OMTM, Volume *17*

Supplemental Information

Preclinical Toxicology of rQNestin34.5v.2:

An Oncolytic Herpes Virus with Transcriptional

Regulation of the ICP34.5 Neurovirulence Gene

E. Antonio Chiocca, Hiroshi Nakashima, Kazue Kasai, Soledad A. Fernandez, and Michael Oglesbee

Supplemental Figure Legends

Supplemental Figure 1. Schematic of the construction of the fHSVQ2-X series of bacterial artificial chromosomes (BAC) that contain the entire HSV1 genome with endogenous HSV1 ICP34.5 and ICP6 deletions and insertion of a large sequence utilized for transfer of exogenous sequences, such as the Nestin/hsp68 promoter/enhancer sequence (marked by X). See main text for more detail.

Supplemental Figure 2- Infection with rQNestin34.5 v.2. suppressed phosphorylation of eIF-2α in human glioma cell lines and human primary glioma cells, but not in human primary normal cells.

Supplemental Figure 3- Panel A: Cytotoxicity of rQNestin34.5v.2 against a panel of human glioma cells vs. a panel of normal cells. rQNestin34.5v.2 was added to a panel of glioma cells, U87ΔEGFR (U87dE), U87, U251 and OG02 glioma "stem-like" cells and to a panel of normal cells, human astrocytes (HA), human fibroblasts (Fibro.), human smooth muscle (SM), human skeletal muscle cells (SkM) and mouse astrocytes (MA). Cells were seeded on 6-well plates in complete medium prepared by following manufacturer's instructions for normal primary cells, BTSC medium for primary glioma cells or DMEM supplemented with 2% FBS for glioma cell lines and allowed to adhere. The medium for normal cells were changed to basal medium a few hours after cell preparation. Next day, viruses were added at MOI=0.1. rQNestin34.5v.2 inactivated with UV radiation was used as mock control. One hour after infection, cells were washed with glycine saline solution (10 mM glycine, 137 mM NaCl, 24.1 mM KCl, 0.49 mM $MgCl_2$. 0.68 mM $CaCl_2$, pH 3) followed by PBS to remove unattached viruses and fresh medium were added. Cells were

incubated at 37 C in an atmosphere containing 5% CO_2. Five days after infection virus cytotoxicity was measured as surviving cells counted with a Coulter counter (Beckman Coulter). The results showed that there were < or = 20% of glioma cells alive and > or = 80% normal cells alive five days after rQNestin34.5v.2 addition. Panel B: Replication potential of rQNestin34.5v2 in different cells. Control rHSVQ1 and wild-type F strain in 4 established glioma cell lines (U251, Gli36, T98G, and U87dE) , 3 glioma stem-like cells (G97, OG02, X12) and 4 normal cells (HUVEC, Skeletal muscle-SKM, Smooth musckle-SM, and Fibroblasts-Fibro. Cells (2×10^5) were plated in 6-well plates. The following day, cells were infected with rQNestin34.5v.2 (v2), parental rHSVQ1 (Q1), or wild-type F strain (F) at MOI = 0.1. One hour after infection, cells were washed with glycine saline solution (10 mM glycine, 137 mM NaCl, 24.1 mM KCl, 0.49 mM $MgCl_2$. 0.68 mM $CaCl_2$, pH 3) followed by PBS to remove unattached viruses and fresh medium were added. Cells were incubated for 3 days at 37 C in an atmosphere containing 5% CO_2. The cells and medium were collected and subjected to three cycles of freeze/thaw with dry ice/Ethanol and 37 C water bath. After pelleting cell debris by centrifuge (35000 x g, 10 min., 4 C), supernatant was transferred to new tubes and stored at – 80 C until titration. The titer of each sample was determined by conventional plaque assay with Vero cells. rQNestin34.5v.2 replication was higher than that of the ICP34.5-negative rHSVQ1 in 4 established glioma cell lines and 3 primary gliomas grown under stem-like condition, but similar to rHSVQ1 in 4 normal cells. F strain replication was higher in all, as expected.

