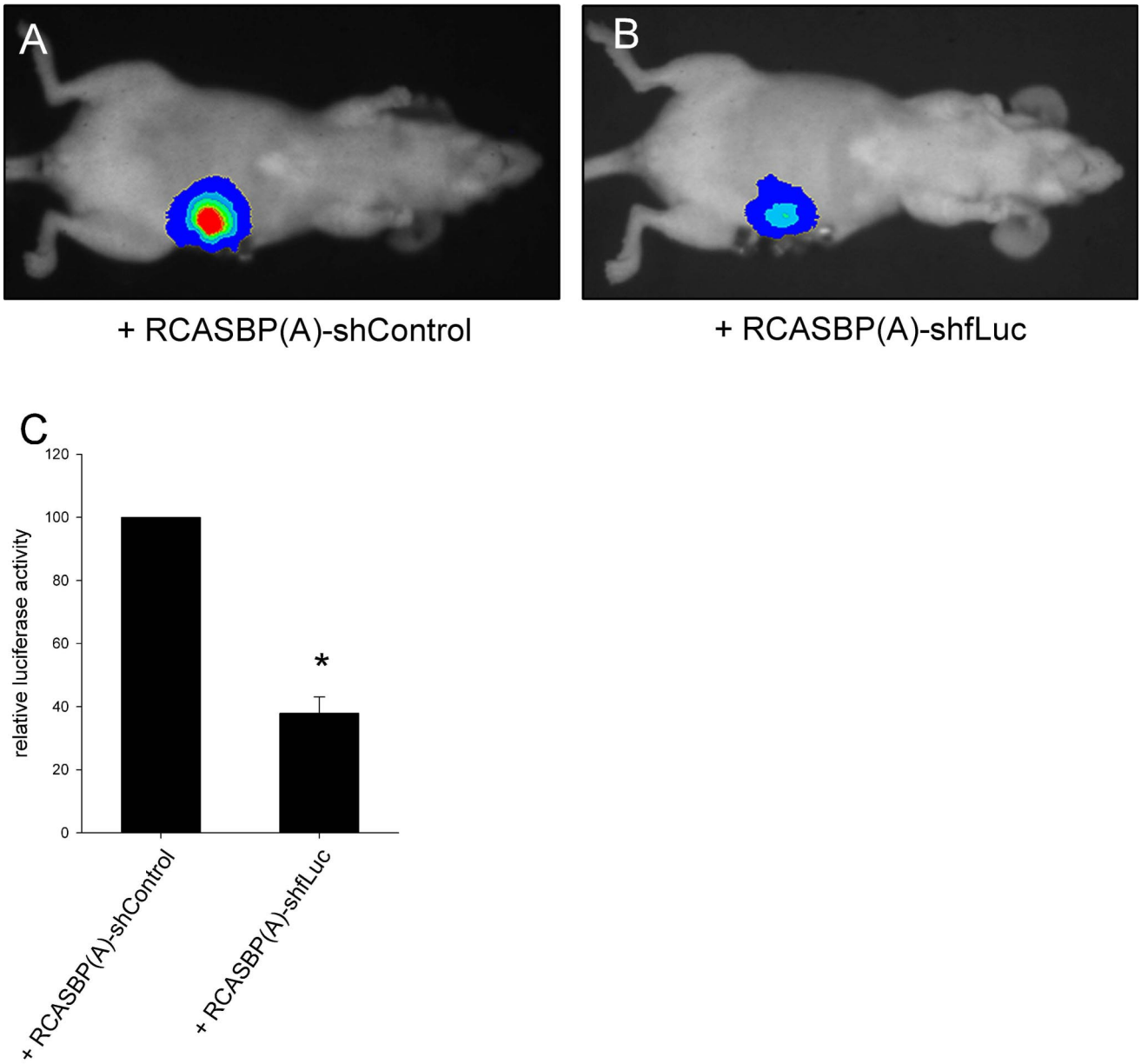

Fig. S7. Down-regulation of firefly luciferase (fLuc) expression by RCAS-mediated RNA interference *in vivo*. MiaPaCa-2$^{fLuc\text{-}IRES\text{-}TVA}$ pancreatic cancer cells with stable expression of fLuc and TVA were orthotopically implanted into the pancreas of nude mice ($n = 3$). Ten weeks later, mice were infected with RCASBP(A) virus carrying a short hairpin RNA directed against fLuc (RCASBP(A)-shfLuc) or a control shRNA (RCASBP(A)-shControl) and imaged for bioluminescence *in vivo* as described in *Materials and Methods*. (*A* and *B*) *In vivo* bioluminescence imaging of tumor-bearing mice after infection with RCASBP(A)-shControl (*A*) or RCASBP(A)-shfLuc (*B*). (*C*) Quantification of luciferase activity revealed knockdown of firefly luciferase expression to ≈40% of controls. Data are expressed as percent luciferase activity of RCASBP(A)-shfLuc infected mice relative to RCASBP(A)-shControl infected animals and represent the means ± SD of three independent experiments. (Student's *t* test: $*P < 0.001$ versus control).

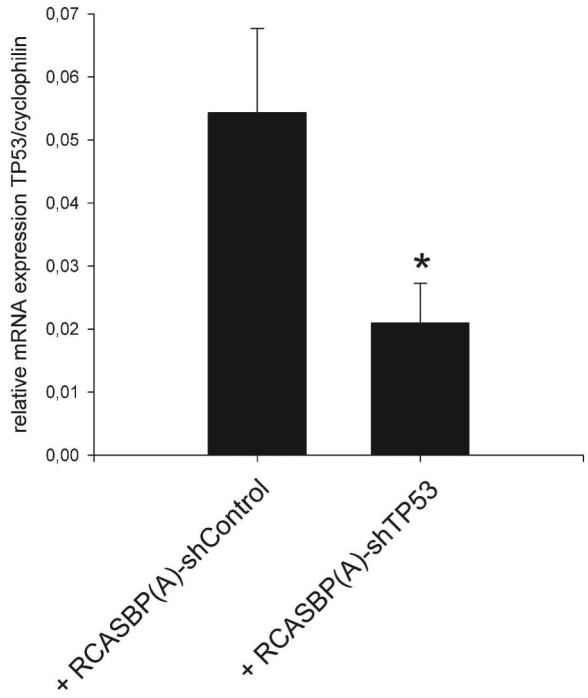

Fig. S8. Down-regulation of TP53 expression by RCAS mediated RNA interference *in vivo*. Three-week-old *Ptf1a/p48*$^{Cre/+}$;*LSL-R26*$^{Tva\text{-}lacZ/+}$;*LSL-Kras*$^{G12D/+}$ mice were infected with RCASBP(A) virus carrying a short hairpin RNA directed against murine TP53 (RCASBP(A)-shTP53) or control shRNA (RCASBP(A)-shControl) as described in *Materials and Methods*. RNA was isolated from primary pancreatic ductal adenocarcinomas of RCASBP(A)-shTP53 ($n = 5$) and RCASBP(A)-shControl ($n = 5$) infected animals and quantitative real-time RT-PCR performed. Relative amounts of TP53 transcripts were calculated by using standard curves and normalized against the globally expressed cyclophilin housekeeping gene measured in the same RNA preparation (Student's *t* test: $*P < 0.001$ versus control).