Pediatric high-grade glioma (pHGG) and diffuse intrinsic pontine gliomas (DIPGs) are aggressive pediatric brain tumors in desperate need of a curative treatment. Oncolytic virotherapy is emerging as a solid therapeutic approach. Delta-24-RGD is a replication competent adenovirus engineered to replicate in tumor cells with an aberrant RB pathway. This virus has proven to be safe and effective in adult gliomas. Here we report that the administration of Delta-24-RGD is safe in mice and results in a significant increase in survival in immunodeficient and immunocompetent models of pHGG and DIPGs. Our results show that the Delta-24-RGD antiglioma effect is mediated by the oncolytic effect and the immune response elicited against the tumor. Altogether, our data highlight the potential of this virus as treatment for patients with these tumors. Of clinical significance, these data have led to the start of a phase I/II clinical trial at our institution for newly diagnosed DIPG (NCT03178032).
P ediatric high-grade glioma (pHGG), including diffuse intrinsic pontine glioma (DIPG), are aggressive solid tumors that develop during childhood. pHGG was traditionally considered similar to adult high-grade gliomas; however, over the last decade, an emerging plethora of genomic data has changed our understanding of both pHGG and DIPG and demonstrated that these cancers are indeed very different entities from their adult counterparts. The knowledge acquired from genomic data has supported the 2016 WHO reclassification of central nervous system (CNS) tumors in which DIPG is included in a new category named diffuse midline glioma (DMG) H3-K27M-mutant.

Regarding their epidemiology, these two malignancies account for ~8–12% of all CNS tumors that arise in children. Because the incidence of this disease is ~85 per 100,000, similar to most pediatric tumors, pHGG and DIPG are considered orphan diseases. The incidence peak of pHGG and DIPG appears in patients aged between 6 and 9 years. The standard treatment for pHGG is maximal surgical resection, followed by radiotherapy and/or temozolomide cycles. Unfortunately, surgery is not a therapeutic option for DIPG, and the standard of care is radiotherapy (RT). RT improves the quality of life and survival of these children; however, RT is not curative. Despite the significant number of clinical trials investigating pHGG and DIPG, patient outcomes have continued to be dismal over the last four decades. The implementation of alternative therapies that challenge the actual therapeutic paradigm could provide a breakthrough in the treatment of these tumors.

Virotherapy is emerging as an alternative treatment for cancer. In fact, the recent approval of T-VEC (Talimogene laherparepvec), which is an oncolytic herpes virus, by the FDA for metastatic melanoma has enabled the possibility to use other oncolytic viruses as standard treatment for cancer.

Delta-24-RGD is a replicative oncolytic adenovirus specifically modified to destroy cancer cells. A genetic modification in the E1A viral gene, which is a 24-base pair deletion responsible for pRB protein binding, confers this adenovirus with selectivity to cancer cells. The addition of an RGD sequence in the viral fiber improves its infectivity, allowing the virus to interact with αvβ3 and αvβ5 integrins that are overexpressed in a wide range of tumors, including glioma cells. In fact, several clinical trials involving adult glioma patients have shown that the intratumoral administration of Delta-24-RGD (DNX-2401 in the clinic) is safe and provides a therapeutic advantage in many patients. Recent studies have demonstrated that the antitumor effect displayed by oncolytic adenoviruses is not only related to their intrinsic oncolytic effect but also to the triggering of an immune response. Tumor infection elicits specific antitumor immunity in adult mouse glioma models. Importantly, these results have been confirmed in patients, and histological analyses of tumor resections after Delta-24-RGD administration have shown an increased number of infiltrated immune populations in the tumors, suggesting that an immune-mediated antiangioma response occurred. These results encourage the translation of Delta-24-RGD as a feasible treatment for pediatric brain tumors.

In this study, we evaluate the antiangioma effect of Delta-24-RGD in pHGG and DIPG cell lines. Our data show that Delta-24-RGD results in a significant antitumor effect in vitro in a panel of cell lines and in vivo in pHGG and DIPG orthotopic immunosuppressed and immunocompetent models. Our results show that in addition to the oncolytic effect, Delta-24-RGD administration triggers an antitumor immune response. These promising preclinical results paved the way for a phase I/II clinical trial investigating DNX-2401 (NCT03178032) for the treatment of newly diagnosed DIPGs at our institution.