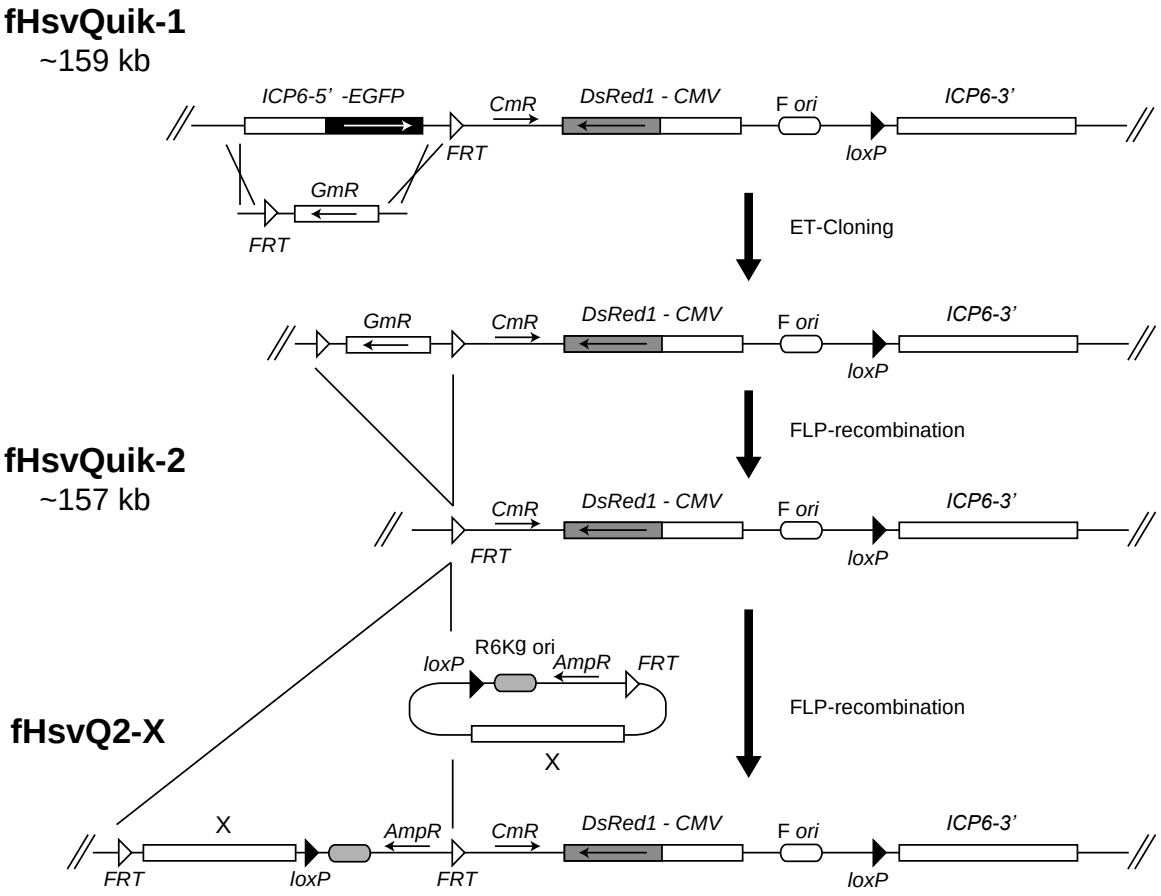
Supplemental Figure 4. H & E (Panel A), HSV IHC (B) and CD45 IHC (C) for brain from mouse 653. Panel A- Large brain tumor in frontal lobe demonstrates necrotic tumor with CPE (listed as 1) and encompassing area delineated by the arrow. Immediately adjacent

there are cells in the tumor area that appear swollen due to rQNestin34.5v.2 infection (listed as 2). Further out, there appears to be an area of uninfected tumor (listed 3). Panel B- IHC for virus antigen demonstrates abundant virus gene expression in tumor areas exhibiting CPE (zones 1 and 2). Panel C- IHC for CD45 shows the inflammatory tumor area, surrounding the area of necrosis, and also some associated inflammation in brain surrounding infected tumor. Mouse 653 underwent planned euthanasia, 4 days after rQNestin34.5v.2 injection in a brain tumor.

