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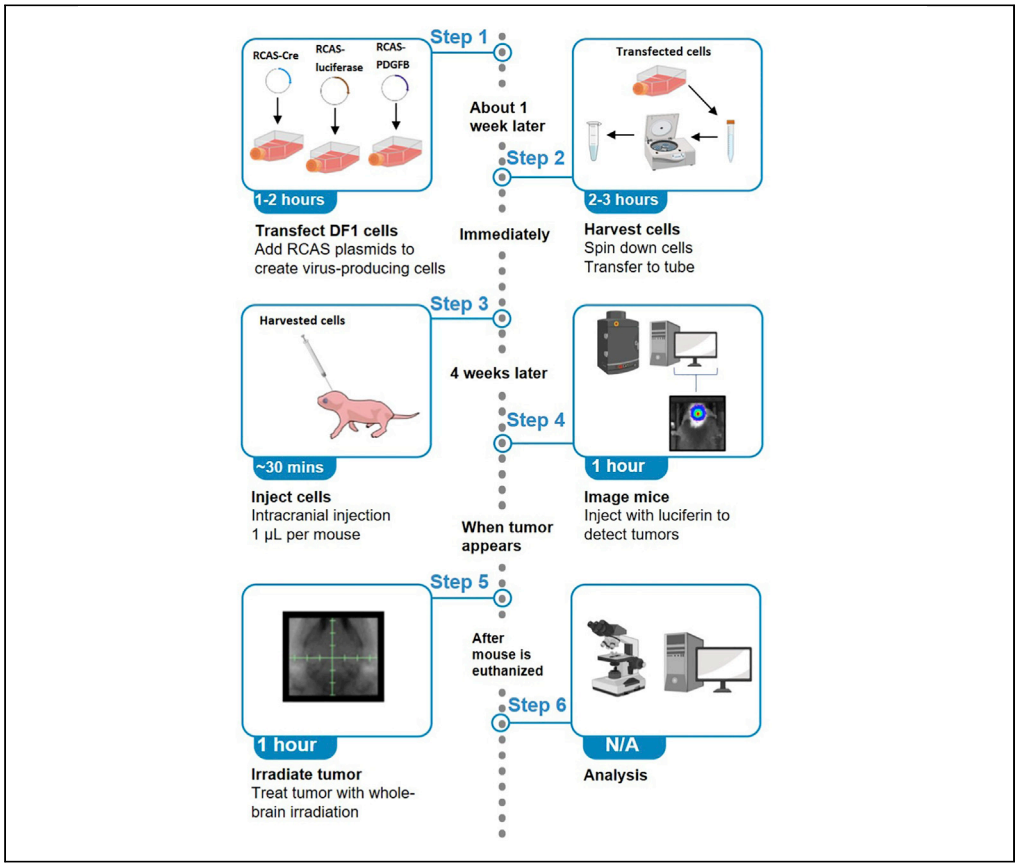
Protocol

Inducing primary brainstem gliomas in genetically engineered mice using RCAS/TVA retroviruses and Cre/loxP recombination



Genetically engineered mice are commonly used to model brainstem gliomas in pre-clinical research. One technique for inducing primary tumors in these genetically engineered mice involves delivering viral vectors containing the code for gene-editing proteins. We present a protocol for generating primary brainstem gliomas using the RCAS-TVA retroviral delivery system and the Cre/loxP gene editing system. We describe steps for transfecting and harvesting chicken fibroblast cells, intracranially injecting cells into mice, imaging primary tumors, and treating primary tumors with focal, image-guided brain irradiation.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol to breed genetically engineered mice for generation of brainstem gliomas

Generation of primary brainstem gliomas using the Cre/LoxP and RCAS-TVA systems

Detection of primary brainstem gliomas using bioluminescence imaging

Treatment of tumor-bearing mice using focal, image-guided whole-brain irradiation

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Inducing primary brainstem gliomas in genetically engineered mice using RCAS/TVA retroviruses and Cre/loxP recombination

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SUMMARY

Genetically engineered mice are commonly used to model brainstem gliomas in pre-clinical research. One technique for inducing primary tumors in these genetically engineered mice involves delivering viral vectors containing the code for gene-editing proteins. We present a protocol for generating primary brainstem gliomas using the RCAS-TVA retroviral delivery system and the Cre/loxP gene editing system. We describe steps for transfecting and harvesting chicken fibroblast cells, intracranially injecting cells into mice, imaging primary tumors, and treating primary tumors with focal, image-guided brain irradiation.

For complete details on the use and execution of this protocol, please refer to Deland et al. (2021).[1]

BEFORE YOU BEGIN

Institutional permissions

All experiments performed on animal models were approved by the IACUC at Duke University. Please acquire all permissions needed for the use of animal models from proper authorities before performing any experiments or procedures on live animals.

Breed mice

Timing: ~24 weeks

This protocol describes induction of brainstem gliomas that are driven by deletion of a tumor suppressor, such as p53, by Cre-mediated recombination and expression of the oncogene PDGF-B. Additionally, tumor-specific deletion of a gene of interest can be examined. For instance, we used this approach to study the gene encoding the apical DNA-damage response kinase *Ataxia telangiectasia mutated* (*Atm*).[1–3] To accomplish these perturbations, mice must be generated that contain the following unique alleles:

Nestin^{TVA}: To achieve lineage-specific gene editing only in neural stem cells, mice containing the *Nestin*^{TVA} allele are used to express the avian retroviral receptor, TVA, on neural stem cells. Since avian RCAS retroviruses specifically transduce cells that express the TVA receptor, they will transduce only neural stem cells in *Nestin*^{TVA} mice.[4]

Floxed tumor suppressor genes: Deletion of tumor suppressors is accomplished by introducing an RCAS retrovirus to express Cre into the brainstem neural stem cells of mice. Mice in which both alleles of a tumor suppressor of interest are flanked by loxP alleles (termed "floxed" or "fl") must be used. Cre then functionally deletes the floxed tumor suppressor. Thus, mice containing p53$^{fl/fl}$ or other floxed tumor suppressors are used. The complex mouse alleles below combine *Nestin*^{TVA} and a homozygous floxed tumor suppressor alleles.

Floxed genes of interest: To study the impact of *Atm* deletion in neoplastic tumor cells, two floxed alleles of *Atm*^{fl} are included. For many experiments, the tumors from *Atm*$^{fl/fl}$ mice (complete loss of *Atm* in tumor cells) are compared to tumors from littermate *Atm*$^{fl/+}$ mice (intact expression of one allele of *Atm* in tumor cells). Similar approaches could be taken to study loss of other genes of interest if floxed alleles are available.

1. Obtain C57BL/6 mice with the following genotypes: *Nestin*^{TVA}, *p53*^{fl}, *Atm*^{fl}, and *Ink4a/Arf*^{fl}.
2. Set up breeding crosses to generate mouse models with the desired combinations of alleles. Examples of such crosses are shown in Figure 1.
   a. Generate the *Nestin*$^{TVA/TVA}$; *p53*$^{fl/fl}$; *Atm*$^{fl/fl}$ and *Nestin*$^{TVA/TVA}$; *p53*$^{fl/fl}$; *Atm*$^{fl/+}$ mouse models.
   b. Generate the *Nestin*$^{TVA/TVA}$; *Ink4a/Arf*$^{fl/fl}$; *Atm*$^{fl/fl}$ and *Nestin*$^{TVA/TVA}$; *Ink4a/Arf*$^{fl/fl}$; *ATM*$^{fl/+}$ mouse models.
   c. Generate the *Nestin*$^{TVA/TVA}$; *p53*$^{fl/fl}$; *Ink4a/Arf*$^{fl/fl}$; *Atm*$^{fl/fl}$ and *Nestin*$^{TVA/TVA}$; *Ink4a/Arf*$^{fl/fl}$; *Atm*$^{fl/+}$ mouse models.