Results
Delta-24-RGD determinants of infection and replication in pHGG/DIPG. To evaluate whether pHGG and DMG/DIPG are susceptible to viral infection, using a cohort of 220 patient samples of pHGG and DMG/DIPG previously published, we performed "in silico analyses" of the expression of the following main receptors that use Delta-24-RGD to enter cells: Coxsackie adenovirus receptor (CAR; CXADR), integrinα5β3 (ITGA5), integrinαVβ5 (ITGA5), and integrinαVβ3 (ITGAV). Our analyses revealed that independently of the molecular group to which the patients were assigned, all patients expressed significant amounts of these genes (Fig. 1a and Supplementary Fig. 1a). These data indicate that these tumors are potentially susceptible to Delta-24-RGD infection. Then, as Delta-24-RGD replication is dependent on an aberrant RB pathway, we analyzed the expression and amplifications/deletions of the genes involved in the RB pathway, specifically cdk4/6, cyclin D1, RB, and E2F1. We found that different RB pathway profiles are related to the different molecular subgroups (H3.3G34R, H3.3K27M, IDH1, BRAF, PXC3, and HGG_GBM_wt) (Fig. 1b, Supplementary Fig. 1b). cdk4/6 had a modest rate in all subgroups; meanwhile, CCND1 amplification was only observed in the H3.3K27M molecular subgroup. RB deletions were only present in the IDH1 group. However, CDKN2A deletions were found in almost all subgroups (Fig. 1b), and this gene encodes p16INK4a and p14arf. As a result of all these deletions, we observed increased E2F1 expression, which, in turn, activates the viral transcriptional program and finally its replication (Supplementary Fig. 1c). In summary, these results highlight the suitability of Delta-24-RGD as a possible therapy for pHGG and DMG, including DIPGs.

Delta-24-RGD antiangioma effect in human pHGG and DIPG cell lines. The analysis of viral receptor (αvβ3, αvβ5, integrin, and CAR) expression in the membrane of a panel of pHGGS and DIPGs revealed that all display these receptors in their membrane, suggesting that they could be infected by an adenovirus. Interestingly, the CAR receptor was abundantly expressed in all cell lines assessed (ranging from 40 to 90%) (Fig. 2a). These results are consistent with the expression data observed in the patients (Fig. 1). Then, we tested the capacity of Delta-24-RGD to infect pHGG (CHLA-03-AA, CHLA-200, PBT-24, SJ-GBM2, and SF188) and DIPG (TP54, TP80, TP83, TP84, and SU-DIPG IV) cell lines. We infected these cell lines with a replication-deficient Ad5-GFP-RGD adenovirus at 10 and 100 MOIs and quantified the % of GFP-expressing cells by flow cytometry. We observed that at the 10 MOI, >50% of the cells were infected, and 100% of the cell lines was achieved at 100 MOI (Fig. 2b, Supplementary Fig. 2a). In general, the pHGG cell lines were more easily infected than the DIPG cell lines.

Following the infection of the indicated cell lines with increasing amounts of Delta-24-RGD, E1A, and fiber, which are an early protein and master regulator of the viral cycle and a late protein, respectively, showed a robust expression. The expression of these proteins was patent even at the smallest dose of five MOI (Fig. 2c and Supplementary Fig. 2b). These results suggest that Delta-24-RGD is able to undergo a viable viral cycle in pHGG and DIPG cells. Furthermore, Delta-24-RGD efficiently replicated with at least an increment of two logarithms in viral progeny compared with the input virus (10⁶ pfu, dashed line) in all cell lines tested after 72 h of infection (Fig. 2d). Overall, the virus showed a better replication in the pHGG cell lines when compared with the DIPG cell lines (P = 0.03; Supplementary Fig. 2c).

Subsequently, we evaluated the antitumor effect of Delta-24-RGD in the pHGG (N = 5) and DIPG (N = 7) cell lines.
The Delta-24-RGD treatment resulted in a significant antitumor effect in all cell lines tested. The IC50 ranged between 3.6 and 68 MOIs in the DIPG cell lines and 0.8 and 32 MOIs in the pHGG cell lines (Fig. 2e, Supplementary Fig. 3 and Table 1). PBT-24, which is a pHGG cell line in which Delta-24-RGD presented the best infectivity and replication capacity, also displayed the lowest IC50. In summary, these results demonstrate that Delta-24-RGD is able to infect, replicate and exert a significant cytotoxic effect in all pHGG and DIPG cell lines tested.