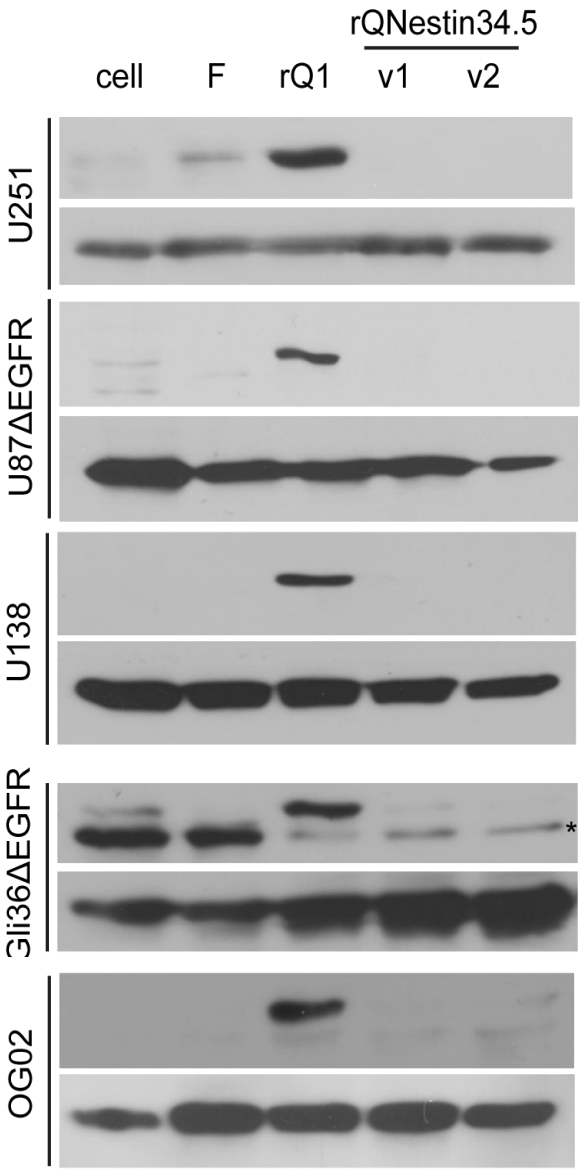
Supplemental Figure 5. Panel A: Series of HSV IHC (top) and CD45 IHC (bottom) for brain sections from mouse 655. Arrow points to HSV infected tumor located in close proximity to ventricular surface. Panel B: HSV IHC of ventricular surface showing some ependymal cells infected with HSV. Panel C: Presence of CD45+ cells associated with the infected ependymal cell layer.

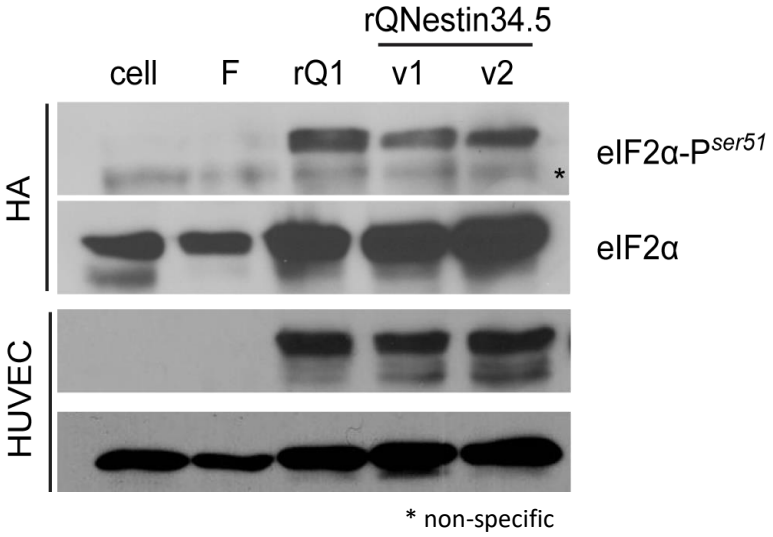
Supplemental Figure 6. Brain from mouse 770. The upper series shows the H and E stained sections. Arrows point at areas of tumor burden located on one side in ventral thalamus and brainstem. Lower series shows the large area of HSV immunohistochemical staining located on the contralateral side of the brain in the ventricle and periventricular areas, with additional involvement of the mesencephalon and hippocampus.

