Brainstem gliomas are aggressive tumors that are more prevalent in pediatric patients. The location of these tumors makes them inoperable, and currently there is no effective treatment. Recent genomic data revealed the unique biology of these tumors. The following protocol provides a method to incorporate these specific genetic lesions in a mouse glioma model. Using this model, the effects of these mutations in tumor progression and response to treatments can be studied within a relevant in vivo context.

**HIGHLIGHTS**

We describe a method to induce spontaneous brainstem high-grade gliomas in mice.

Genetic drivers can be selected to recapitulate human pediatric brainstem lesions.

Tumors develop in an immunocompetent host, providing a relevant preclinical model.

Cells isolated from the tumors can be implanted, generating a transplantable model.

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SUMMARY

Brainstem gliomas are aggressive tumors that are more prevalent in pediatric patients. The location of these tumors makes them inoperable, and currently there is no effective treatment. Recent genomic data revealed the unique biology of these tumors. The following protocol provides a method to incorporate these specific genetic lesions in a mouse glioma model. Using this model, the effects of these mutations in tumor progression and response to treatments can be studied within a relevant \textit{in vivo} context.

For complete details on the use and execution of this protocol, please refer to Mendez et al. (2020).

BEFORE YOU BEGIN

The Sleeping Beauty (SB) transposase system was used to generate a murine model of brainstem high-grade glioma (Mendez et al., 2020). Plasmids are constructed to encode oncogenic drivers and the genetic alterations of interest are flanked by inverted and directed repeat (IR/DR) sequences. (Refer to Calinescu et al., 2015; Koschmann et al., 2016; Nunez et al., 2019 and Mendez et al., 2020 for additional information regarding the plasmids used and the cloning procedures).

The constructs are injected into the fourth ventricle of neonatal mice with an additional plasmid encoding the SB transposase and luciferase (Figure 1). After injection, the SB transposase recognizes the IR/DR sequences flanking the transposons and facilitates their stable integration into the host genome by a “cut and paste” mechanism (Wiesner et al., 2009; Calinescu et al., 2015) (Figure 1B). The luciferase enzyme allows for monitoring of plasmid uptake and integration and of tumor growth through bioluminescent imaging (Figure 1D). Additionally, plasmids are designed so that a unique fluorescent protein is expressed concurrently with each gene of interest. Tumors will form in the brainstem and animals will reach endpoint between 2–6 months post injection, depending on the genetic alterations used. Furthermore, the SB induced tumors arise \textit{de novo} and demonstrate the histological characteristics of brainstem high-grade glioma (Figure 2C). At endpoint stage, the tumors can be harvested and used to generate primary 3D cell cultures of tumor neurospheres. Furthermore, neurospheres derived from the sleeping beauty induced tumors can be orthotopically implanted in mice. The genetic characteristics of implanted cells and implantation location can be tailored to provide the most relevant anatomical context (e.g., Implanting H3.3K27M-mutant cells in the brainstem). This provides a consistent preclinical platform to study tumor initiating capacity, progression, histopathology, survival, immune microenvironment, and treatment efficacy \textit{in vivo}.
Establish Breeding Cages

**Timing:** 20–30 days (operational time 1 h)

1. Establish a breeding cage 3–4 weeks prior to date of SB plasmid injections. Mice should be between 4–6 months of age.
2. Confirm pregnancy of female and arrange a separate cage to house the male mouse.
3. Monitor female mouse daily throughout gestation until pups are born.
4. Proceed with the SB injection protocol at post-natal day 1.

**Preparation of SB Plasmids for Brainstem High-Grade Glioma**

**Timing:** 1 h

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**Figure 1. Sleeping Beauty Transposase Model of Brainstem High-Grade Glioma in Mice**

(A) Plasmid maps used to generate the brainstem glioma model: (i) base plasmids for the SB model, (ii) plasmids used for modeling brainstem glioma expressing mutant histone H3.1 K27M with co-occurring mutation in ACVR1, (iii) plasmid used for modeling a brainstem glioma with histone H3.3 K27M mutation, (iv) plasmid used for modeling a brainstem high-grade glioma with mutant PDGFRA D842V. DNA transposons containing genes of interest, and fluorescent proteins (BFP, GFP, Katushka) are flanked by inverted/direct repeat (IR/DR) sequences.

(B) The Sleeping Beauty transposase recognizes the IR/DR sequences and then integrates the transposon into the host’s chromosomal DNA between thymine (T) and adenine (A) bases.

(C) Schematic of a post-natal day 1 mouse pup demonstrating the coordinates for SB plasmid injection targeting the fourth ventricle (3 mm posterior to the lambda suture at a depth of 3 mm).

(D) Bioluminescence imaging of a mouse pup 24-h post SB plasmid injection.
5. Mix plasmid DNA with in vivo Jet-PEI transfection reagent for injection into the fourth ventricle of neonatal mice.

