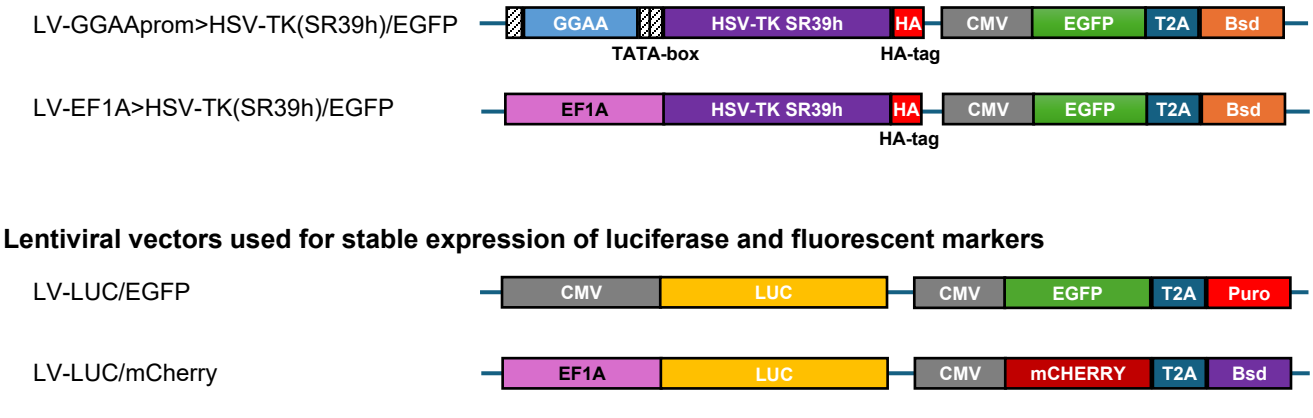
Supplementary Information

Suicide gene therapy targeting Ewing sarcoma via an Ewing-specific GGAA promoter
S.T. Cervera, S. Martinez, M. Iranzo-Martínez, R.M. Melero-Fernández de Mera, J. Alonso.

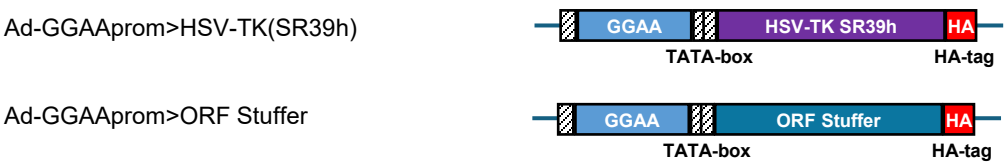
Figure S1 Supplementary

Lentiviral vectors used for stable expression of HSV-TK(SR39h)



Lentiviral vectors used for stable expression of luciferase and fluorescent markers

Adenoviral vectors used in *in vitro* and *in vivo* experiments



Supplementary Figure S1. Schematic representation of vectors used in this article. See material and methods section for details. Genes, promoters, etc… are not to scale.

GGAA: GGAA promoter Ewing-specific.
EF1A: EF1A promoter. Constitutive.
CMV: CMV promoter. Constitutive.

HSV-TK SR39h: Herpes simplex virus thymidine kinase, mutant SR39h, codon optimized.
EGFP: Enhanced green fluorescent protein.
mCherry: Variant of monomeric RFP1 (red fluorescent protein) protein.
LUC: Humanized firefly luciferase.
ORF Stuffer: Amino acids 2-83 of E. coli beta galactosidase.

Puro: Puromycin resistance gene.
Bsd: Blasticidin resistance gene.