Culture DF1 chicken embryo fibroblast cell lines

Timing: 2–3 weeks

3. Prepare DF1 cell media.
   a. Mix Dulbecco's Modified Eagle Medium (DMEM) with the following additives: 10% Fetal Bovine Serum, 100 U/mL Penicillin, 100 μg/mL Streptomycin, and 2 mM L-Glutamine.
4. Culture DF1 cells in prepared DF1 cell media.
   a. Grow at 39°C and 5% CO_2.
   b. Grow to confluency and do not let the cells overgrow.
   c. Subculture at 1:6 (for 2 days); 1:12 (for 3 days); 1:24 (for 4 days).
   d. Trypsinize with 0.25% Trypsin/EDTA for 3–5 min (at 39°C if necessary).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal HA-probe	Santa Cruz Biotechnology	Cat# sc-805 RRID: AB_631618
Mouse monoclonal Ser1981 phosphorylated ATM	MilliporeSigma	Cat# 05740 RRID: AB_2062670
Rabbit anti-mouse Ser824 phosphorylated Kap1	Thermo Fisher	Cat# A300-767A RRID: AB_2779445
Rabbit polyclonal Ser15 phosphorylated p53	Cell Signaling Technology	Cat# 9284 RRID: AB_10721303

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Rat monoclonal p19ARF/CDKN2A (clone 5-C3-1)	Novus Biologicals	Cat# NB200-174 RRID: AB_10822123
Mouse monoclonal IgG p53 mAb	Cell Signaling Technology	Cat# 2524
Rabbit polyclonal IgG p21	Santa Cruz Biotechnology	Cat# sc-471 RRID: AB_383248
Rabbit polyclonal cleaved caspase-3	Cell Signaling Technology	Cat# 9661S RRID: AB_2341188
Rabbit polyclonal IgG anti-NQO1	Abcam	Cat# ab34173 RRID: AB_11081045
Mouse monoclonal anti-actin	BD Biosciences	Cat# 612656 RRID: AB_2861213
Mouse monoclonal IgG GAPDH antibody	Proteintech	Cat# 60004-1-Ig RRID: AB_1874649
Goat anti-Mouse IgG IRDye 680RD	Li-Cor Biosciences	Cat# 926-68070 RRID: AB_10956588
Goat anti-Rabbit IgG IRDye 800CW	Li-Cor Biosciences	Cat# 926-32211 RRID: AB_621843
Chemicals, peptides, and recombinant proteins		
D-luciferin, potassium salt	Gold Biotechnology	Cat# LUCK-1G
Papain	Worthington Biochemical	Cat# LS003118
Ovo	Worthington Biochemical	Cat# 3087
DNase	MilliporeSigma	Cat# D4527-40KU
Ethylenediaminetetraacetic acid tetrasodium salt dihydrate	MilliporeSigma	Cat# ED4SS
NeuroCult™ Basal Medium	Stem Cell	Cat# 05700
10% proliferation supplements	Stem Cell	Cat# 05701
Human EGF Recombinant Protein	Thermo Fisher	Cat# PHG0311
Human FGF-basic (FGF-2/bFGF) Recombinant Protein	Thermo Fisher	Cat#13256-029
Heparin solution	Stem Cell	Cat#07980
X-tremeGENE™ 9	MilliporeSigma	Cat# 6365779001
Critical commercial assays		
PicoPure DNA extraction kit	Thermo Fisher	Cat# KIT0103
Direct-zol RNA Kit	Zymo Research	Cat# R2051
iScript™ Advanced cDNA Synthesis Kit	Bio-Rad	Cat# 1725037
Experimental models: Cell lines		
UMNSAH/DF-1 chicken fibroblast cells	ATCC	CRL-12203™
Primary glioma cell lines	This paper	N/A
Experimental models: Organisms/strains		
Mouse: *Nestin*^{TVA}: Tg(NES-TVA)J12Ech/J: breeding age: male and female	The Jackson Laboratory	MGI:2663944
Mouse: *p53*^{fl}: B6.129P2-Trp53tm1Brn/J: breeding age: male and female	The Jackson Laboratory	MGI:1931011
Mouse: *Atm*^{fl}: 129-Atmtm2.1Fwa/J: breeding age: male and female	The Jackson Laboratory	MGI:3851145
Mouse: *Ink4a/Arf*^{fl}: Cdkn2atm1Rdp: breeding age: male and female	Mouse Genome Informatics	MGI: 1857942
Oligonucleotides		
Primers for *Atm*^{fl} alleles, see Table S1	This paper	N/A
Primers for *p53*^{fl} alleles, see Table S1	This paper	N/A
Primers for *Ink4a/Arf*^{fl} alleles, see Table S1	This paper	N/A
Atm recombined probe, 5′-ACACATGCATGC AGGCAGAGCATCCCT-3′	This paper	N/A
Atm floxed probe, 5′-AGCTGTTACTTTTGC GTTTGGTGTGGCG-3′	This paper	N/A
p53 recombined probe, 5′-CTTGATATCG AATTCCTGCAGCCCGGG-3′	This paper	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
p53 floxed probe, 5′-ATGCTATACGAA GTTATCTGCAGCCCGG-3′	This paper	N/A
Ink4a/Arf recombined probe, 5′-CATTATACGAA GTTATGGCGCGCCC-3′	This paper	N/A
Ink4a/Arf floxed probe, 5′-CTCTGAAAACCTCC AGCGTATTCTGGTA-3′	This paper	N/A
Recombinant DNA		
Plasmid: RCAS-Cre	Laboratory of Oren Becher[1]	N/A
Plasmid: RCAS-Luc	Laboratory of Oren Becher[1]	N/A
Plasmid: RCAS-PDGFB	Laboratory of Oren Becher[1]	N/A
Plasmid: RCAS-GFP	Laboratory of Oren Becher[1]	N/A
Software and algorithms		
ImageJ	NIH	https://imagej.nih.gov/ij/
Prism 7	GraphPad Software Inc.	https://www.graphpad.com/scientific-software/prism/
QuantaSoft	Bio-Rad	https://www.bio-rad.com/en-us/life-science/digital-pcr/qx200-droplet-digital-pcr-system/quantasoft-software-regulatory-edition?ID=1864011
Other		
Biological safety cabinet	Thermo Fisher Scientific	Cat#13-261-222
IVIS Lumina III In Vivo Imaging System	PerkinElmer	Cat#CLS136334
CO_2 incubators	Thermo Fisher Scientific	Cat#4110
EVOS M7000 Imaging System	Thermo Fisher Scientific	Cat#AMF7000
Hamilton syringe	Hamilton	Cat#84851
Isoflurane vaporizer	Kent Scientific	Cat#VetFlo-1205S
Oxygen concentrator	Fisher Scientific	Cat#04-777-122
Sure-Seal Large Mouse/Rat Induction Chamber	World Precision Instruments	Cat#EZ-1785
Sterile sleeves	VWR	Cat#414004-510

MATERIALS AND EQUIPMENT

Lysis Buffer		
Reagent	Final concentration	Amount
1 M Tris-HCl (pH 7.5–8.5)	0.1 M	10 mL
0.5 M Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)	0.005 M	1 mL
20% Sodium dodecyl sulfate (SDS)	0.2%	1 mL
5 M NaCl	0.2 M	4 mL
ddH_2O	N/A	84 mL
Total	**N/A**	**100 mL**
Store at 20°C–22°C for a maximum of 2 weeks.		