△ CRITICAL: Plasmid DNA solutions must be sterile, endotoxin free and highly concentrated (2–7 μg/μL) for a minimal injection volume. (To generate high quality-concentrated plasmid preparations, we recommend to use a maxiprep column-based kit, such as QIAGEN Plasmid Maxi Kit [cat. nos. 12162, 12163, and 12165].) A maximum of five plasmids can be introduced per Jet-PEI solution.

a. Plasmid DNA preparation: Mix the SB transposase and luciferase plasmid preparations (pT2/SB100x-Luc) with the plasmids containing oncogenic DNA or genes of interest (pT2-GeneX) at a mass ratio of 1(pT2/SB100x-Luc):2(pT2-GeneX). Additionally, the total mass of plasmid should be adjusted to 20 μg of total plasmid DNA in 40 μL plasmid mixture. For
example, for a mix containing four plasmids: 2.85 μg of pT2/SB100x-Luc, 5.71 μg pT-Caggs-NRASV12, 5.71 μg of pT2-shP53-GFP4 and 5.71 μg of pKT-H3.1-K27M-ires-Katushka (Figure 1A).

b. In vivo transfection reagent preparation: Calculate the appropriate amount of Jet-PEI transfection reagent using the formula below.

\[
\text{In vivo Jet-PEI (μL)} = \frac{\left(3 \times 10^6 \text{ μg DNA}\right) \times (N/P \text{ ratio})}{150}
\]

The N/P ratio represents the optimal ionic proportions of nitrogen residues (N) in the polyethyleneimine (PEI) solution to the phosphate residues (P) in the plasmid DNA. The mass of plasmid DNA is multiplied by 3 to account for the nanomoles of anionic phosphate per micromole of nucleic acid. The product of the numerator in the equation above is then divided by 150 (mM), which is the concentration of the nitrogen residues in the in vivo Jet-PEI solution. The optimum N/P ratio for in vivo transfections is between 6 and 8. Add sterile glucose to achieve a final concentration of 5% (w/v).

c. Pipet to mix DNA and Jet-PEI solutions, vortex, spin down for 5 s on a bench microcentrifuge (~500 x g), and maintain mixture at room temperature (24°C) for 20 min prior to injection.

6. Prepare the stereotaxic frame to be used for injection of SB plasmids.
   a. Fit a sterile 10 μL syringe with a 30-gauge hypodermic needle into the micropump.
   b. Verify that the injection system is functioning properly by drawing up and then releasing 10 μL water from the syringe.
   c. Use a slurry of dry ice in alcohol to cool the stereotactic stage to 2°C–8°C.

7. Prepare a bucket of ice to induce anesthesia by hypothermia on the pups.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Experimental Models: Organisms/Strains** | | |
| Mouse (C57BL/6J) | Jackson Laboratory | Cat# 000664 |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| Accutase | Innovative Cell Technologies, Inc. | Cat # AT1104 |
| Anti-Anti (100×) | Corning | Cat# 30-004-C1 |
| B27 Supplement (50×) | Thermo Fisher Scientific - Gibco | Cat# 17504044 |
| D-Luciferin Potassium Salt | Goldbio | Cat# LUCK-3G |
| DMEM/F12 | Thermo Fisher Scientific - Gibco | Cat# 11330032 |
| DPBS, no calcium, no magnesium | Thermo Fisher Scientific - Gibco | Cat# 14190144 |
| Epidermal growth factor (EGF) | PeproTech | Cat# AF-100-15 |
| Fibroblast growth factor (FGF) | PeproTech | Cat# 100-188 |
| In vivo Jet-PEI (with 10% glucose solution) | Polypus | Cat# 201-10G |
| N2 Supplement (100×) | Thermo Fisher Scientific - Gibco | Cat# 17502048 |
| Normocin | InvivoGen | Cat# ant-nr-1 |
| Tyrode’s salts | SIGMA-ALDRICH | T2145 |
| Sodium bicarbonate | SIGMA-ALDRICH | SB761 |
| Heparin | SIGMA-ALDRICH | H5515 |
| Ketamine (Ketaset) | MWI Animal Health | #000680 |

(Continued on next page)
## REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Dexmedetomidine HCl | Zoetis | NADA 141-267 |
| Carprofen           | Zoetis | NADA 141-199 |
| Sodium Chloride 0.9% 10 mL | MWI Animal Health | #033500 |
| Buprenorphine       | MWI Animal Health | #060969 |

### Recombinant DNA

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| pT2-SB100x-Luc      | Addgene| Plasmid #20207 |
| pT-Caggs-NRASV12    | Addgene| Plasmid #20205 |
| pT2-shP53-GFP4      | Addgene| Plasmid #124261 |
| pKT-ACVR1-G328V-IRES-Katushka | N/A | N/A |
| pKT-H3.1-K27M-IRES-Katushka | N/A | N/A |
| pLVX-3X-FLAG-H3.3K27M-IRES-BFP | N/A – Generated in laboratory | N/A |
| pLVX-3X-FLAG-H3.3WT-IRES-BFP | N/A – Generated in laboratory | N/A |