Supplemental Figure 1



Supplemental Figure 2





* non-specific

Human glioma cell lines:

- U251
- U87ΔEGFR
- U138
- Gli36ΔEGFR

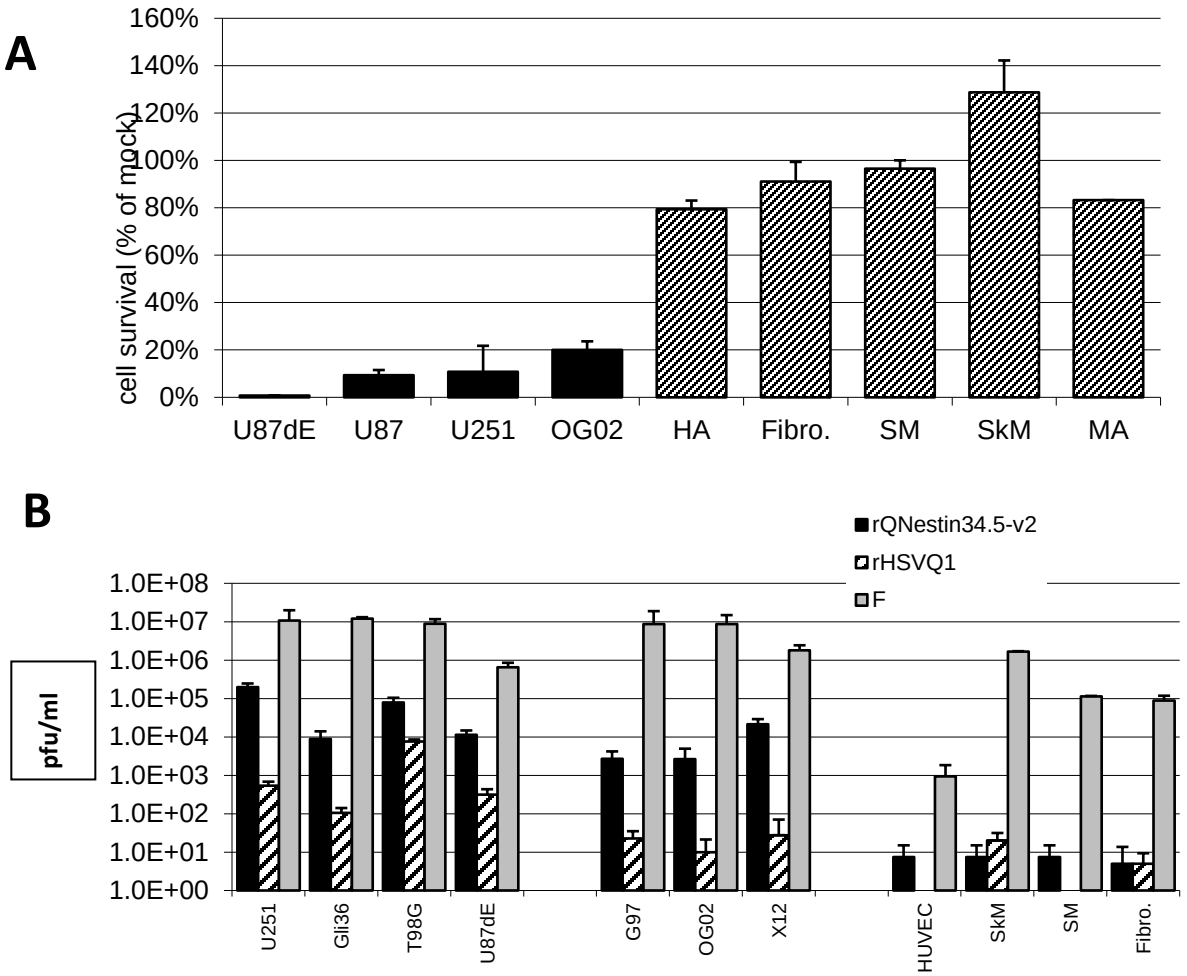
Human primary glioma cells:

- OG02

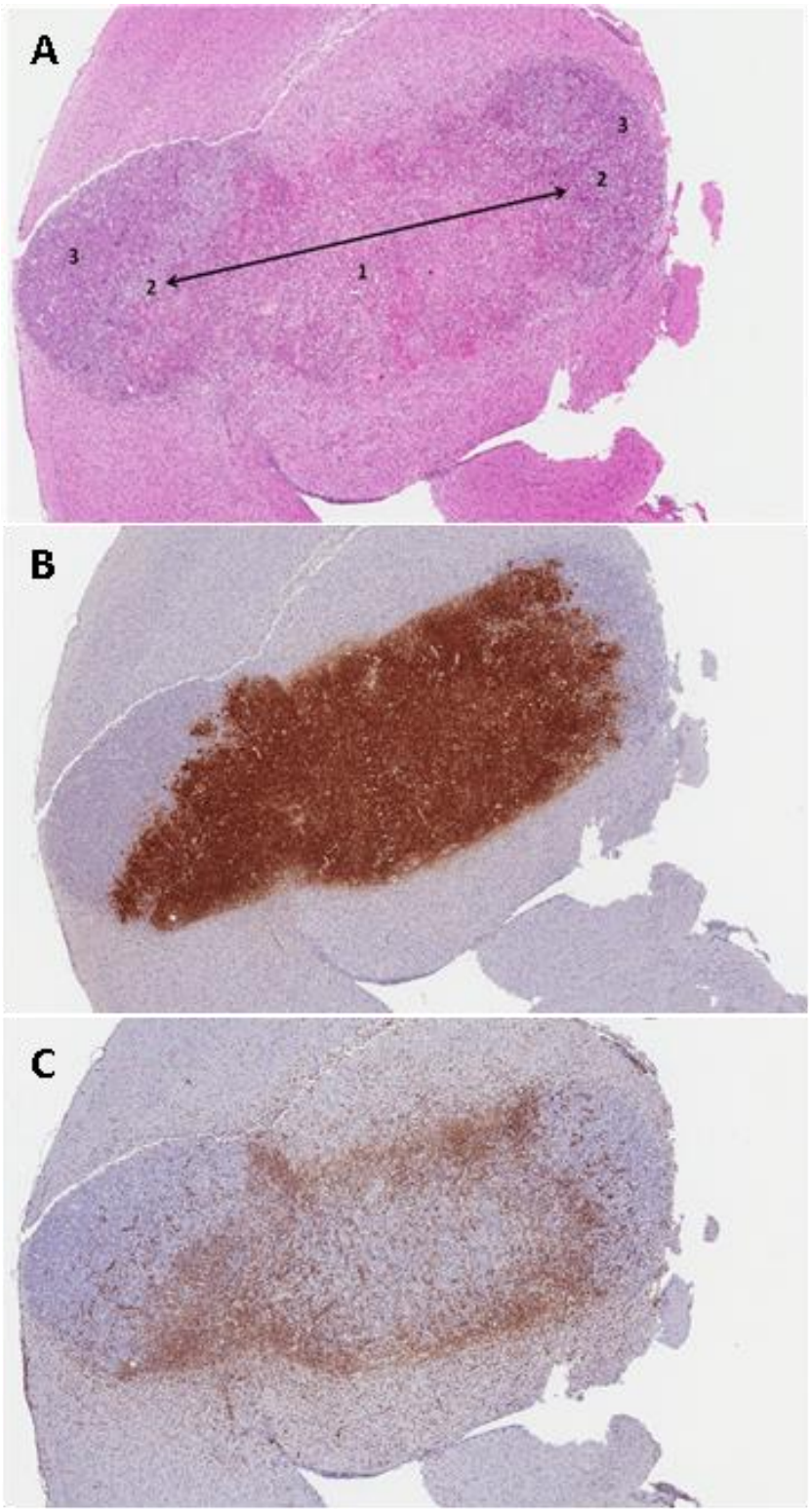
Human primary normal cells:

- human astrocyte (HA)
- human umbilical vein endothelial cells (HUVEC)