Delta-24-RGD extends survival in pHGG and DIPGs orthotopic models. Given the encouraging in vitro results, we evaluated the antitumor effect of Delta-24-RGD in vivo in two orthotopic models of DIPG (TP80 and TP54) and two models of pHGG (CHLA-03-AA and PBT-24). A single dose (10^8 pfu per animal) of Delta-24-RGD was intratumorally injected on day 3 after cell implantation. The mice were monitored during the experiment until physical decline, such as weight loss or ataxia, was observed. The survival analyses showed that Delta-24-RGD was able to significantly increase (P = 0.024, Log-rank test) the overall survival of mice bearing TP80 with a median survival of 217 days. The Delta-24-RGD administration increased survival by an average of 40 days (P = 0.024, Log-rank test), and the treatment resulted in 44% of long-term survivors in the treated group (Fig. 3a and Supplementary Fig. 4a). Mice bearing the TP54 cell line in the pons exhibited a median survival of 83.5 days, whereas the mice treated with Delta-24-RGD exhibited a median survival of 95.5 days (P = 0.04, Log-rank test) (Fig. 3b and Supplementary Fig. 4a). The median survival of the mice bearing CHLA-03-AA was 46 days, whereas the Delta-24-RGD-treated mice had an increased overall survival by 53 days, resulting in 36% of long-term survivors free of disease (P < 0.0001, Log-rank test) (Fig. 3c and Supplementary Fig. 4a). Finally, compared with the mice bearing the PBT-24 control (survival of 75 days), the treated mice displayed a median survival of 100.5 days (Log-rank test; P = 0.0013), and there were 33% of long-term survivors in the treatment group (Fig. 3d and Supplementary Fig. 4a).

**Table 1 Median-effect doses (IC50) of Delta-24-RGD in pHGG and DIPG cell lines**

| Cell lines | IC50 (MOIs ± SD) |
|------------|------------------|
| pHGG       |                   |
| CHLA-03-AA | 10.22 ± 3.1      |
| PBT-24     | 0.8 ± 0.06       |
| SF188      | 9.6 ± 0.5        |
| SJ-GBM2    | 32 ± 7.1         |
| CHLA-200   | 5 ± 0.06         |
| TP54       | 9.6 ± 3.1        |
| DIPG IV    | 3.6 ± 1.8        |
| TP80       | 39.7 ± 10.9      |
| TP83       | 51.9 ± 13.7      |
| TP84       | 68 ± 4.1         |
| JHH-DIPG1  | 26.2 ± 1.5       |
| JHH-DIPG16A| 9.6 ± 5.9        |

| DIPG       |                   |
| CHLA-200   | 5 ± 0.06          |
| SF188      | 9.6 ± 0.5         |
| SJ-GBM2    | 32 ± 7.1          |
| TP54       | 9.6 ± 3.1         |
| DIPG IV    | 3.6 ± 1.8         |
| TP80       | 39.7 ± 10.9       |
| TP83       | 51.9 ± 13.7       |
| TP84       | 68 ± 4.1          |
| JHH-DIPG1  | 26.2 ± 1.5        |
| JHH-DIPG16A| 9.6 ± 5.9         |

The IC50 value (MOIs) is the median-effect dose (dose affecting 50% of the cells, i.e., 50% survival). IC50 = 50% inhibitory concentration; MOIs = multiplicity of infection.
The pathological analyses of the tumors showed the highly infiltrative nature of the TP80 cell lines recapitulating the invasive phenotype displayed by midline gliomas. The hematoxylin/eosin staining revealed that the tumor cells infiltrated the whole-brain parenchyma, including other structures, such as the cerebellum (Fig. 3e). TP54, CHLA-03-AA, and PBT-24 are very proliferative tumors with defined borders. The treatment with Delta-24-RGD resulted in smaller tumors and long-term survivors free of disease (Fig. 3f). E1A and hexon expression was detected in the brains of mice treated with the virus (Fig. 3g and Supplementary Fig. 4b, c). The long-term free of disease survivors did not display the expression of viral proteins.