### Other

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Syringe (10 μL)     | Hamilton| 700 Series |
| Hypodermic needle (30-gauge) | Hamilton | Cat# 7803-07 |
| Cell strainer (70 μm) | Alkali Scientific | Cat# MT4070 |
| Vacuum Filtration Flasks PES membrane | CellPro | V05022 |
| Plastic pestle      | Alkali Scientific | Cat# MT4010 |
| Just for Mouse Stereotaxic Instrument | Stoelting | Cat# 51730 |
| Quintessential Stereotaxic Injector (QSI) | Stoelting | Cat# 53311 |
| Small Animal Heat Lamp | Morganville Scientific | Cat# HL0100 |
| SZX7 Stereomicroscope System (with fluorescence unit) | Olympus | n/a |
| Covidien Monoject tuberculin syringe (1 mL) | Fisher Scientific | Cat# 22-257-154 |
| IVIS Spectrum In Vivo Imaging System | Perkin Elmer | Cat# 124262 |
| Friedman Bone Rongeurs (5.5 inch, curved, delicate) | Stoelting | Cat# 52160P |
| Micro Dissecting Scissors (4.25 inch, straight, 24 mm, sharp) | Stoelting | Cat# 52132-22P |
| Brown-Adson Forceps (4.7 inch, side teeth) | Stoelting | Cat# 52104-31P |
| Premium polypropylene sutures, size 4-0 | AD Surgical | Cat# PSP-418R13 |
| Fisherbrand Gauze Sponges (non-sterile, 4 ply) | Fisher Scientific | Cat# 22-246069 |
| Kimwipes            | Fisher Scientific | Cat# 06-666A |
| Colibri Retractors  | Fine Science Tools | Cat# 17000-03 |
| Webster Needle Holder, 5 inch, Delicate, Smooth | Stoelting | Cat# 52122-90P |
| Povidone Iodine swab | Fisher Scientific | Cat# NC0436628 |
| Rodent Warmer X1 with Mouse Heating Pad | Stoelting | Cat# 53800M |
| Cordless Micro Drill | Stoelting | Cat# 58610 |
| Precise Trim, Cordless/Corded trimmer w/#40 blade | Stoelting | Cat# 51472 |
| Surgical Blades (scalpel No.15) | Fisher Scientific | Cat# 14-840-24 |
MATERIALS AND EQUIPMENT
Luciferin Solution for Tumor Monitoring

| Reagent                  | Final Concentration | Amount |
|--------------------------|---------------------|--------|
| Luciferin                | 30 mg/mL            | 1 g    |
| DPBS (without Ca\(^{2+}\) or Mg\(^{2+}\)) | 1×                  | 33 mL  |

Prepare 1 mL aliquots of Luciferin and store at −80°C. The preparation is stable for up to 1 year.

Neural Stem Cell Media for Culturing Brainstem High-Grade Glioma Neurospheres

Neurosphere (NS) Media Base

| Reagent           | Final Concentration | Amount |
|-------------------|---------------------|--------|
| DMEM/F12          | –                   | 480 mL |
| Anti-Anti 100×     | 1×                  | 5 mL   |
| N2 supplement 100× | 1×                  | 5 mL   |
| B27 supplement 50× | 1×                  | 10 mL  |
| Normocin          | 100 μg/mL           | 1 mL   |

NS Media Growth Factors

| Reagent                  | Final Concentration | Amount |
|--------------------------|---------------------|--------|
| Epidermal growth factor (EGF) | 20 ng/mL           | –      |
| Fibroblast growth factor (FGF) | 20 ng/mL           | –      |

Prepare 500 mL of NS media and store at 4°C. The base media can be stored for up to 4 months. Immediately prior to tumor harvesting, prepare 50 mL of complete NS media supplemented EGF and FGF. Aliquot 5 mL of complete NS media for tumor collection and use remaining media for tumor dissociation.

Tyrode's Solution

| Salt             | Final Concentration |
|------------------|---------------------|
| NaCl             | 137 mM              |
| KCl              | 2.7 mM              |
| MgCl\(_2\)       | 1 mM                |
| CaCl\(_2\)       | 1.8 mM              |
| Na\(_2\)HPO\(_4\) | 0.2 mM              |
| NaHCO\(_3\)      | 12 mM               |
| D-glucose        | 5.5 mM              |

Preparation: Resuspend the content of Tyrode’s Salts to a volume of 900 mL with ultrapure water. Add 1.0 g sodium bicarbonate. Adjust pH to 7.4 with of 1 N HCl or 1 N NaOH. Add additional water to bring the solution to 1 L. Sterilize immediately by filtration using a membrane with a porosity of 0.22 μm.