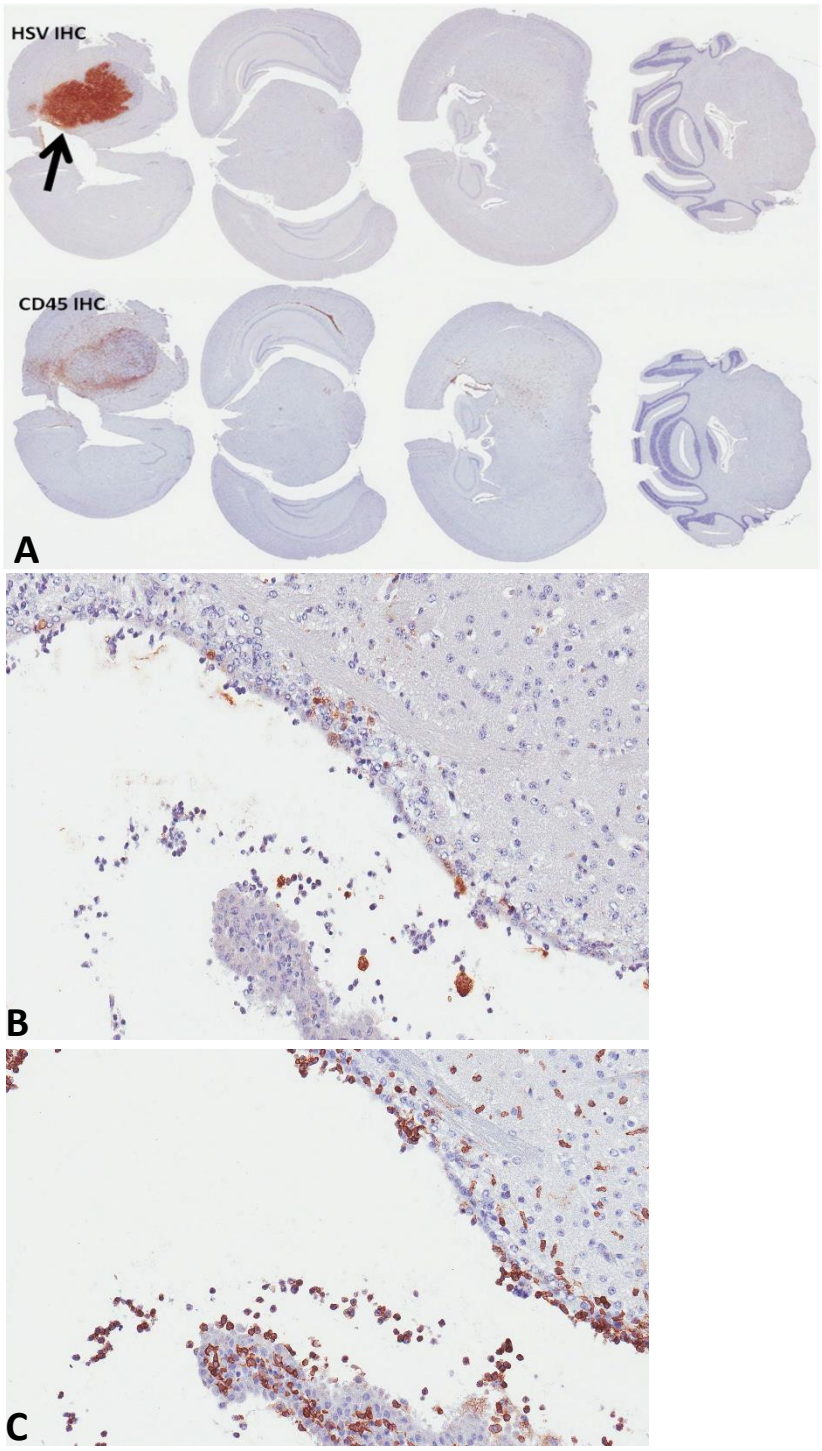
Supplemental Figure 3



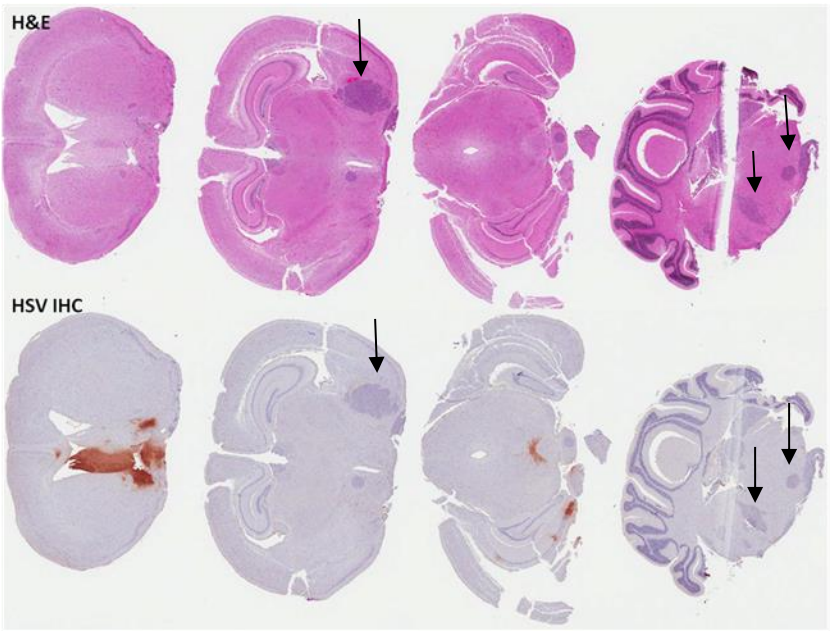
Supplemental Figure 4



Supplemental Figure 5



Supplemental Figure 6



Supplemental Table 1- Pilot dose-effect for rQNestin34.5v.2 or wild-type F strain lethality after different routes of administration in athymic and immunocompetent Balb/c mice.

Route	Strains	Virus	Dose of virus (PFU)	Dose of CPA (mg/kg)		
				0	300	200
IC	Athymic nude	**rQNv2**	1.0E+03		0 / 5	
	8-week		3.0E+03		1 / 17 @d4[A]	
			1.0E+04		3 / 25	0 /15
			1.2E+04	0 / 9	0 / 9	
			3.0E+04		0 / 4	
			1.0E+05		10 / 23	
			1.2E+05	0 / 9	0 / 9	
			3.0E+05	1/11@d51 [B]	2 / 13	
			1.0E+06	11 / 48		
			3.0E+06	8 / 29		
			1.0E+07	6 / 27		
			3.5E+07	2 / 8	5 / 8	
		F	1.0E+04	15 / 15		
		Vehicle		0 / 5	1 / 12	0/5
IC	Balb/c	**rQNv2**	1.0E+06		0 / 18	0/10
