Supplementary Figure 1: *In vitro* characterization of pro-apoptotic Ads and their targets. A, Expression of therapeutic targets were determined *in vitro* in CNS-1 cells by immunofluorescence (green) using antibodies specific for TNF-α receptor 1 (TNFR1), TRAIL receptor 2 (TRAILR2), Fas and Ki67, a cellular marker of proliferating cells. Nuclei were stained using DAPI (blue). Arrows indicate immunopositive cells. B, CNS-1 cells were infected with the Ads expressing the pro-apoptotic transgenes and 24 h later, transgene expression was determined by immunofluorescence using antibodies specific for TNF-α, TRAIL, FasL and TK. Arrows indicate immunopositive cells. C, Transgene expression of pro-apoptotic cytokines was also assessed by ELISA in the supernatant of CNS-1 cells infected with Ad-TNF-α and Ad-TRAIL 48 h after infection. Release of FasL was detected by using conditioned media from Ad-FasL-infected CNS-1 cells to induce LN18 cell death, which have high sensitivity to FasL cytotoxicity. Cell viability was assessed by flow cytometric analysis of Annexin-V-stained LN18 cells 24 h after incubation with conditioned media. As controls, LN18 cells were incubated with fresh media or conditioned media from CNS-1 cells non-treated or infected with an Ad without transgene (Ad0). * p<0.05 vs mock; ^ p<0.05 vs Ad0 (One-way ANOVA followed by Tukey’s test).
Supplementary Figure 2. Tumor DNA fragmentation *in vitro* and *in vivo* upon administration of pro-apoptotic Ads. **A.** CNS-1 cells were infected with Ads expressing pro-apoptotic transgenes, i.e. HSV1-thymidine kinase (Ad-TK), TNF-α (Ad-TNF-α), FasL (Ad-FasL) or TRAIL (Ad-TRAIL). 24h after infection, cells infected with Ad-TK were incubated with GCV. Untreated cells and cells infected with an Ad containing no transgene (Ad0) were used as controls. DNA fragmentation was determined by agarose gel electrophoresis 72h after infection or addition of GCV. **B.** Rats were implanted with CNS-1 cells in the brain and treated 4 days later with intratumoral injection of saline, Ad-TK, Ad-TNF-α, Ad-FasL or Ad-TRAIL. Ad-TK-treated rats received GCV. Tumor DNA was purified 5 days after the treatment and DNA fragmentation was assessed by agarose gel electrophoresis.
Supplementary Figure 3. Single channel images of therapeutic targets of TNF-α and TRAIL within intracranial CNS-1 tumors and peritumoral brain tissue. Rats were implanted in the striatum with CNS-1 tumors and 9d later brains were processed for immunocytochemistry. Confocal microphotographs show staining of therapeutic targets (TNFR1 and TRAILR2, green) and tumor cells (vimentin, red), neurons (NeuN, red) and astrocytes (GFAP, red). Nuclei were stained with DAPI (blue). T: tumor area. N: necrotic patch. Arrows indicate cells expressing the therapeutic target indicated. Dashed line represents tumor border. Scale bars: 10 μm.
Supplementary Figure 4. Single channel images of therapeutic targets of FasL and TK within intracranial CNS-1 tumors and peritumoral brain tissue. Rats were implanted in the striatum with CNS-1 tumors and 9d later brains were processed for immunocytochemistry. Confocal microphotographs show staining of therapeutic targets (Fas and Ki67, green) and tumor cells (vimentin, red), neurons (NeuN, red) and astrocytes (GFAP, red). Nuclei were stained with DAPI (blue). T: tumor area. N: necrotic patch. Arrows indicate cells expressing the therapeutic target indicated. Scale bars: 10 μm.
Supplementary Figure 5. Chronic neurotoxicity of pro-apoptotic Ads after injection into normal brain. Lewis rats (n=4/treatment) were injected in the striatum with saline, Ad-TNF-α, Ad-TRAIL, Ad-FasL or Ad-TK. Rats treated with Ad-TK received GCV. Sixty days post-vector delivery, neuropathological analysis of the brain was assessed by Nissl staining and immunocytochemistry using antibodies against tyrosine hydroxylase (TH), myelin basic protein (MBP), major histocompatibility complex II (MHCII), CD68 (macrophages) and CD8 (cytotoxic T cells). Scale bars: 2 mm.
Supplementary Figure 6. Chronic neurotoxicity of combined gene therapy after injection into normal brain tissue. Lewis rats were injected in the striatum with saline, Ad0, Ad-FasL+Ad-Flt3L, Ad-TK+Ad-Flt3L. Rats treated with Ad-TK+Ad-Flt3L received GCV. Sixty days after delivery, neuropathological analysis was assessed by Nissl staining and immunocytochemistry using antibodies against tyrosine hydroxylase (TH), myelin basic protein (MBP), major histocompatibility complex II (MHCII), CD68 (macrophages) and CD8 (cytotoxic T cells). Scale bars: 2 mm.
## Supplementary Table I