STEP-BY-STEP METHOD DETAILS
Injection of SB Plasmids into the Fourth Ventricle of Neonatal Mice

© Timing: 1–1.30 h/10 mice

This protocol is adapted from Mendez et al., 2020 and has been used by our laboratory to generate reproducible models of brainstem high-grade glioma. This non-viral method induces tumors in situ via Sleeping Beauty transposase mediated integration of plasmid DNA into the host genome (Figure 1A). To generate high-grade brainstem glioma, plasmids encoding DNA transposons, encoding
the SB transposase and luciferase, fluorescently labeled oncogenic drivers and genes of interest are injected into the fourth ventricle of post-natal day 1 pups (Figure 1). The resulting tumors exhibit the salient features of brainstem high-grade gliomas.

1. Load syringe with 10 μL of plasmid DNA/Pei solution prepared previously.
2. Anesthetize first pup prior to plasmid injection by hypothermia. Wrap the pup in gauze and place it on ice for 2 min. Anesthesia will continue on the cooled stereotaxic frame throughout the procedure.
   
   **△ CRITICAL:** Maintain the temperature of the frame above freezing (2°C–8°C) to prevent burn injury to the pups. Use Parafilm to prevent direct contact of the pups with the metallic frame.

3. Place pup on pre-cooled stereotaxic stage and immobilize head using the gauze covered ear bars.

   **△ CRITICAL:** Ensure the pup’s head is secured by the ear bars and is parallel to the surface of the stage.

4. Disinfect injection surface by wiping the pup’s head with 70% ethanol.
5. Lower the needle to injection surface and adjust the stereotaxic coordinates to target the fourth ventricle (3 mm posterior to the lambda suture; Figure 1C).
6. Lower needle further until a dimple has formed on the skin and record the coordinates.
7. Lower the needle to a depth of 4 mm, and then raise the needle 1 mm to get to a depth of 3 mm below the skull.
8. Inject 0.75 μL plasmid solution using the automatic injection at a rate of 0.5 μL/min.
9. After the injection is completed, leave needle in place for 1 min.
10. Slowly raise the needle from the skull.
11. Remove the pup from the stereotaxic frame, and place it under a heating lamp. Monitor pup’s recovery to ensure normal breathing, mobility, and rosy color have returned (5–7 min). Gentle lung massage and limb stimulation can be performed to aid in the recovery.

   **△ CRITICAL:** To ensure pup survival, it is important to limit the length of time between anesthesia and post-injection warm up. If the time between hypothermia and heating lamp less than 10 min, survival of the pups is typically 100%.

12. Repeat steps above to proceed with remaining injections.
13. Return pups to mother’s cage and monitor her response for 30 min to ensure nurturing behavior resumes.

   **△ CRITICAL:** The mother is typically accepting of the pups after the injection procedure. However, a surrogate mother can be added to the cage if feeding behavior does not resume.

14. Return cage to vivarium.

   **△ CRITICAL:** The plasmid encoding for the SB transposase (pT2/SB100x-Luc) also harbors a transposon that mediates Luciferase gene genomic integration. Once integrated into the host’s genome (24 h post injection) the expression of the Luciferase enzyme allows for tumor monitoring by bioluminescent imaging (Figure 1D) (Calinescu et al., 2015; Koschmann et al., 2016; Nunez et al., 2019; Mendez et al., 2020). Frequently, for a successful injection procedure, we detect luciferase signal on >80% of the injected mice after 24 h post injection.
15. Monitor plasmid uptake in pups using an IVIS bioluminescence imaging system (Figure 1D).
   a. Remove pups from the mother’s cage 24–48 h after injection. Anesthetize pups prior to imaging by hypothermia. Wrap the pups in gauze and place them on ice for 2 min. After this, place pups individually into wells of a sterile 6-well tissue culture plate.
   b. Use a 1 mL syringe with a 30-gauge hypodermic needle to subcutaneously inject 10 μL of luciferin (30 mg/mL) between pup’s shoulder blades. Pinch and lift the skin gently prior to injection to prevent penetration of organs.
   c. Wait for 5 min to allow for luciferin to penetrate the brain.
   d. Image pups immediately on IVIS bioluminescence imaging system (IVIS Spectrum settings: automatic exposure, large binning, aperture f = 1).

\[ \text{\textcopyright CRITICAL: Plasmid uptake must be verified no later than 24 h post injection, as the process relies on the detection of transient expression. The transient expression decreases after 24 h, and the growth of fur on the head of the pups makes it more difficult to detect the luciferase expression at time points later than 24 h post injection.} \]