	8-week		3.0E+06		3 / 27	
			1.0E+07	1 / 28	4 / 28	0/24
		F	1.0E+03		4 / 4	
			1.0E+04	3 / 5		
			1.0E+05	19 / 22		
		Vehicle		0 / 15	0 / 5	0/ 5
IT	Balb/c	**rQNv2**	1.0E+05	0 / 5		
			1.0E+06	0 / 5	0 / 5	
	8-week		3.0E+06		0 / 5	
			1.0E+07	1 / 11	0 / 5	0/ 12
			2.0E+07	2 / 5		
		F	1.0E+05	1 / 5		
		Vehicle		0 / 4	0 / 3	0/ 3
	Balb/c	**rQNv2**	1.0E+07	0 / 9	0 / 9	0/11
	>6-month	F	1.0E+05	3 / 5		
		Vehicle		0 / 7	0 / 4	0 / 3
IH	Balb/c	**rQNv2**	1.0E+07	0 / 10	2 / 10	0 /12
			2.0E+07	0 / 5		
	8-week	F	1.0E+05	0 / 9	4 / 5	
		Vehicle		0 / 10	0 / 5	0 / 3
IV	Balb/c	**rQNv2**	1.0E+07	0 / 15	1 / 10	0 / 12
			2.0E+07	0 / 5		
	8-week	F	1.0E+05	0 / 4	2 / 5	
			1.0E+07	2 / 5		
		Vehicle		0 / 10	2 / 5	0 / 3

[A]Reason for mouse death at day4 is not known; [B]Reason for the single mouse death at day 51 was established to be due to viral-induced encephalitis.