Ovomucoid Solution		
Reagent	Final concentration	Amount
Ovo	0.71 mg/mL	10 mg
Neurocult (basal)	1×	14 mL
DNase in EBSS	10 mg/mL	20 µL
Total	**N/A**	**14.02 mL**
Incubate at 37°C until ready to use.		

Papain Solution		
Reagent	Final concentration	Amount
Papain	0.94 mg/mL	23.6 mg
EDTA	0.18 mg/mL	4.5 mg
Cysteine:	0.18 mg/mL	4.5 mg
Earle's Balanced Salt Solution	1×	25 mL
Total	**N/A**	**25 mL**
Store at −20°C for a maximum of 24 h.		

- **Proteinase K solution:** add 100 mg of Proteinase K to 5 mL of ddH_2O.

Store at −20°C.

- **D-Luciferin Stock Solution:** dissolve 1 g of D-Luciferin Potassium Salt in 66.6 mL of Dulbecco's Phosphate Buffered Saline without Ca^{2+} and Mg^{2+}.

Store at −80°C; expires after 1 year.

Alternatives: D-Luciferin Sodium Salt and L-Luciferin Potassium Salt can be substitute for D-Luciferin, Potassium Salt.

STEP-BY-STEP METHOD DETAILS

Step 1: DF1 transfection

Timing: Approximately 1 h and 45 min

This section describes the procedure for transfecting DF1 chicken embryo fibroblast cells with RCAS plasmids, which will enable the DF1 cells to generate RCAS viruses. The RCAS plasmids relevant to this protocol include RCAS-Cre, RCAS-Luc, RCAS-PDGFB, and RCAS-GFP (Figure 2). Transfection with the RCAS-GFP plasmid is recommended for confirmation of a successful transfection procedure.

Note: The day prior to transfecting, passage DF1 cells at a 1:12 ratio into a T25 flask—one flask for each plasmid. Cells should be about 30% confluent on the day of transfection.

Note: If you want to induce tumors by deleting p53 with Luciferase and PDGFB expression, then use the following plasmids for transfection individually: RCAS-Cre, RCAS-Luc and RCAS-PDGFB.

1. Warm DMEM null (i.e., DMEM containing no additives) in a warm water or pebble bath at 37°C for 30 min to 1 h. During the wait time:
   a. Prepare the workspace.
      i. Turn on the biological safety cabinet and disinfect gloved hands and the work surface with 70% ethanol.
      ii. Gather Eppendorf tubes (2 per plasmid), T25 flasks containing DF1 cells (one per plasmid), transfection reagent X-tremeGENE™ 9, and RCAS-Cre, RCAS-Luc, RCAS-PDGFB, and RCAS-GFP plasmid stocks in 1.7 mL Eppendorf tubes, then spray the exteriors with 70% ethanol and place inside the biological safety cabinet.
      iii. For each plasmid, label one Eppendorf tube with the plasmid name (e.g., RCAS-Cre), and label the second Eppendorf tube with "DMEM null". Make sure that the T25 flasks are appropriately labeled.

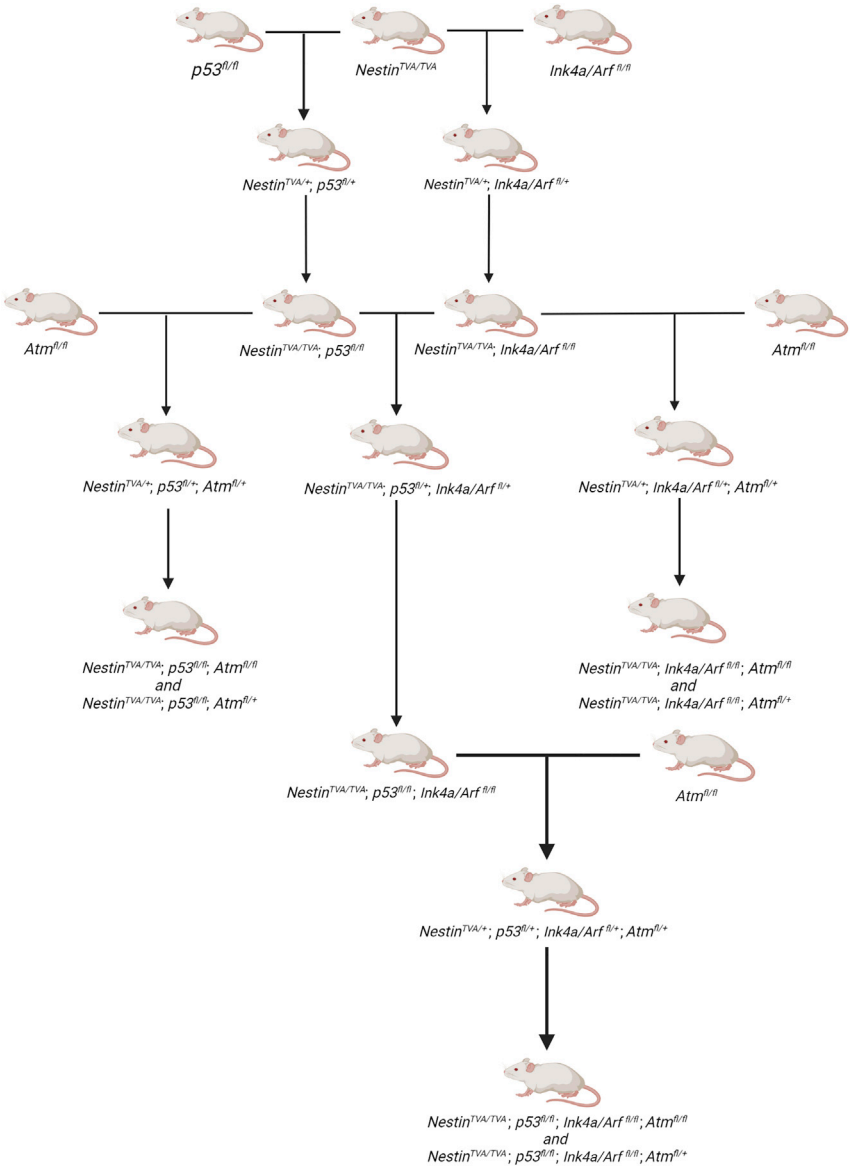

Figure 1. Breeding plan diagram

Diagram depicting the overall mouse breeding plan to generate (i) *Nestin*$^{TVA/TVA}$; *p53*$^{fl/fl}$; *Atm*$^{fl/fl}$; (ii) *Nestin*$^{TVA/TVA}$; *Ink4a/Arf*$^{fl/fl}$; *Atm*$^{fl/fl}$ and (iii) *Nestin*$^{TVA/TVA}$; *p53*$^{fl/fl}$; *Ink4a/Arf*$^{fl/fl}$; *Atm*$^{fl/fl}$ and littermate controls with intact *Atm*$^{fl/+}$. Created with BioRender.com.

iv. Ensure that the workspace is sufficiently stocked with conical tubes, serological pipettes, micropipette tips, lab markers, etc. Ensure that the pipettors are functioning.

b. Disinfect the DMEM null container exterior with 70% ethanol and place inside the biological safety cabinet when finished warming.

2. Aliquot 242.5 µL of DMEM null into each Eppendorf tube labeled "DMEM null".
3. Add 7.5 µL of X-tremeGENE™ 9 into each Eppendorf tube containing DMEM null.
   a. Add X-tremeGENE™ 9 directly to the DMEM null instead of letting it come into contact with the inside plastic of the Eppendorf tube.
   b. When finished adding X-tremeGENE™ 9 to one Eppendorf tube, change pipette tips before moving on to the next Eppendorf tube.