16. Monitor tumor formation and progression using IVIS bioluminescence imaging system. According to the model and the genetic lesions incorporated, tumors will appear between 2 months and 4 months post injection of the plasmids (Figure 2A).
   a. Place animals in chamber with flowing oxygen and isoflurane (1.5%–2.5% isoflurane, with an oxygen flow of 1.5 L/min [LPM]) to anesthetize.
   b. Remove mouse from chamber and use a 1 mL syringe with a 26-gauge needle to intra-peritoneally inject 100 μL of luciferin (30 mg/mL in PBS or saline). Set a timer for 5 min. Up to five animals may be injected at a time.
   c. Place animal back into anesthesia chamber for 3–4 min.
   d. Initiate flow of oxygen and isoflurane (1.5%–2.5% isoflurane, with an oxygen flow of 0.25 LPM) to the bioluminescence chamber.
   e. Position animals into imaging slots and glass nose cones in the bioluminescence chamber.
   f. When the timer sounds, obtain a series of six images at 2-min intervals (IVIS Spectrum settings: automatic exposure, median binning, open aperture f = 1).

17. For each animal imaged, define the area of interest (placing the oval over the head) and measure the intensity of luminescence using the calibrated units (photons/s/cm²/sr) (Figure 2A).

**Generation of Primary Neurospheres from Brainstem High-Grade Glioma**

\[ \text{\textcopyright Timing: 1 h} \]

Sleeping beauty induced tumors can be harvested and used to generate stable primary cell cultures that harbor the key mutations of high-grade brainstem glioma. These tumor neurospheres (NS) represent a valuable model of high-grade brainstem glioma and facilitate the study of the biology of brainstem tumors in vitro and in vivo (Figure 2B).

18. Monitor tumor bearing animals for signs of tumor burden.

\[ \text{\textcopyright CRITICAL: The specific genetic alterations delivered to the brainstem will determine the length of time before animals display neurological deficits as a symptom of tumor burden. For SB induces brainstem tumors symptoms of morbidity include hunched posture, impaired mobility, scruffy fur, as well as ataxia and seizures.} \]

19. Euthanize tumor bearing mice by transcardial perfusion with Tyrode’s solution supplemented with Heparin (15 Units/L).
CRITICAL: Given their location, brainstem tumors can be relatively small at the time mice demonstrate significant signs of tumor burden. It is important to preserve as much tumor tissue as possible when generating NS cultures; therefore, be extremely careful and precise when dissecting to preserve the tumor tissue.

20. Decapitate mouse and gently dissect brain from skull.
21. Place brain in sterile Petri dish resting on damp ice.
22. Visualize tumor using fluorescent dissecting stereo microscope (e.g., Olympus SZX16) at the appropriate wavelength for fluorescent markers used in SB plasmid mixture.
23. Use a scalpel to isolate fluorescent tumor from surrounding tissue.
24. Transfer tumor into a 1.5 mL microcentrifuge tube containing 300 μL of neural stem cell media.
25. Use plastic pestle to dissociate mechanically the tumor tissue.
26. Add 10 mL of neural stem cell media to the dissociated tumor suspension and filter it through 70 μm strainer.
27. Centrifuge at 2,000 × g for 2 min. Wash pellet twice with 1 mL of Dulbecco’s phosphate-buffered saline (DPBS) (without Ca²⁺ or Mg²⁺), resuspending by pipetting and centrifuging at 2,000 × g for 2 min in each wash.
28. Incubate tumor suspension with 1 mL of pre-warmed (37°C) Accutase. Incubate for 2–3 min at 37°C.
29. Add 10 mL of DPBS to the cells suspension and centrifuge cells at 2,000 × g for 2 min. Discard supernatant.
30. Resuspend cells in 1 mL of NS complete media. After resuspension, add NS complete media (supplemented with EGF and FGF to final concentration of 20 ng/mL) to a final volume of 10 mL. Plate cells in a T25 flask and culture at 37°C, 5% CO₂ in neural stem cell media. For the first 2 weeks, centrifuge the neurospheres (500 × g, 2 min), resuspend with 10 mL NS media supplemented with growth factors to a final concentration of 20 ng/mL (FGF and EGF) and replate to a new T25 flask twice a week.
31. Monitor cells for formation of neurospheres. Once visible, collect spheres (discard freely floating single cells or cells adhering to flask), passage, and replate.
   a. Size of flask will depend on volume of cells recovered at this time.
   b. If neurospheres are small or not forming readily, change media and replace growth factors as needed until well formed spheres appear.
32. Maintain cells in culture. Passage and expand cells as needed when flasks contain neurospheres and media becomes orange/yellow. Use cells for implantation and other experiments at a passage below 10, since over-passaging can lead to accumulation of genetic rearrangements.

**Orthotopic Implantation of Glioma Neurospheres to Generate Brainstem High-Grade Glioma**

**Timing:** 1–1.5 h

Neurospheres from the SB tumors can be implanted orthotopically to generate a 100% penetrant and reproducible model of brainstem high-grade glioma located in the correct anatomical location (Figure 3). This is important because the brain has unique components including the blood-brain barrier, cellular microenvironment, and tissue/organ architecture.