Next, we assessed the antitumor effect of the viral treatment in already established tumor tumors generated by the CHLA-03-AA and PTB-24 (15 days after cell injection; Supplementary Fig. 4d). The median survival of the mice bearing CHLA-03-AA was 40 days, whereas the Delta-24-RGD-treated mice had an increased median survival of 14.5 days, resulting in 33% of long-term survivors free of disease (Log-rank test; $P = 0.005$) (Fig. 3h). Mice bearing PBT-24 cell line and treated with Delta-24-RGD also displayed a significant increase in overall survival (82 days), compared with the mice bearing the PBT-24 control (76 days; $P = 0.04$, Log-rank test) (Fig. 3i).

**Delta-24-RGD antitumor effects in murine DIPG cell lines.** Clinical trials investigating oncolytic viruses have uncovered that the efficacy of these viruses is partially owed to their potential to awaken the immune system$^{14,16}$. Therefore, our following step was to assess the immune response to Delta-24-RGD treatment in
Delta-24-RGD administration in murine DIPG-immunocompetent models. We have clinical evidence of the safety of Delta-24-RGD in supratentorial tumors. However, Delta-24-RGD administration into the pons could lead to inflammation and, if uncontrolled, fatalities specifically in DIPG patients. Therefore, we performed a dose escalation study to confirm the lack of toxicity in this model. Previously, we had performed kinetics studies to evaluate the most suitable number of cells for these experiments (Supplementary Fig. 5a). Mice bearing DIPG cells in the pons were intratumorally treated with virus at doses ranging from $10^6$ to $10^8$ pfu in increments of a half logarithm and followed for 15 days to assess toxicity. No dose analyzed caused a lethal reaction (Supplementary Table 2). In addition, we evaluated one injection versus three viral injections every other day at the maximum dose ($10^8$ pfu). Again, under this schedule, we did not observe any toxicity (Supplementary Table 2).
The animals did not display a significant weight variation related with PBS or Delta-24-RGD administration (Supplementary Fig. 5b). The histology analyses of the DIPG tumors in vivo in mice bearing either NP53 or XFM cells showed that after administration of Delta-24-RGD E1A could be detected in the tumor in both models (Supplementary Fig. 5c). In addition, administration of Delta-24-RGD significantly increased (two-tailed Student t test; \(P < 0.0001\)) the number of CD3-positive cells infiltrating the tumor mass compared with that in their counterpart controls (Fig. 5a, b and Supplementary Fig. 6a). Moreover, higher CD3+ levels corresponded to higher numbers of CD4+ and CD8+ cells (two-tailed Student t test; \(P = 0.0002, P = 0.0005\), respectively) and a decrease in FoxP3+/CD4+, which was not significant in the NP53-bearing mice (two-tailed Student t test, \(P = 0.07\)) but was significant in the XFM-bearing animals (two-tailed Student t test \(P = 0.03\)) (Fig. 5c–e, and Supplementary Fig. 6b, c). The mRNA expression analysis in the tumors treated with Delta-24-RGD revealed higher IFN gamma, CD8a, and CD4 mRNA levels compared with those in the PBS-treated tumors (Fig. 5f and Supplementary Fig. 6d). Moreover, splenocytes extracted from mice treated with the adenovirus were co-cultured with tumor cells. The splenocytes from the Delta-24-RGD-treated NP53 and XFM-bearing mice expressed significantly more IFN gamma than those extracted from the PBS-treated mice (two-tailed Student t-test; \(P = 0.001, P = 0.0005\), respectively) (Fig. 5g and Supplementary Fig. 6e), suggesting that there is a specific antitumor immune response.