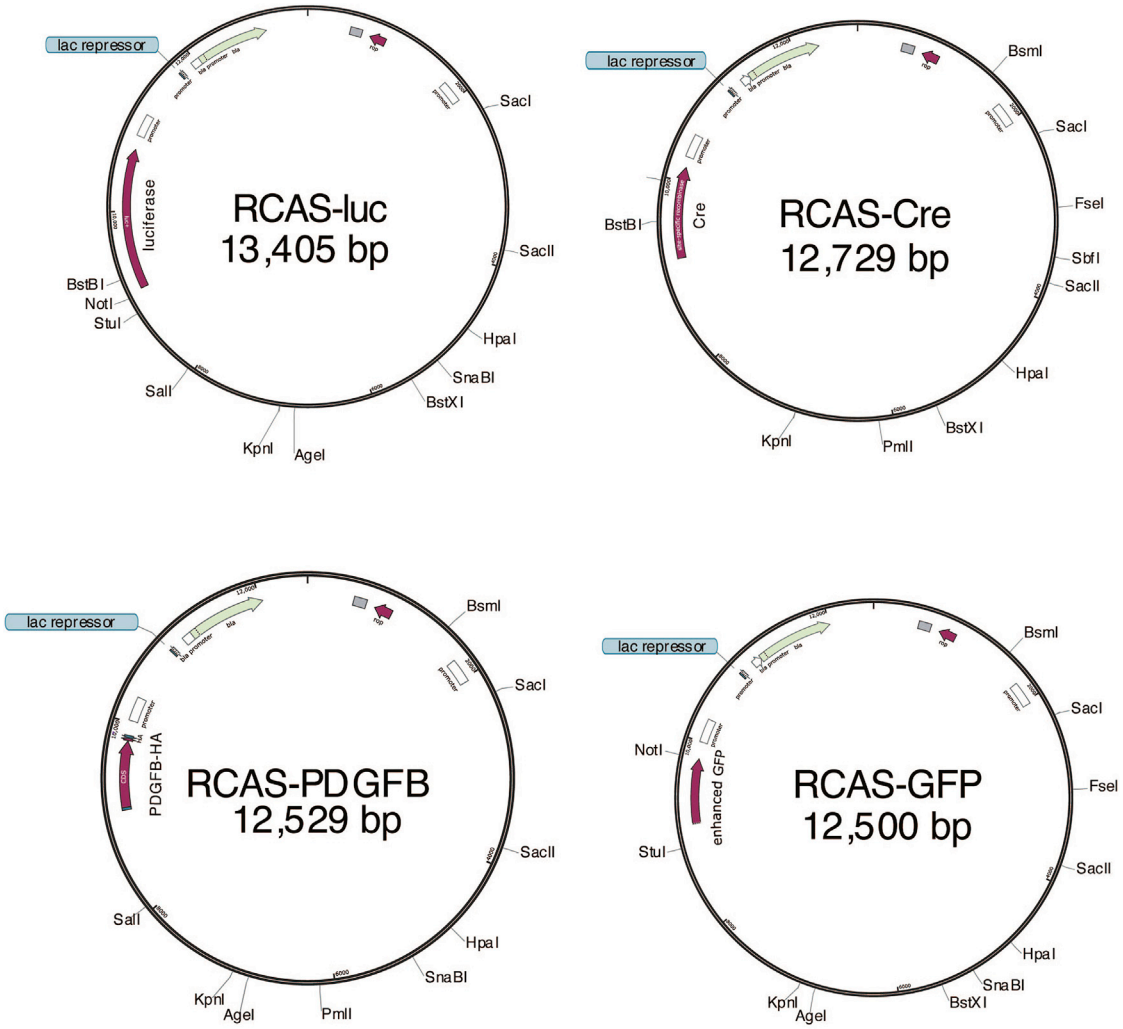

Figure 2. Maps of RCAS plasmid vectors

Maps of the RCAS-Luc (top left), RCAS-Cre (top right), RCAS-PDGFB (bottom left), and RCAS-GFP (bottom right) plasmid vectors, displayed using SnapGene software v6.2 (www.snapgene.com).

c. Let the DMEM null and X-tremeGENE™ 9 mixture incubate at 20°C–22°C for 5 min.

4. Aliquot 2.5 μg of each plasmid to its appropriate Eppendorf tube.
5. After the 5-min incubation period is finished, add the DMEM null and X-tremeGENE™ 9 mixture to each plasmid.
   a. One 250 μL mixture of DMEM null and X-tremeGENE™ 9 should be added to each plasmid aliquot.
   b. Mix each tube containing DMEM null, X-tremeGENE™ 9, and plasmid by flicking for 10 s.
6. Let each tube containing DMEM null, X-tremeGENE™ 9, and plasmid incubate at 20°C–22°C for 20 min.
7. Add each DMEM null, X-tremeGENE™ 9, and plasmid mixture to its appropriate T25 flask.
   a. Add the mixture dropwise to the cells in the flask.
8. Place the T25 flasks of cells back into the incubator.

⚠ CRITICAL: There should be GFP expression in the DF1 cells transfected with RCAS-GFP within 48 h after completion of the procedure (Figure 3). Use a fluorescent microscope to visualize GFP expression in order to confirm the success or failure of the procedure.

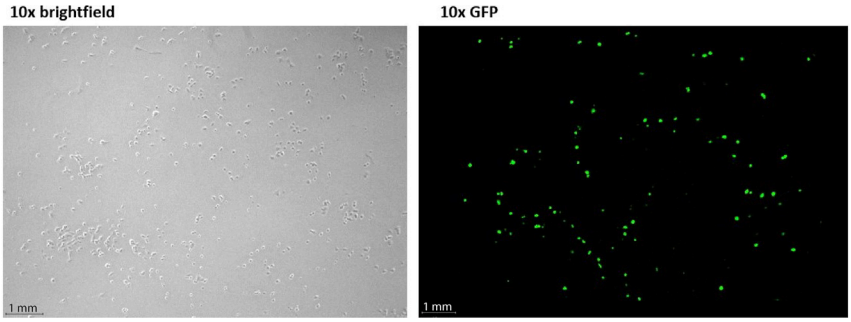

Figure 3. Microscopy image of DF1-GFP cells 24 h post-transfection

DF1 cells transfected with RCAS-GFP seen under brightfield (left) and under fluorescence microscopy (right). Scale bar = 1 mm.

The percentage of cells that are GFP-positive increases subsequently with time and passage. troubleshooting 1.

Note: X-tremeGENE™ 9 can be substituted with other lipid-based transfection reagents for this procedure.

Step 2: Harvesting of transfected DF1 cells

Timing: Approximately 2 h and 30 min

This section describes the procedure for harvesting transfected DF1 cells. This allows for the cells to later be injected into the mouse brainstem to eventually induce a brain tumor. Harvesting DF1 cells transfected with RCAS-GFP is optional for this procedure.

Note: Cells should be confluent before being harvested.

Note: Cells can be harvested from passage 3 to passage 20 after transfection. Cells usually need to be passaged every 2 days.

Note: Check the DF1-transfected cells for GFP expression using a fluorescent microscope to verify virus production prior to starting this procedure. The cells transfected with RCAS-GFP should show GFP expression, while the cells transfected with the RCAS-Cre, RCAS-Luc, and RCAS-PDGFB plasmids should show no GFP expression.

9. Warm DF1 media, PBS, and 0.25% trypsin in a warm water or pebble bath at 37°C for 30 min to 1 h. During the wait time:
   a. Find a Styrofoam box or cooler, fill it with ice, and have it ready nearby.
   b. Prepare the workspace.
      i. Turn on the biological safety cabinet and disinfect gloved hands and the work surface with 70% ethanol.
      ii. Ensure that the workspace is sufficiently stocked with conical tubes, serological pipettes, micropipette tips, lab markers, tissue culture flasks, etc. Ensure that the pipettors are functioning.
   c. Place the prewarmed DF1 media, PBS, and trypsin inside the biological safety cabinet.
10. Wash and passage DF1 cells when cells are highly confluent.
   a. Remove spent media from cells using aspiration.
   b. Wash cells with 5 mL of PBS.
   c. Trypsinize cells with 0.25% trypsin (0.7 mL for a T25 flask and 1 mL for a T75 flask).