33. Take a 70%–90% confluent T-75 flask of neurospheres and transfer to a 15 mL conical tube with a serological pipet. Centrifuge at 350 × g for 5 min.
34. Remove supernatant and resuspend cell pellet in 1 mL of pre-warmed (37°C) Accutase. Incubate for 2–3 min at 37°C.

CRITICAL: Make sure neurospheres are properly dissociated by gently pipetting the solution post incubation, leave cells at 37°C longer if not dissociated. Additionally, cells can be filtered through a 70 μm strainer to discard cell clumps.
35. Add 9 mL of PBS and centrifuge at 1,500 rpm (~350 g) for 5 min to wash cells.

36. Remove supernatant and resuspend in 100 μL PBS.

⚠️ CRITICAL: For higher implantation concentrations, it may be necessary to resuspend in a smaller volume.

37. Count cells and dilute to the proper concentration with PBS in a 0.6 mL Eppendorf tube for implantation (e.g., 30,000 cells/μL if implanting 30,000 cells). Maximum injection volume is 5 μL. Keep cells on ice (4°C).

⚠️ CRITICAL: Higher concentrations increase the likelihood of clogging the injection needle. Limit to a concentration of 50,000 cells/μL.
38. To maximize reproducibility, mice implanted should be 6–8 weeks of age. Anesthetize mouse.
   a. Intraperitoneally inject mouse with mixture of ketamine (120 mg/kg) and dexmedetomidine
      (0.5 mg/kg), dissolved in sodium chloride 0.9% solution
   b. Wait until animal is properly sedated.

∆ CRITICAL: When sedated, mouse should not be responsive to pain stimuli (e.g., foot pinch)
and breathing should slow down. Sedation parameters should be checked throughout sur-
gery. Ketamine can be re-dosed if necessary, using half the original dose. It should not be
mixed with dexmedetomidine.

39. Shave top of head with animal hair clippers.
40. Inject each mouse intraperitoneally with carprofen (5 mg/kg) dissolved in sodium chloride 0.9%
solution.
41. Place mouse on stereotaxic frame.
   a. Set mouse on platform with heat support (should be set to around 37°C).
   b. Open mouth with forceps.
   c. Place mouth over the bite bar, making sure front teeth go through hole on bar and pull
tongue to the side.
   d. Press ear bars lightly against side of head and adjust so they have the same reading. After
      adjusting, they should be similar lengths and be pressing with enough pressure to stabilize
      head.
   e. Tighten nose clamp (Not too hard, until you feel some resistance).
42. With a cotton swab, apply iodine to the top of head and wipe off. Repeat three times, after the
   last application wait 5 min before wiping off.
43. Make 0.25 cm incision on top of skull with scalpel.
44. Using forceps, carefully pull back skin from incision and insert retractors.

∆ CRITICAL: Make sure that skull is in the correct orientation. To do this move needle along
the sagittal and coronal sutures (raised slightly above the skull surface so it does not touch
it). The needle movement should be aligned (in parallel) with the sagittal and coronal suture
lines, and maintaining the same distance to them along its movement.

45. Locate the lambda coordinate and move the needle slightly above this location. Lambda can be
visualized easier if the skin is slightly dry and by pressing down lightly on top of skull near the
suture.
46. From the lambda coordinate, move the needle 1 mm posterior and 1 mm lateral to the target
site (Figure 3A).
47. Mark the location with a tuberculin syringe equipped with a 26-gauge needle by gently scraping
the peristomeum.
48. Raise Hamilton syringe and drill through the skull using a #7 or #8 bit connected to a Dremel drill
at marked location (1 mm posterior, 1 mm lateral from lambda). Drill until you reach the dura
mater.

∆ CRITICAL: When drilling, use gentle pressure and take breaks to make sure area does not
get too hot.

49. Load the syringe with cell suspension.
   a. Flick or gently pipette tube with tumor cells to resuspend cells.
   b. Draw up cells (volume depends on concentration and number of cells to be injected).
   c. Eject 1 μL of volume to ensure that there is no air in syringe and that cells can be dispensed
      properly.
**CRITICAL:** Prior to loading, flush injection syringe with PBS to ensure that it is not clogged and dispenses volume correctly. When loading cell suspension, make sure to draw more volume than needed (e.g., if injecting 1 μL, draw at least 3 μL into syringe). Maximum injection volume per mouse is 3 μL.

50. Lower the syringe to target site until tip barely touches the dura mater.
51. Lower the syringe 5 mm inferior to the dura mater.
52. Raise syringe 0.5 mm (Should now be at 4.5 mm inferior to the dura mater) and wait 2 min.
53. Slowly deliver the cells at a rate of 1 μL/min.
54. Wait 5 min, and then slowly retract the syringe from the brain.

**CRITICAL:** Be patient when retracting the syringe. Removing it too quickly can cause injected cells to come out.

55. Eject the remaining volume in the syringe into a tube containing 10% bleach solution and flush needle twice by fully loading it with PBS, and then twice with ultrapure sterile water.