Once we had proof of the capacity of the virus to trigger an antitumor immune response we evaluated the therapeutic efficacy of Delta-24-RGD in immunocompetent models. First, animals bearing NP53 subcutaneous tumors and treated with Delta-24-RGD displayed a significant lower tumor volume than those treated with PBS (Two-tailed Student t test; \(P < 0.05\); Fig. 5h). Next, we evaluated the antitumor effect of Delta-24-RGD in orthotopic immunocompetent mice bearing either NP53 or XFM DIPG murine cell lines. The survival analyses showed that Delta-24-RGD was able to significantly increase the overall survival of mice bearing NP53 or XFM (Log-rank Test; \(P = 0.01\) and \(P = 0.0002\), respectively) (Fig. 5i and Supplementary Fig. 6f). NP53-bearing mice treated with the virus showed a mean survival of 26 days in comparison with 21 days in PBS-treated mice and led to one (10%) long-term survivor mice (Fig. 5i). Most
importantly, Delta-24-RGD treatment of XFM-bearing mice led to 80% of long-term, compared with a median survival of 18 days in PBS-treated XFM-bearing mice (Supplementary Fig. 6f). To demonstrate the generation of an immune memory we performed the reimplantation of NP53 and XFM cell lines in the long-term survivors of the previous experiments. Our data showed that animals previously treated with Delta-24-RGD did not develop tumors (Fig. 5j and Supplementary Fig. 6g). To demonstrate that the antitumor response observed in the murine immunocompetent model was owing to an immune response, we performed similar experiments in athymic nude mice bearing either NP53 or XFM orthotopic tumors, as the replication of the virus is highly attenuated in murine cell lines. As expected, treatment with the virus did not present a survival benefit in this model (Log-rank test; \( P = 0.98 \) and \( P = 0.52 \), for NP53 and XFM, respectively) (Fig. 5k and Supplementary Fig. 6h). These data underscore the importance of the immune response in the antitumor effect of the virus.

In summary, Delta-24-RGD infects, replicates, and kills DIPG murine cells in vitro. Of importance, the in vivo administration...
triggers an immune response against DIPG tumors, leading to a significant increase in survival.

Discussion
The potential of oncolytic adenoviruses in the treatment of brain tumors has been shown by various studies. In fact, several clinical trials have evaluated the suitability of different oncolytic viruses in adult patients with glioblastoma and showed promising results. The ability of oncolytic virotherapy to trigger an immune response and the possibility of developing new armed viruses with molecules that modulate the immune response have opened new opportunities in cancer therapy.

Regarding pediatric brain tumors, several preclinical studies published during the prior 5 years have evaluated the efficacy of different oncolytic viruses, and most of these works focused on neuroblastoma and gliomas. For example, the intratumoral injection of HSV1716, which is an oncolytic herpes virus, showed safety and evidence of an immune response and viral replication in young cancer patients. In all of these studies, the oncolytic virus administration displayed no toxicity at the specific doses administered to the pediatric patients. Unfortunately, studies performed in pHGG and DIPG are limited, and the use of oncolytic adenoviruses in high-grade pediatric brain tumors continues to be vastly unexplored.

Delta-24-RGD has been widely studied in preclinical and, recently, clinical trials (DNX-2401), demonstrating its safe profile and efficacy against gliomas, but almost all studies were performed in adult patients (NCT00805376, NCT01956734, NCT02197169, NCT02798406). Recent studies have shown that pHGG, including DIPG, differ from their counterparts in adults. Therefore, assessing Delta-24-RGD efficacy in these tumors is timely, as therapeutic options remain suboptimal. In this work, we evaluate the suitability of Delta-24-RGD for the treatment of pediatric gliomas, specifically pHGG and DIPG. Our data show that all cell lines are susceptible to treatment with Delta-24-RGD. We observed that the adenovirus had better antitumor effects in pHGG and was correlated with a tendency to have a higher replication in these cell lines. In Supplementary Fig. S2c there was a statistically significant difference in the replication between the DIPG cell lines and the pHGG cells displayed. Delta-24-RGD contains a 24 bp deletion in the E1A region; thus, viral replication preferentially occurs in cells with a mutation in the RB pathway. Genetic studies have shown that the RB1 mutation is more frequent in hemispheric high-grade gliomas than brain stem tumors, which might explain the higher susceptibility to Delta-24-RGD replication and the higher cytotoxic effect observed in the pHGG cells compared with those in the DIPG cells. In fact, Delta-24-RGD has the strongest antitumor effect against PBT-24 cells, which is a pediatric cell line derived from a child with hemispheric high-grade glioma in which the RB protein was completely absent. Regarding DIPGs, in an elegant study performed by Becher's group they showed that H3.3K27M repressed p16 tumor suppressor (CDKN2A), leading to an increase in proliferation. In addition, other study has shown that PDGF, highly expressed in DIPGs, has the ability to stabilize E2F1. Therefore, even though maybe the RB pathway is not always aberrant at the genomic level (deletions of RB or p16/CDKN2A, amplifications of cyclins or CDKs), there is always E2F1 free owing to uncontrollable replication of tumor cells. Therefore, this fact warrants viral replication.