Figure 4. Injection of mouse pup brainstem with DF1 cells

DF1 cells injected into the brainstem of P4 pups (left), with zoom-in showing needle target on skin surface with red crosshair (right).

d. Incubate cells at 39°C for 5 min. Make sure cells are detached before proceeding.
e. Neutralize the 0.25% trypsin inside the flask with DF1 media.
f. Triturate the cells three times and passage at the desired ratio.
g. Place the flasks containing the newly passaged cells into the incubator. Do not discard the flask(s) of cells from which the passaged cells were taken from, as the cells in these flasks will be harvested.

11. Determine the cell counts.
    a. For manual cell counting, use trypan blue solution and a C-Chip disposable hemocytometer kit and follow the instructions provided by the kit. Only calculate the cell counts for the cells of the cell lines that will be used for mouse injections.
12. Add the RCAS-Cre, RCAS-Luc, and RCAS-PDGFB transfected cells together.
    a. Add together the cells transfected with RCAS-Cre, RCAS-Luc, and RCAS-PDGFB in a 1:1:1 mixture. To calculate the volume of cells of each group needed to reach this ratio, divide the smallest cell count out of all the groups by the cell count of the group of interest. Multiply that value by the volume of media remaining in the flask containing the cells of the group with the smallest cell count.
    b. Transfer the appropriate volume of cells of each group—calculated previously—together into a 50 mL conical tube.
    c. Spin the cells in a centrifuge at 300 × *g* for 5 min.
        i. Check that a pellet has formed. troubleshooting 2.
    d. Discard the supernatant.
13. Resuspend the cells in DF1 media.
    a. To calculate the volume of DF1 media needed to resuspend the cells, divide the total number of cells by 100,000.
        i. To calculate the total number of cells, find the sum of the cell count multiplied by the volume added to the 50 mL conical tube for each group.
        ii. The final desired concentration of cells should be about 100,000 cells per μL of DF1 media.
14. Place the cells on ice.

Note: The harvested cells should be in ice for a maximum of 1 h.

Step 3: Intracranial injection of transfected DF1 cells

Timing: Approximately 30 min

This section describes the procedure for performing an intracranial injection on a mouse pup (Figure 4). This allows for the transfected DF1 cells to generate the RCAS viruses inside the mouse brainstem and eventually induce a tumor.

Note: This procedure should be performed on mouse pups between age 3 through 5 days.

15. Prepare work space.
    a. Clean a laminar-flow hood with disinfectant, then retrieve all necessary materials and the mice to be injected.
    b. Clean the inside of a Hamilton syringe with de-ionized water.
16. Sedate mouse pups (e.g., litter of $Nestin^{TVA/TVA}$; $p53^{fl/fl}$; $Atm^{fl/fl}$ and $Nestin^{TVA/TVA}$; $p53^{fl/fl}$; $Atm^{fl/,+}$ mice).
    a. Carefully remove the mouse pups from their cage and sedate them using hypothermia. This can be done by filling a container with ice, placing a sheet of aluminum foil on top of the ice, and placing each pup on top of the aluminum foil.
    b. Wait until pups stay still before performing the injection. This usually takes about 5 min.
17. Inject harvested DF1 cells into the sedated mouse pups.
    a. Use a Hamilton syringe to withdraw 1×10^5 virus-producing DF1 cells resuspended in 1 μL of cell media.
    b. Inject 1 μL of the resuspended DF1 cells into the brainstem of a mouse pup by placing the needle tip between the ears and in the middle of the pup's head with the syringe tilted at a 15° angle. The needle should then be driven about 2 mm below the skin to enter the pup brainstem. Cells should then be gently injected.
18. Repeat the previous step for every mouse pup in the litter.
19. Warm the mouse pups.
    a. Warm all sedated pups and confirm that they have awakened before placing them back into their cage.
20. Clean the work area and equipment.

Step 4: Luciferase imaging of mice injected with transfected DF1 cells

Timing: Approximately 1 h per six mice

This section describes the procedure for bioluminescence imaging of brainstem gliomas in mice using luciferin. This allows the mice to be monitored for glioma formation prior to radiation treatment. Mice should be imaged on a weekly basis starting 4 weeks after intracranial injection until tumor is detected.

21. Thaw luciferin solution in a heating block.
    a. Remove the 15 mg/mL luciferin solution from the heating block immediately upon thawing and place in a cooler of ice.
    b. 1.5 mL of luciferin should be thawed for every three mice imaged.
    c. Bring the thawed luciferin to the IVIS Lumina III In Vivo Imaging System.
22. Start-up the IVIS Lumina III.
    a. Open the LivingImage 4.5 software.
    b. Log into the appropriate user account.
    c. Click "initialize" to warm-up the IVIS Lumina III.
        i. The IVIS Lumina III will finish initializing once the "Temperature" bar turns from red to green.
23. Set-up the work area.
    a. Gather all the necessary materials while the IVIS Lumina III is initializing. These include a scale, cotton swabs, nair hair removal cream, water, antibiotic, insulin syringes, luciferin, and paper towels.
    b. Make sure that the oxygen concentrator, the isoflurane vaporizer, and the mouse induction chamber are set up appropriately, with all the necessary tubes and connections secured.
    c. Check to make sure the isoflurane level in the vaporizer is full or near full.
24. Turn on the oxygen concentrator and isoflurane vaporizer.

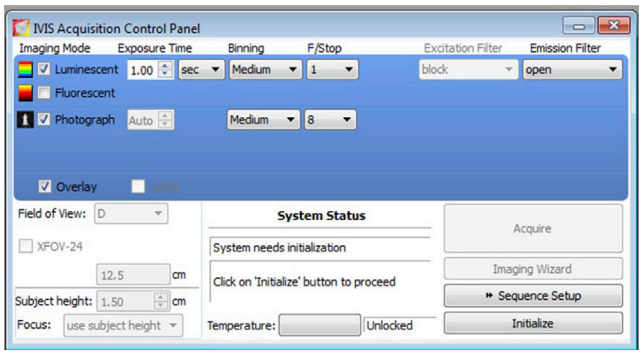

Figure 5. Image of control panel for the LivingImage 4.5 software

a. Adjust the flow rate of isoflurane between 2.5 and 3% v/v.