HA: Hemagglutinin epitope tag
T2A: Self-cleaving 2A peptide from Thosea asigna virus

Figure S2 Supplementary

Suicide gene therapy targeting Ewing sarcoma via an Ewing-specific GGAA promoter

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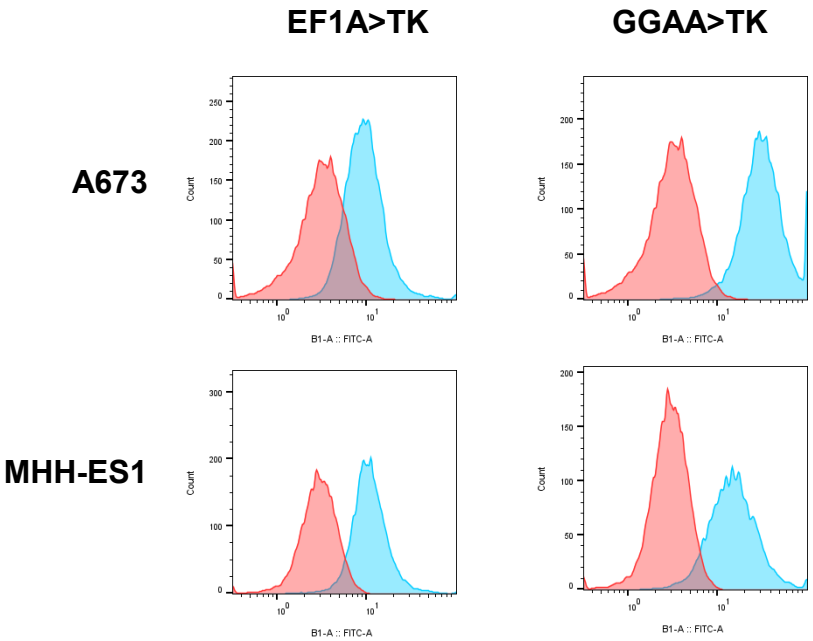

Supplementary Figure S2. Transduction levels of EF1A>TK and GGAA>TK lentiviral vectors in A673 and MHH-ES1 Ewing sarcoma cells. Quantification of EGFP positive cells by flow-cytometry in stably transduced cells (MOI=0.5). The percentage of transduced cells was similar between cell lines with each lentiviral vector.

Figure S3 Supplementary

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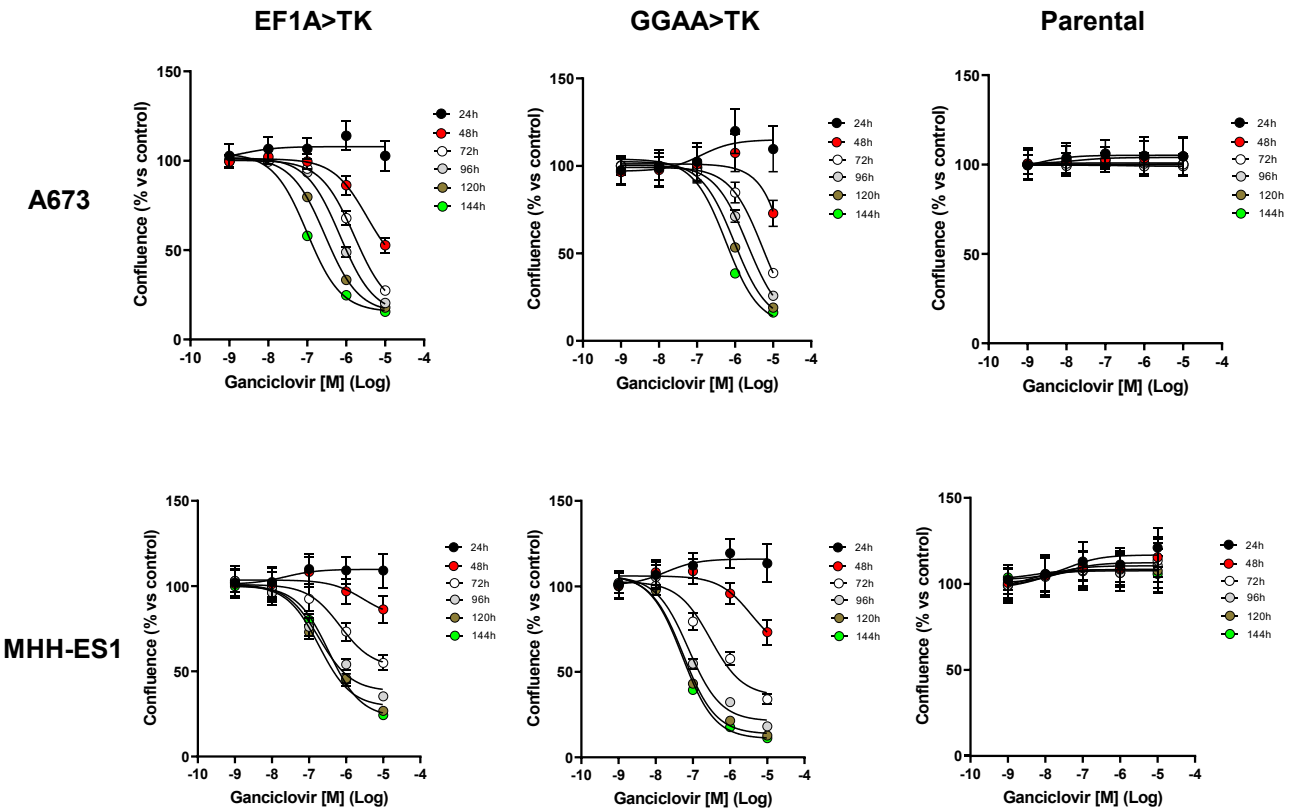