**CRITICAL:** It is important to flush syringe quickly after removal to make sure it does not clog between injections, especially when using higher cell concentrations.

56. Wash the injection site three times with sterile PBS.
57. Remove the mouse from stereotaxic frame by loosening nose clamp and ear bars. Then, gently remove the mouth from the bite bar with forceps.
58. Close incision with 4-0 sutures.
59. Perform subcutaneous injection of buprenorphine (0.01 mg/kg) followed by intraperitoneal injection of atipamezole (1 mg/kg).
60. Place the animal in fresh cage with heat support and monitor until full recovery.

**EXPECTED OUTCOMES**

One-day post SB injections, a bioluminescent signal will be detected by IVIS imaging in the brain of pups (Figure 1D). At this point, pups without signal (<20% of total injected pups) can be discarded, as lack of signal indicates that transfection failed. Mice will develop normally until they begin to display adverse neurological symptoms. The length of time post injection to moribund state is variable and depends on the specific genetic lesions injected and the degree of integration into the host genome. The tumor will demonstrate a positive signal on IVIS bioluminescent imaging (Figure 2A), and be detectable by fluorescent microscopy in the brain after dissection from the skull (Figure 2B), and by histological analysis (Figure 2C). Dissociated tumor cells will form neurospheres in culture, and these neurospheres will retain the genetic lesions of the tumor (visible by fluorescence). Following orthotopic surgery, incision will remain intact, clean, and dry. Mice will show signs of recovery (increased alertness, responsiveness, and activity) in the days following procedure. If neurospheres express luciferase, tumor growth can be monitored by measuring bioluminescent signal. After some time (20–40 days depending on the genetic lesions present) mice will start to show signs of brainstem tumor burden (e.g., loss of balance, running in circles, rolling, decreased activity). If implanting a group of mice, these symptoms will occur around the same time. Upon brain extraction, tumor should be visible in the brainstem. Tumor will be visible via fluorescent microscopy if using constructs expressing fluorescent proteins. Histopathological analysis will reveal tumor presence in the brainstem region, specifically the pons (Figure 3B). Tumors will also continue to express the same set of genetic lesions present in neurospheres at implantation.

**LIMITATIONS**

While human brainstem high-grade gliomas consistently express specific hallmark mutations (in genes encoding H3F3A, HIST1B/C), these tumors also demonstrate variable expression of oncoproteins and other genetic alterations that may contribute to tumorigenesis. It is not feasible for the
SB model to reflect this variability, but it does successfully recapitulate the most salient features of brainstem high-grade glioma.

The rate of tumor progression in the SB model is relatively slow (2–6 months) and can be very variable depending on the efficiency of transfection and accuracy of the injection, as well as the progression of the HGG, which may vary among mice.

Tumors arising from the orthotopic model may not be as diffuse and histopathologically accurate as the tumors arising de novo generated by the SB model. This may be due to the faster development of the orthotopic model-derived tumors compared to the SB model. SB de novo tumors develop over a longer time and evolve from a pre-neoplastic stage to a high-grade glioma, thus exhibit a more complex histology that likely reflects the interaction of the tumor cells with the environment, like migration around blood vessels, immune infiltration, etc. Nevertheless, orthotopically implanted tumors still exhibit diffusion and characteristics of pHGG such as necrosis, pseudopalisades around necrotic areas, and cellular atypia.

The surgical procedure for orthotopic injection may damage the brain architecture and lead to an increase in inflammatory signaling in the brain. This could influence tumor development depending on the extent of inflammation and type of neurosphere implanted.

The brainstem is a particularly sensitive structure, since it controls processes related to the autonomic nervous system. For this reason, tumors of small size in this area compromise the health of the animal. This limits the tumor sample size, making it difficult to obtain a large amount of cells to develop cell cultures and tissue sections for histopathological analysis. This can be overcome by collecting tumor samples from several animals.

A recent study reports that the implantation of adult mice with cells expressing luciferase may elicit an increased pro-inflammatory immune response, when compared with the implantation of Wild type glioma cells (Sanchez et al., 2020). In this study, luciferase expressing cells were implanted in adult animals, which have a fully developed immune system. In contrast, the SB system is used to induce HGG in neonatal mice, which have an immature immune system, therefore the luciferase expression is not able to elicit an immune response in these animals. For this reason, we think that the SB model is suitable to study the immune microenvironment. On the other hand, if the orthotopic model is used to study the TME, the effects of the luciferase expression on the immune response have to be properly accounted for.

TROUBLESHOOTING

Problem 1
Mother not showing nurturing behavior (step 13).

Potential Solution
This may occur due to a variety of factors. However, take cautions and minimize handling of the pups and decrease stress around mother (e.g., quiet location, minimal movement of cage, etc). If neglecting behavior continues, a surrogate mother may be used.