**Median survival of intracranial tumor models**

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>CNS-1 TUMOR IN LEWIS RATS</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>4-DAY TUMOR</td>
</tr>
<tr>
<td>SALINE</td>
<td>17 d (n=9)</td>
</tr>
<tr>
<td>Ad.TNF-α</td>
<td>18 d (n=5)</td>
</tr>
<tr>
<td>Ad.TRAIL</td>
<td>16.5 d (n=8)</td>
</tr>
<tr>
<td>Ad.FasL</td>
<td>21 d&lt;sup&gt;a&lt;/sup&gt; (n=9)</td>
</tr>
<tr>
<td>Ad.TK+GCV</td>
<td>6/7 long term survivors&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ad.FasL+Ad.Flt3L</td>
<td>N/A</td>
</tr>
<tr>
<td>Ad.TK+GCV+Ad.Flt3L</td>
<td>N/A</td>
</tr>
</tbody>
</table>

<sup>a</sup>: p<0.05 vs saline; <sup>b</sup>: p<0.05 vs Ad.FasL; <sup>c</sup>: p<0.05 vs Ad.TK+GCV (Mantel log-rank test)

Numbers between brackets indicated the number of animals per group.
SUPPLEMENTARY DATA

MATERIALS AND METHODS

**Immunofluorescence**

Transgene expression of the proapoptotic Ads was evaluated *in vitro* in CNS-1 cells fixed with 4% PFA using the following antibodies: anti-TNF-α in rabbit (1:100, Pierce P350), anti-FasL in Armenian hamster (1:50, BD Biosciences 555022), anti-TRAIL in mouse (1:25, R&D systems MAB375), and anti-TK in rabbit (1:10,000, developed in our lab5), followed by FITC-conjugated anti-Armenian hamster goat IgG (1:800, Jackson labs 127-095-160) or Alexa Fluor488-conjugated anti-rabbit or anti-mouse goat IgG (1:1000, Invitrogen Molecular Probes, A11034 and A11029, respectively). Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI) (5µg/ml, Invitrogen Molecular Probes, Carlsbad, CA, USA) and coverslips were mounted with ProLong Antifade (Invitrogen Molecular Probes).