25. Weigh the mice (e.g., *Nestin*$^{TVA/TVA}$; *p53*$^{fl/fl}$; *Atm*$^{fl/fl}$ and *Nestin*$^{TVA/TVA}$; *p53*$^{fl/fl}$; *Atm*$^{fl/+}$ mice intracranially injected with transfected DF1 cells) and record their weights.
    a. Place the mice in the induction chamber and wait until they are anesthetized.
    b. Weigh the mice and record the weight in grams.
26. Remove hair from the mice.
    a. Apply nair to the head of the mouse using a cotton swab.
    b. Place the mouse back into the induction chamber for 3–5 min.
    c. Remove as much of the nair and loose hair as possible using a wet cotton swab.
    d. Repeat sub-steps a-c if there is hair remaining on the head.
    e. Thoroughly rinse the mouse's head with water and iodine to prevent burns.
27. Adjust the IVIS Lumina III settings as needed (Figure 5).
    a. Make sure no lens is attached to the inside top of the imager. If there is a lens present, remove it by pulling downward.
    b. Set the field of view to "D".
    c. Make sure that "XFOV-24" is unchecked.
    d. Make sure the "luminescence" and "photograph" boxes are checked.
    e. Make sure the nose cones are positioned correctly inside the IVIS Lumina III.
    f. Make sure the exposure time is set to "60 s".
28. Inject 1 to 3 mice with 15 mg/mL luciferin *via* intraperitoneal injection.
    a. The volume of luciferin that each mouse should receive is equal to 10 μL per gram of body weight.
    b. Draw the appropriate volume of luciferin solution into an insulin syringe.
        i. If any bubbles appear on the inside of the syringe, remove them by flicking the syringe and slowly dispensing the solution.
        ii. Repeat if necessary until no bubbles are present.
    c. Place the insulin syringe needle tip at the lower abdomen of the mouse, very slightly angle the syringe, and carefully push the needle into the mouse.
    d. Dispense the appropriate volume of luciferin into the mouse by slowly depressing the syringe plunger, then carefully remove the needle. troubleshooting 3.
    e. Place the mouse back into the induction chamber and record the time of injection.
29. Wait 15 min after injecting each mouse.
    a. Position the mouse inside the *in vivo* bioluminescent imaging system chamber a couple of minutes before the 15-min wait period ends. Instructions for the IVIS Lumina III In Vivo Imaging System are provided here, but other *in vivo* imaging devices capable of measuring bioluminescence on anesthetized mice can also be used.
        i. The mouse should be positioned so that it is lying flat and its head is clearly visible.
30. Begin the luminescence imaging by clicking "Acquire".

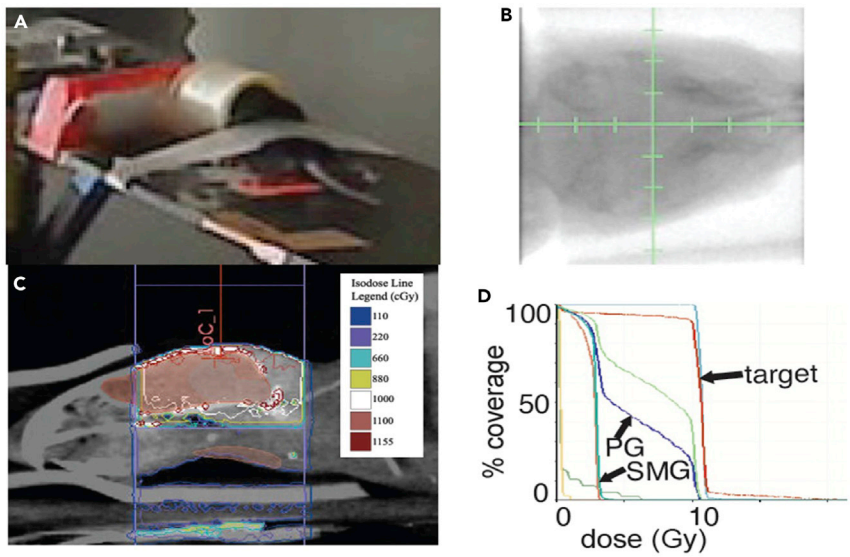

Figure 6. Focal image-guided irradiation of mouse tumors

(A) Example of mouse anesthetized with isoflurane on irradiator couch.

(B) kV image guidance prior to radiation therapy.

(C) Sagittal image from a cone-beam CT with isodose lines showing lateral beams targeting brainstem.

(D) Dose-volume histogram demonstrates >95% of brainstem target is covered by radiation dose prescription with minimal radiation dose to the parotid and submandibular glands (PG, SMG). Reproduced under MDPI open access policy.[3]

a. Make sure the door to the IVIS Lumina III is closed before clicking "acquire".

31. Save the image file.
32. Remove the sedated mice and return them to their cage(s).
33. Repeat steps 25 through 32 for every set of mice to be imaged.
34. When finished, close the software and turn off the oxygen concentrator and isoflurane vaporizer.

Note: Bioluminescent imaging setups other than the IVIS Lumina III can be used with optimization according to the manufacturer's instructions.

Step 5: Focal, image-guided brain irradiation of tumor-bearing mice

Timing: Approximately 1 h

This section describes the procedure for subjecting mice to focal brain irradiation (Figure 6). This procedure is needed to dissect the impact of tumor irradiation on mice.

35. Prior to carrying out experimental mouse irradiations, characterize dosimetry of the below focal brain irradiation treatment in collaboration with small animal radiation physics experts and Radiation Safety officers.
36. Anesthetize mice (e.g., litter of *Nestin*$^{TVA/TVA}$; *p53*$^{fl/fl}$; *Atm*$^{fl/fl}$ and *Nestin*$^{TVA/TVA}$; *p53*$^{fl/fl}$; *Atm*$^{fl/+}$ mice) with brainstem gliomas confirmed by *in vivo* imaging.
    a. Turn on the oxygen concentrator and isoflurane vaporizer.
    b. Adjust the flow rate of isoflurane between 2.5 and 3% v/v.
    c. Place the mice in the induction chamber and wait until they are anesthetized (approximately 10 min).
37. Place anesthetized mouse on X-Rad 225 cx stage in a prone position.

38. Acquire orthogonal X-ray images using fluoroscopy with 40 kVp, 2.5 mA x-rays filtered through 2 mm aluminum.
39. Irradiate mice with 225 kVp, 13 mA x-rays filtered through 0.3 mm copper using a 15 × 40 mm rectangular radiation field. The average dose rate was calculated to be 282 cGy/min using an ionization chamber.
40. Deliver right and left lateral fields.

Alternatives: Other image-guided small animal irradiation platforms can be used if an X-Rad 225cx is not available.

Note: Image-guided setups that use focal beams, rather than beams that cover the entire head, are likely needed to achieve high doses such as 10 Gy × 3 without causing significant toxicity to the salivary glands. We have found that a parallel opposed beam configuration optimizes brainstem coverage while sparing the salivary glands.

Note: Radiation treatment dosimetry should be confirmed using metal–oxide–semiconductor field-effect transistor (MOSFET) in collaboration with institutional Radiation Safety officers.

Optional step: Derivation of cell lines from primary mouse brainstem gliomas

Timing: Approximately 4 h

This section describes the procedure for deriving cell lines from mouse brainstem gliomas generated as described above. Such cell lines can be used to confirm floxed allele recombination in neoplastic cells from the primary tumor. Additionally, such cell lines can be used to examine the biology of mouse in primary brainstem gliomas using *in vitro* assays such as clonogenic survival assays to examine the effects of specific genotypes or perturbagens on primary mouse brainstem glioma cells.

41. Remove tumor, place in PBS, cut up into small pieces, and transfer to a 15 mL tube.
42. Sterile filter papain solution.
    a. Before filtering, vortex the solution, incubate at 37°C for a few minutes, then vortex again.
    b. Use a 0.2 μm filter for sterile filter, then incubate at RT for 30 min to activate (active for 1 h).
43. Aspirate PBS off of tumor tissue, then resuspend in 5 mL papain + 30 μL DNase.
44. Triturate 30× with a 5 mL pipet.
45. Incubate for 15 min at 37°C.
    a. During this incubation time, prepare and sterile filter the ovomucoid solution.
    b. Use a 0.2 μm filter for sterile filter.
46. Triturate tissue 30× with a 5 mL pipet.
47. Add 2 mL of ovo solution, then invert.
48. Centrifuge for 5 min at 162 × *g*.
49. Aspirate supernatant.
50. Add 500 μL of ovo to pellet, then triturate with P1000 30×.
51. Add 5 mL Neurocult (basal).
52. Centrifuge for 5 min at 48 × *g*.
53. Collect supernatant, if desired, into a 50 mL conical tube.
54. Repeat steps 50–53, two more times (not necessary).
55. Resuspend pellet in Neurocult complete media and strain through a cell strainer to remove clumps of cells and tissue that did not dissociate.
56. Combine with supernatant collected from the three spins if desired (will contain more cells, but will also contain dead cells and debris); spin again at 162 × *g* for 5 min.
57. Count cells and place in appropriate vessel.