Supplementary Figure S3. Effect of ganciclovir (GCV) treatment on Ewing sarcoma cells stably expressing HSV-TK (SR39h). Cell viability (confluence) was continuously monitored using a live-cell analysis system. Briefly, cells were seeded onto 96-well plates at a density of 3 000 cells/well, and cell growth was monitored for six days in an Incucyte SX5 live-cell imaging device. Five images of each well were recorded every 6 hours for a total of 144 hours. At 24 and 96 hours, the cells were treated with various concentrations of GCV or 0.1% DMSO (vehicle). The percentage of confluent cells at each time point was quantified using Incucyte 2022B Rev2 software and compared with that of control cells. The effect of GCV on cell growth was detected as soon as 48 hours and was maximal at 120–144 hours of treatment in Ewing sarcoma cells transduced with EF1A>TK or GGAA>TK lentivirus. No toxic effect of GCV was observed on parental cells at any tested concentration.

Figure S4 Supplementary

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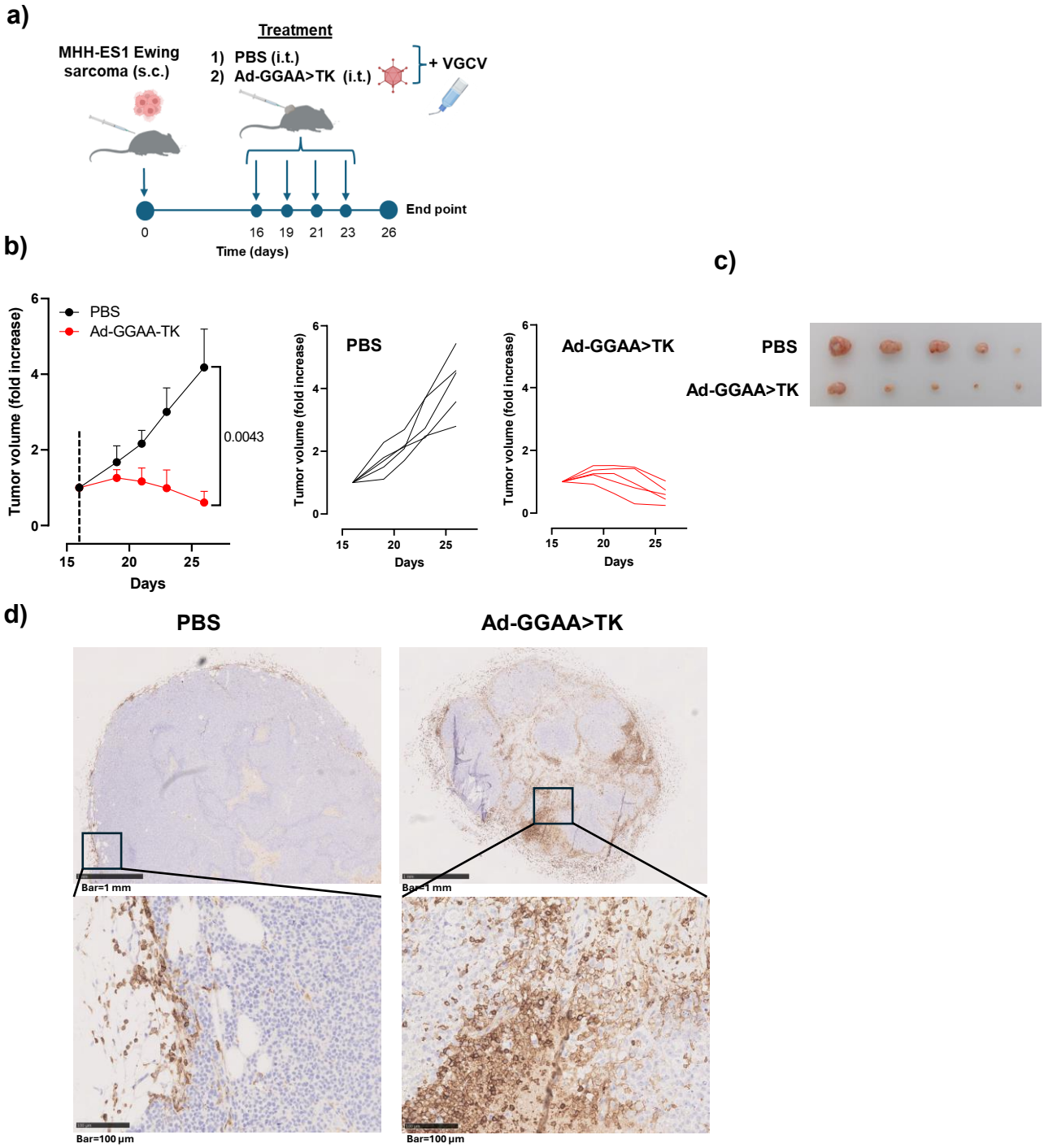

Figure S4. The combination of adenovirus GGAA>TK (Ad-GGAA>TK) and VGCV reduces tumor growth and induces immune cell infiltration *in vivo*. a) Schematic representation of the experimental design. Nude mice were inoculated s.c. with MHH-ES1 Ewing sarcoma cells and split into two groups when the tumors reached a mean volume of approximately 200 mm^3 (dashed line). One group received intratumoral injections of Ad-GGAA>TK ($2x10^{10}$ VP/mL dose, four doses at 2–3-day intervals) (n=5), whereas the control group (n=5) received intratumoral injections of PBS on the same days. Both groups received VGCV dissolved in drinking water. **b)** The tumor volume normalized with respect to the start of treatment for each group (mean±s.d.) and each animal are shown. Statistical significance between groups is shown (2-way ANOVA). **c)** Micrograph of tumors excised at the end of the experiment. The tumor volume (mean±s.e.m.) for the PBS control group was 857.2±376.6 mm^3 and for the group treated with Ad-GGAA>TK 178.3±116.9 mm^3. **d)** Tumor sections were stained with an anti-mouse CD45 antibody to assess immune infiltration. Representative images of a tumor treated with Ad-GGAA>TK and a tumor treated with PBS (control) are shown. The control tumor was negative for CD45+ cells except at the periphery of the tumor, where some CD45+ cells accumulated. In contrast, Ad-GGAA>TK treatment induced massive death of Ewing sarcoma cells and immune cell infiltration (CD45+ cells). Images are shown at the same magnification.

Original western-blot (Figure 1A)

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