Expression of therapeutic targets was performed *in vitro* in PFA-fixed CNS-1 cells and *in vivo* in PFA-fixed free-floating coronal sections from rat brain 9 days after tumor implantation. Immunofluorescence was performed using the following antibodies: anti-TNFR1 in rabbit (1:50, Assay Design Inc. CSA-815E), anti-TRAILR2 in rabbit (1:50, Prosci. Inc. 2019), anti-Fas in goat (1:100, R&D systems AF2159), anti-Ki67 in rabbit (1:25, Vector labs VP-RM04) followed by Alexa Fluor488-conjugated anti-rabbit goat IgG or Alexa Fluor488-conjugated anti-goat chicken IgG (1:1000, Invitrogen Molecular probes A11034 and A21467, respectively). Before staining with anti-Fas and anti-Ki67, cells and tissues underwent antigen retrieval by microwave irradiation in 200 ml of 10 mM citrate buffer (pH=6) for 10 min at medium power. Cell types in the brain were identified using
the following antibodies: anti-vimentin in mouse (1:1000, Sigma V6630), anti-NeuN in mouse (1:1000, Chemicon MAB377), anti-GFAP in Guinea pig (1:500, Advance immunochemical 31223-200) or anti-GFAP in rabbit (1:1000, Chemicon AB 5840) followed by Alexa Fluor594-conjugated anti-mouse or anti-Guinea pig goat IgG (1:1000, Invitrogen Molecular probes A11032 and A11076, respectively) or Alexa Fluor594-conjugated anti-mouse or anti-rabbit chicken IgG (1:1000, Invitrogen Molecular probes A21201 and A21442, respectively).

Neuropathological analysis

Neuropathological analysis was performed in naïve the rat brain 7 and 60 days after Ad injection. Briefly, endogenous peroxidases were inactivated with 0.3% hydrogen peroxide, followed by blocking in 10% horse serum/phosphate-buffered saline. Sections were incubated for 72 h with the following antibodies: anti-tyrosine hydroxylase in rabbit (TH; 1:5000, Calbiochem 657012), anti-myelin basic protein in mouse (MBP; 1:1,000; Chemicon MAB1580), anti-rat CD68 in mouse (clone ED1 to identify macrophages/activated microglia; 1:1,000, Serotec MCA341R), anti-rat major histocompatibility complex II (MHC II, 1:1,000, Serotec MCA46GA), and anti-rat CD8α (to identify cytotoxic T cells; 1:1,000, Serotec MCA48G). Then, the sections were incubated for 4 h with biotin-conjugated anti-rabbit goat IgG or anti-mouse rabbit IgG (1:800, DAKO, Denmark, E0432 and E0464, respectively). Binding of biotinylated secondary antibodies was detected using the Vectastain Elite ABC horseradish peroxidase method (Vector Laboratories, Burlingame, CA, USA) followed by the glucose oxidase and nickel ammonium sulfate-intensified diaminobenzidine method. Sections were mounted on
gelatinized glass slides and dehydrated through graded ethanol solutions and coverslipped using DPX mounting media for histology (Sigma, St. Louis, MO, USA).

For Nissl staining brain sections were mounted on gelatinized glass slides and incubated in cresyl violet (0.1%; Sigma). Sections were passed through destain solution (70% ethanol, 10% acetic acid), dehydrated (100% ethanol and xylene) and coverslipped using DPX mounting media for histology.

Tumor histology was studied by free-floating immunocytochemistry using anti-vimentin antibodies as described above.

**DNA ladder**

Tumor DNA was purified using DNeasy blood and tissue kit (QIAGen 69506) following manufacturer’s protocol. Columns were eluted with 100 µl AE buffer and eluates were incubated with RNAse (1mg/ml) for 20 min at RT. DNA (100 µl) was subjected to electrophoresis on 2% agarose gel for 90 minutes at 45 V. DNA was stained with ethidium bromide.

**Flow cytometry**

To determine levels of cell death, human and rat GBM cells were harvested with with AccutaseTM Enzyme Cell Detachment Medium (eBiosciences, 00-4555-56) and resuspended in 100 µl binding buffer (150mM NaCl, 18mM CaCl₂, 10mM HEPES, 5mM KCl, 1mM MgCl₂). Cell death was detected by incubating the cell suspension with 5µl Annexin V-FITC (Bender MedSystems, Burlingame CA, BMS306FI/a) for 5 min followed by addition of 10 µl propidium iodide (50 µg/ml, Sigma, St Louis MO, P4864) before analysis using a FACScan (Becton–Dickinson). Cells that were positive for Annexin V-FITC and/or propidium iodide were quantified to determine the percentage of
cell death using WinMDI 2.9 software (J. Trotter, Scripps Research Institute, La Jolla, CA).