Recent studies have shown the importance of the adenovirus triggering of the immune response in the antitumor response achieved in patients. The lack of relevant models of these types of tumors until recently has also hindered the development of more-tailored approaches. The development of immunocompetent models that mimic the disease, at least partially, have allowed us to test our approach. Our study is important as we evaluate the immune response triggered by an oncolytic adenovirus in two different DIPG murine models. Our results show that Delta-24-RGD administration in the pons of animals is safe and that oncolytic adenovirus administration unleashes a potent immune infiltration in the brain that is mainly circumscribed in the tumor mass, most likely leading to tumor recognition by the immune system. Our results are consistent with data obtained by Dr. Juan Fuego’s group. The capacity of Delta-24-RGD to increase lymphocyte infiltration in the brain opens the opportunity for developing new combined strategies with immunotherapy. Immunotherapy developed against DIPG has shown limited results in patients owing to its incapability to cross the blood–brain barrier and reach the tumor along with the strong immunosuppressive microenvironment found in brain tumors. Among the immunotherapies that are currently being tested in solid pediatric cancer, are the immune checkpoint inhibitors and the CAR T cells. In addition to insufficient therapeutic effects, treatment using antibodies blocking CTLA-4 have induced the same side effects in pediatric patients that are observed in other tumors together with insufficient therapeutic effects. In the case of treatment utilizing CAR T cells, despite the encouraging responses observed in liquid tumors, the therapeutic effect has been modest in solid tumors and severe side effects have been reported including (1) the cytokine release syndrome also found in therapeutic monoclonal antibody (2) neurological toxicity, patients receiving CD19-specific CAR T cells presented symptoms of neurological damage, and (3) on-target/off-tumor recognition, when selected targets of CAR T cells are also expressed in normal tissue; a fatal side-effect example was a patient treated with HER-2/neu CAR T cell that developed a respiratory failure causing patient death. In contrast, administration of DNX-2401 in the context of clinical trials did not show any severe adverse event in either adults nor in DIPG patients (and our unpublished results). All together, the therapeutic effect of the Delta-24-RGD is a combination of the virus replication capacity and the anti-tumor effect triggered by it.

The encouraging results obtained in this work supported the opening of a phase I/II clinical trial at our institution to evaluate the safety and efficacy of Delta-24-RGD administration followed by RT in patients with newly diagnosed DIPG. Importantly, as a proof of principle, the administration of Delta-24-RGD has proven to be safe in the first cases and (our unpublished results), with no further toxicity associated with either the biopsy or injection of the virus, supporting the feasibility of this treatment. The lack of severe side effects in this trial is in accordance with the other Delta-24-RGD clinical trials where no adverse effect grade IV has been recorded.

In summary, here, we present evidence that Delta-24-RGD could constitute a therapeutic option for the treatment of pHGG and DIPG. The use of oncolytic viruses for the treatment of pediatric brain tumors alone or in combination with other strategies could provide a breakthrough in the treatment of these lethal diseases.

Methods
In silico analyses of pHGG and DMG/DIPG patient’s samples. Gene expression data from 220 pHGos and diffuse midline H3-K27M gliomas were collected and integrated as previously described. In brief, gene expression data from an Affymetrix U133 plus 2 microarray, Agilent 44K transcriptome arrays and RNA sequencing were annotated, normalized, and centered independently before being combined, loss renormalized to remove platform bias and median centered. The gene expression data were annotated by subgroup based on the integration of the whole-genome and whole-exome sequencing and application of the Heidelberg methylation classifier for CNS tumors, resulting in six major subgroups (H3.3G34R, H3.K27M, H3.K27M, IDH1, BRAF, PIK3CA, and
HGG, GBM, H3wt). To compare the absolute levels of expression in the Affymetrix microarray data, rank measurements of the expression of cell surface markers were compared with the present/absent thresholds of gene expression following RNA and mass normalization in the appropriate package in R.