Note: Cell lines will take up to seven passages to establish a continuous culture. Many cell lines will not result in established cultures that are capable of indefinite self-renewal. Growth rates of cell lines can be variable. Early passage cell lines likely contain non-neoplastic brain parenchymal cells, which disappear from the culture over time.

Note: Mouse brainstem glioma cell lines can also be cultured as neurospheres, which may more faithfully model certain aspects of tumor biology. To do so, cells should be cultured in Neurosphere Media and routinely passaged by dissociating the cells. Neurosphere Media contains Neurocult with the following additives: 10% Proliferation Supplements, 20 ng/mL EGF, 10 ng/mL bFGF, 2 μg/mL Heparin.

Note: If using the cells for FACS, do not combine with the supernatant; if using for cell culture, you can combine with the supernatant, but change media out soon after to get rid of all the dead cells/debris in your culture.

Note: For continuous culture as adherent cells, brainstem glioma cell lines can be passaged using Adherent Cell Media, which is identical to DF1 cell media (DMEM 10% FBS 1% Pen/Strep 1% L-Glutamine) using the same techniques to continuously grow, trypsinize, and passage the cells.

Optional step: Polymerase chain reaction (PCR)

Timing: Approximately 6 h

This section describes the procedure for conducting a polymerase chain reaction and gel electrophoresis on genomic DNA isolated from mouse tails or from cell lines derived from the mouse tumors. This procedure is used to genotype mice and cell lines to validate them for breeding or experiments. This can also be applied to cell lines derived from the mouse tumors to confirm floxed allele recombination by using PCR probes that yield specific products in the presence of recombined or non-recombined genomic DNA.

58. Isolate DNA from mouse tails.
    a. Cut mouse tail about 2–3 mm long and place in 1.5 mL Eppendorf tube.
    b. Add 500 μL of Lysis buffer and 8 μL Proteinase K solution, mix well.
    c. Incubate in 55°C–60°C oven for 3 h.
    d. Vortex for 10 s and spin for 3 min at 13,000 × *g* in centrifuge.
    e. Add 1,000 μL of 100% EtOH and mix by inverting three times.
    f. Spin for 5 min at 13,000 × *g* in centrifuge.
    g. Pour out supernatant and air-dry DNA for 30 min.
    h. Add 200 μL–300 μL dH2O to each DNA tube.
    i. Incubate in 55°C–60°C oven for 20 min.
    j. Vortex for 10 s and spin DNA for a second in centrifuge, then add 1.5 μL–2 μL for PCR.
    k. Store DNA at 4°C.
59. Run PCR amplifications.
    a. Add appropriate mix and DNA to PCR vials. See "PCR reaction master mix" for recipe.
    b. Run PCR vials in thermocycler. See "PCR cycling conditions" for PCR program.
60. Prepare electrophoresis gel.
    a. Prepare 1% agarose in TAE buffer in an Erlenmeyer flask.
    b. Microwave the flask for 70 s. If the agarose has not fully dissolved, microwave the flask for an additional 25 s.

⚠ CRITICAL: Use protective gloves when handling hot flasks.

c. Once agarose has fully dissolved, add 8–10 μL of SYBR safe to the solution.
d. Add the appropriate well combs to the gel mold.
e. Gently pour the agarose SYBR safe solution into the mold.
f. Cool for 40 min for the gel to solidify.

Note: The gel can be placed at 4°C to cool more rapidly.

g. Carefully remove combs.
h. Remove gel from mold.

61. Prepare PCR vials for gel electrophoresis.
    a. Add 8 μL of 5× orange gel loading dye to each PCR vial.
    b. Flick vials to mix the contents.
    c. Spin down the vials to bring liquids to the bottom.
62. Place gel in electrophoresis chamber filled with 1× TAE.
63. Add 15 μL of a ladder solution to the first well of each row.
64. Add 12 μL of the contents of each PCR vial to a well.
65. Run the gel at 120 V for 25 min.

Optional: You can add two combs to the gel mold.

Note: Amount of agarose added to TAE buffer changes depending on the size of the DNA (longer DNA requires smaller agarose ratio).

Pause point: After thermocycling, PCR vials can be kept at 4°C for 24 h.

PCR reaction master mix

Reagent	Amount
DNA template	1–2 μL
Taq DNA Polymerase	0.3 μL
Primer 1 (10 μM)	0.5 μL
Primer 2 (10 μM)	0.5 μL
10× LA buffer	2.6 μL
dNTPs	4.2 μL
ddH_2O	16.4 μL

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	1 min	1
Denaturation	98°C	10 s	35 cycles
Annealing	60°C	15 s	
Extension	68°C	2 min	
Final extension	72°C	5 min	1
Hold	4°C	forever	

Optional step: Droplet digital PCR to assess recombination efficiency

Timing: Approximately 7 h

This section describes the procedure for conducting a droplet digital polymerase chain reaction (ddPCR). This procedure quantifies the percentage of cells that exhibit recombination of the floxed allele of genes of interest. This can be used to validate that the majority of tumor cells exhibit expected gene recombination.

66. Extract genomic DNA from flow-sorted primary glioma cells using PicoPure DNA extraction kit (Applied Biosystems.).
67. Design hexachloro-fluorescein and fluorescein amidite-conjugated TaqMan probes.
68. Perform droplet digital PCR using QX200 Droplet Digital PCR System (Bio-Rad.).
    a. Dilute Genomic DNA to 26 ng/μL.
    b. Prepare master mix. See "ddPCR master mix".
    c. Generate droplets using the QX200 Automated Droplet Generator according to manufacturer's protocol.
    d. Perform PCR amplification on generated droplets.
    e. Analyze data.
        i. Use QuantaSoft™ Software (Bio-Rad) for data analyzation.

Pause point: After thermocycling, PCR vials can be kept at 4°C for 24 h.

ddPCR master mix

Reagent	Amount
DNA template	1 μL
2× DreamTaq Green PCR Master mix (Thermo Scientific)	10 μL
Primer 1 (10 μM)	1 μL
Primer 2 (10 μM)	1 μL
ddH_2O	7 μL

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	3 min	1
Denaturation	94°C	30 s	25–35 cycles
Annealing	56°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	5 min	1
Hold	4°C	forever	

EXPECTED OUTCOMES

Confirmation of successfully induced brainstem tumors in the genetically-engineered mice is essential. A mouse with a positive tumor signal detected through *in vivo* bioluminescence imaging is shown (Figure 7). For the animal models described in this protocol, brainstem tumors are anticipated to occur between four to fifteen weeks post-injection with transfected DF1 cells (Figure 8). Depending on the experimental parameters, we often sacrifice mice at 12–15 weeks post-injection if there is no evidence of tumor formation.

Once tumors are generated, mice can be subjected to treatments such as focal brain irradiation (described above) or pharmacologic treatments to examine the effect of these perturbagens on primary mouse brainstem gliomas. Further, mice can be assayed for overall survival, which reflects local tumor progression. Parallel cohorts of mice can be used to collect tumors for immunohistochemical analyses to confirm PDGF-B expression *via* an anti-HA epitope tag, and to confirm functional deletion of floxed alleles using antibodies against their protein product (for instance, anti-p53 in mice containing $p53^{fl/fl}$). Immunohistochemistry, RNA-seq, quantitative PCR, and myriad other techniques can also be applied to characterize tumors and to examine the effects of specific perturbagens. Tumor cell lines derived from the brainstem gliomas provide a renewable model for *in vitro* studies. These molecular tumor analyses are outside the scope of this protocol for mouse brainstem glioma generation.