Problem 2
Mice developing hydrocephaly (step 16).

Potential Solution
Hydrocephaly can develop based on the volume, the speed, and the accuracy of the injection. If hydrocephaly occurs, euthanize animals according to institutions guidelines. If injected animals become hydrocephalic frequently, adjust your injection procedure by decreasing the speed of injection of the transfection mix and removing needle as slowly as possible.
Problem 3
The tumor cells are not dissociating well (step 28).

Potential Solution
Cell dissociation can be improved by gently pipetting the solution or briefly incubating the cells at 37°C. Caution: Leaving cells too long in Accutase can be harmful to cells.

Problem 4
Cells not maintaining fluorescence and/or oncogenic transgenes in culture (step 32).

Potential Solution
A selection marker (e.g., puromycin or geneticin) can be added in order to facilitate maintenance of oncogenic transgenes. Alternatively, cells can be sorted via flow cytometry to maintain or enhance the population of cells that contain the desired genetic lesions.

Problem 5
Neurosphere culture is contaminated following tumor transfer and dissociation (step 32).

Potential Solution
It is possible that neurosphere cultures may become contaminated with other cells, bacteria, or fungi. Typically, additional brain cells that are inadvertently transferred with the tumor to the flask will not be an issue due to the fast growth rate of the tumor-derived neurospheres (i.e., they will be diluted exponentially throughout passages). To prevent the growth of bacteria and fungi, additional anti-bacterial and fungicidal compounds can be added to the media. Additionally, standard preventive measures must be taken to avoid contamination, such as testing for mycoplasma regularly. We also recommend to perform STR (short tandem repeat) profiling to unequivocally identify the different primary cell cultures, and to test for the presence of fluorescence regularly.

Problem 6
Neurospheres are adhering to the bottom of the flask (step 32).

Potential Solution
Sometimes neurospheres can adhere to the bottom of the flask when growth factors are depleted. To prevent this, make sure to frequently change the media and replenish growth factors.

Problem 7
Animal not sedated properly (step 38).

Potential Solution
If this occurs, first you should wait to allow the anesthesia to take full effect. If the mouse is still not properly sedated, you may need to administer more anesthetic (e.g., if using ketamine and dexmedetomidine, administer half of the original dose of ketamine alone). As always, make sure to abide by your institution’s animal protocol.

Problem 8
Cannot set mouse properly on the frame (step 41).

Potential Solution
Mouse size may vary when using different strains and ages. Therefore, one may encounter problems to correctly situate the mouse on the stereotaxic frame. If this occurs, adjust the ear bars and nose clamps to the proper height so that the top of the mouse head is flat and parallel to the base of the frame.
Problem 9
Difficulty inserting retractors (step 44).

Potential Solution
Depending on the size of the retractors and experience of the user, it may be difficult to properly insert the retractors. If this occurs, make the incision slightly larger to allow for more room.

Problem 10
Cannot locate lambda (step 45).

Potential Solution
Sometimes it may be difficult to see the suture lines on the skull. In this case, allow the tissue to dry slightly and lightly press on the skull near the area with a tuberculin syringe needle.

Problem 11
Needle is clogged (step 49).

Potential Solution
Sometimes, injecting high concentrations of cells can cause the needle to clog. If this happens, try flushing the needle with a saline solution. If this does not work, use a Hamilton needle cleaning solution and/or sonicate the needle.

Problem 12
Mice are not recovering quickly (step 60).

Potential Solution
It may take some time for the mice to recover from the surgery. For this process, it is important to use a heating pad underneath the recovery area.

Problem 13
The sutures have been removed from surgical area (step 60).

Potential Solution
If this occurs, it may be necessary to re-suture the surgical area. First check with your mouse husbandry staff, but typically if the incision is still healing it must be re-sutured. Prior to re-suturing, sedate the mouse similarly to pre-surgery. Clean the surgical area with iodine and rinse it with PBS. Create small cuts in the tissue on the sides of the opening (this is important for proper healing). Suture as done previously. Allow mice to recover, similarly to post surgery, and place mouse in a cage by itself until fully healed.

RESOURCE AVAILABILITY
Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Maria G. Castro, mariacas@med.umich.edu.

Materials Availability
Catalog numbers for the available plasmids used to generate the genetically engineered mouse models of brainstem high-grade glioma using the Sleeping Beauty method are detailed in the Key Resources Table. Those plasmids that are not currently available in Addgene will be provided by the Lead Contact upon request with no restrictions.

Data and Code Availability
No datasets or code were generated during this study.
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AUTHOR CONTRIBUTIONS
M.G.C. and P.R.L. conceived the research question and provided funding; J.C.G., S.H., and F.M.M. performed experiments; J.C.G., F.M.N., and S.H. prepared the figures, J.C.G., F.M.N., and S.H. wrote the manuscript under the supervision of M.G.C. and P.R.L. All authors read and edited the final version of this manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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