Cell lines and culture conditions. Pediatric glioma CHLA-03-AA cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). CHLA-200 and SJ-GM2 were obtained from the Children’s Oncology Group (COG Cell Line & Xenograft Repository). The PBT-24 pHGG cell line was developed from a biopsy obtained at the University Clinic of Navarra from a 13-year-old boy. The tumor samples were obtained with signed informed consent. Ethical approval for the obtaining tumor samples was granted by the Ethical Committee of the University of Navarra (CE: Comité Etico de Investigación) under the protocol numbers CEI-UN 2016-014. Tumors were cut into small pieces, and they were enzymatically disaggregated. The cells obtained from dissociation were cultured with Rosewell Park Memorial Institute medium supplemented with 10% fetal bovine serum and 1% antibiotic. The cell lines obtained from the ATCC or COG were cultured following the manufacturer’s specifications. The SF188 cell line was kindly provided by Dr. Chris Jones (Cancer Research Institute, Sutton, UK). The DIPG cell lines TP54, TP80, TP83, and TP84 were generously provided by Dr. Eric Raabe (John Hopkins, Baltimore). All DIPG cell lines were maintained as neurospheres cultured in a specialized serum-free basal medium supplemented with a human neural stem cell proliferation supplement (Neuro Cult™ NS-A Proliferation Kit, #05751, STEMCELL Technologies), basic fibroblast growth factor and epidermal growth factor (20 ng/mL Sigma-Aldrich, St Louis, MO). All cell lines were maintained in a humidified atmosphere containing 5% CO2 at 37 °C. All cell lines were routinely tested for mycoplasma (Mycoalert detection kit; Lonza) and authenticated at the CIMA Genomic Core Facility (Pamplona, Spain) using DNA profiling. A description of the cell lines, including the H3 mutational status, has been summarized in the Supplementary Table 1.

The murine DIPG cell lines NP53 and XFM were provided by Dr. Becher (Northernwestern University, Illinois, USA)17,18. The cell lines were generated from DIPG arising in genetically modified mice. The NP53 cell line was generated from a tumor arising in a DIPG mouse model induced by PDGF-B signaling and INK4A and ARF loss18. The XFM cell line was generated from a tumor arising in a DIPG mouse model induced by PDGF-B signaling, TP53 loss, and ectopic H3.3K27M17. The XFM cell line was generated from a tumor arising in a DIPG mouse model induced by PDGF-B signaling, TP53 loss, and INK4A and ARF loss18.

Viral reactivation assays. The pHGG and DIPG cell seeds were seeded at a density of 2 × 10^5 cells per well in six-well plates and infected with 10 MOIs of Delta-24-RGD. After 3 days, the cells were collected, and the final amount of virus was determined by a method based on anti-hexon staining in HEK293 cells45.

Cell viability assay. The TP54, TP80, TP83, TP84, SU-DIPG IV, JHH-DIPG1, and JHH-DIPG16A cells were seeded at a density of 1 × 10^5 cells/well, and PBT-24, CHLA-03-AA, CHLA-200, and SJ-GM2 were seeded at a density of 2 × 10^5 cells/well in 96-well plates. On the following day, the cells were infected with Delta-24-RGD at different MOIs (5, 10, 25, 50, 100, and 300). The cell viability was assessed after 5 days using an MTS assay (Promega) as previously described46. The dose–response curves were analyzed using GraphPad software. The IC50 value was the median-effect dose (dose affecting 50% of the cells, i.e., 50% survival).

Immunoblotting. For the immunoblotting assays, the samples were subjected to sodium dodecyl sulphate-Tris-glycine gel electrophoresis. The membranes were incubated with the following antibodies: E1A, (1:1000; sc-430, Santa Cruz Biotechnology), E7, (1:1000; 19107-1, Cell Signaling), E7, (1:1000; 19107-1, Cell Signaling), JUN, (1:1000; ab2490, Abcam, Cambridge, MA) CD98 (1:1000, (DAW22) #99841 Cell Signaling, Danvers, MA) and FOX3 (1:400; clon IFK-16s, ref. 14–5773 eBiosciences, Thermo Fisher, Waltham, MA) following conventional procedures. For the immunohistochemical staining, Vectastain ABC kits (Vector Laboratories Inc., Burlingame, CA) were used according to the manufacturer’s instructions.

Statistical analysis. For the in vivo experiments, the data were expressed as the mean ± SD, and the comparisons were performed using two-tailed Student t tests. The in vivo cytopathic effect of Delta-24-RGD on pHGG and DIPG xenografts was assessed by plotting survival curves according to the Kaplan–Meier method. The survival in different treatment groups was compared using a log-rank test. The program GraphPad Prism 5 (Statistical Software for Sciences) was used for the statistical analyses.

Data availability. The data that support the findings of this study are available within the paper or Supplementary Information, or source data file or available from the corresponding author upon request.

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**Additional information**

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