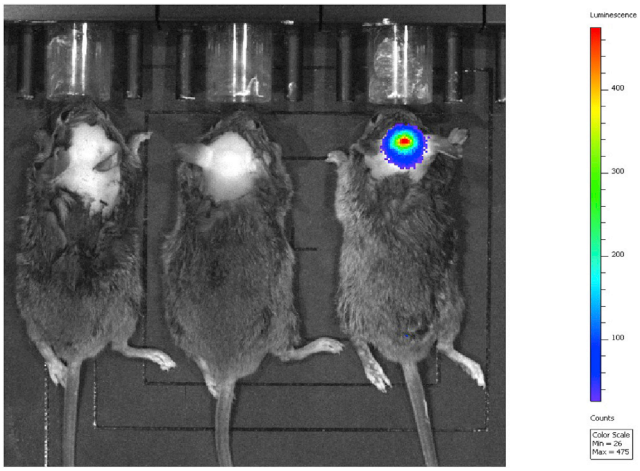

Figure 7. Brainstem tumor detected with bioluminescence imaging

Photograph of mice intraperitoneally injected with D-Luciferin solution and placed on the stage inside the IVIS Lumina III In Vivo Imaging System. Mice without brainstem tumors (leftmost and middle) and a mouse with a brainstem tumor (rightmost) are shown. A maximum luminescence signal of 100 was the designated threshold value for positive tumor detection.

LIMITATIONS

The methods described in this protocol do not result in brainstem tumors in 100% of the experimental mice. In some cases, mice injected with virus-producing DF1 cells may develop brain tumors that do not originate in the brainstem. For instance, we have observed tumors centered in the thalamus, cerebellum, or in the leptomeningeal space (Figure 9). In our *in vivo* experiments, about 20%–30% of all mice injected with virus-producing DF1 cells developed tumors outside of the brainstem. Therefore, mice that develop such tumors may need to be excluded from studies depending on the experimental design.

Some genetically-engineered mice injected with DF1 cells transfected with the RCAS-Cre, RCAS-Luc, and RCAS-PDGFB will develop tumors that do not express luciferase. This likely happens

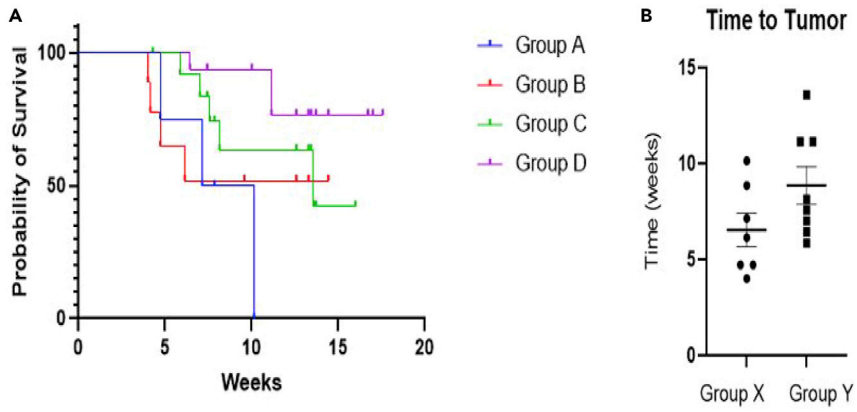

Figure 8. Example data plots

(A) Example plot of Kaplan-Meier curves tabulating the probability of mouse survival post injection with transfected DF1 cells. Groups A-D are four different genotypes containing different combinations of inducible conditional deletions of p53 and *Atm* alleles.

(B) Example dot plot tabulating the time to tumor detection post injection with transfected DF1 cells. Groups X and Y are two different genotypes containing different combinations of inducible conditional deletions of p53 and *Atm* alleles. Data are represented as mean ± SEM.

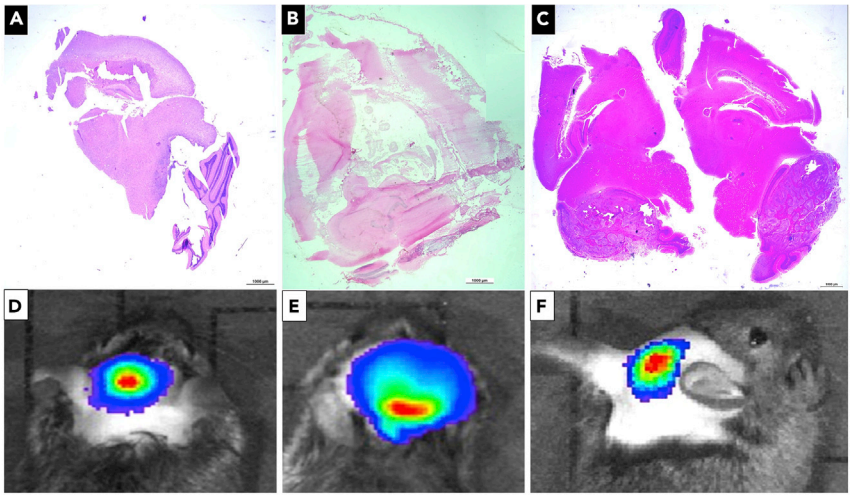

Figure 9. Images of tumors formed inside and outside the brainstem

Bioluminescence and H&E images of mouse brains whose tumors developed either inside or outside of the brainstem.
(A) H&E stain of a mouse brain with a tumor in the brainstem. Tumor location indicated by black circle.
(B) H&E stain of a mouse brain whose tumor developed in the leptomeningeal space.
(C) H&E stain of a mouse brain with a tumor in the cerebellum. Tumor location indicated by black circle.
(D) Bioluminescent image of the tumor displayed in panel A.
(E) Bioluminescent image of the tumor displayed in panel B.
(F) Bioluminescent image of the tumor displayed in panel C. Scale bars = 1,000 μm.

when the tumor cells do not take up the RCAS-Luc plasmid. Therefore, the tumors in these mice will not display a luminescent signal in the presence of luciferin.

TROUBLESHOOTING

Problem 1

Low transfection efficiency.

Potential solution

Increase plasmid concentration and/or plasmid purity.

Problem 2

No cell pellet present.

Potential solution

Spin the cells again. If this does not work, then harvest cells when they are at a higher confluency.

Problem 3

Luciferin is coming out of the injection site.

Potential solution

Hold the syringe at a slightly larger angle so that the needle is not too shallow in the mouse skin.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Zachary J. Reitman (zjr@duke.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102094.

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AUTHOR CONTRIBUTIONS

L.B.W. wrote the manuscript. H.Q.L. wrote the PCR and ddPCR sections. L.L., N.W., K.D., and D.G.K. provided critical feedback. Z.J.R. revised the manuscript.

DECLARATION OF INTERESTS

D.G.K. is a cofounder of and stockholder in XRAD Therapeutics, which is developing radiosensitizers. D.G.K. is a member of the scientific advisory board and owns stock in Lumicell Inc, a company commercializing intraoperative imaging technology. None of these affiliations represents a conflict of interest with respect to the work described in this manuscript. D.G.K. is a coinventor on a patent for a handheld imaging device and is a coinventor on a patent for radiosensitizers. XRAD Therapeutics, Merck, Bristol Myers Squibb, and Varian Medical Systems provide research support to DGK, but this did not support the research described in this manuscript. Z.J.R. receives honoraria from Oakstone publishing and Eisai Pharmaceuticals for educational seminars. Z.J.R. receives royalties for intellectual property that is managed by Duke Office of Licensing and Ventures which has been licensed to Genetron Health. These sources did not support the research described in this manuscript.

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