Supplemental Table 2- Early Deaths by Group and Day for intracerebral administration in athymic mice brains– GLP Toxicity Assessment

Group	Animal – Death Status (Day)	
Control (group 1)	No early deaths	
CPA (group 2)	Males	Females
	215[A] – found dead (52)[B]	265 – found dead (54)
	218 – found dead (55)	264 – found dead (59)
	216 – found dead (59)	
4.2×10^4 pfu virus (group 3)	Males	Females
	309 –found dead (6)	365 – found dead (54)
	316 – found dead (53)	364 – found dead (55)
3×10^3 pfu virus + CPA (group 4)	Males	Females
	418 – found dead (53)	467 – found dead (53)
	413 – found dead (54)	

[A]Number refers to mouse identification number ; [B]Number in parenthesis refers to day of death

Supplementary Table 3- Unscheduled lethality in athymic mice with human GBM xenografts treated with oHSV+/- CPA or vehicle.

Group	Animal – Death Status (Day)	
Tumor	551-580[A]- Day 4 – 9[B]	
Tumor + virus	688[A] - found dead (5)[C] 680 – MSAC[D] (7) 684 – found dead (10) 676 – found dead (14) 678 – found dead (16) 669 – MSAC (18) 661 – MSAC (19) 675 – MSAC (19) 687 – found dead (20) 666 – found dead (22) 685 – MSAC (22) 665 – found dead (23)	670 – found dead (23) 689 – MSAC (23) 664 – MSAC (24) 673 – found dead (24) 691 – found dead (26) 663 – MSAC (28) 668 – found dead (31) 683 – found dead (32) 690 – MSAC (39) 682 – MSAC (41) 686 – found dead (48)
Tumor + virus + CPA	778 – found dead (5) 767 – MSAC (10) 761 – found dead (17) 766 – MSAC (17) 781 – found dead (17) 774 – found dead (19) 762 – found dead (20) 763 – MSAC (20) 770 – found dead (20) 785 – MSAC (22)	769 – found dead (23) 775 – found dead (23) 782 – found dead (24) 776 – MSAC (27) 780 – found dead (27) 768 – MSAC (28) 783 – MSAC (28) 773 – found dead (29) 779 – found dead (29) 777 – found dead (35)

[A]– Number refers to the animal identification number; [B]- 5 Mice were necropsied as scheduled. The remaining were found dead or humanely euthanized by Day 9 because of tumor growth.; [c]- Number in parenthesis refers to day of death or sacrifice after oHSV injection; [D]- MSAC = **M**ouse humanely **Sac**rificed due to moribund condition.

Supplemental Table 4. qPCR for rQNestin34.5 ICP22 gene (Mice with Brain Tumors inoculated with rQNestin34.5v.2)

Animal ID	Day	Bone Marrow	Blood	Brain	Heart	Kidney	Scalp Tumor	Gonads	Liver	Lungs	Spinal Cord	Spleen	Lymph Node
651	4	ND	-	4.6E6	SC	1.4E1	No	SC	1.6E2	1.2E2	SC	SC	SC
652	4	ND	4.7E3	1.4E7	2.2E4	ND	No	SC	1.1E2	ND	SC	SC	SC
653	4	ND	-	-	ND	ND	No	SC	1.2E2	ND	1.7E3	SC	SC
654	4	ND	1.7E4	-	SC	ND	No	SC	SC	ND	SC	SC	SC
655	4	ND	-	-	SC	1.3E1	No	SC	SC	ND	SC	5.9E1	SC
656	5	ND	9.6E3	6.6E5	SC	ND	No	SC	ND	ND	1.5E4	SC	SC
657	5	ND	6.3E4	4.7E6	SC	ND	No	SC	7.6E1	8.3E1	SC	SC	SC
658	5	1.3E3	-	4.2E7	SC	ND	No	SC	8.5E1	ND	SC	SC	SC
659	5	ND	2.3E5	-	SC	2.6E1	No	SC	ND	2.5E1	SC	SC	SC
660	5	ND	-	-	SC	ND	No	SC	3.1E2	5.5E1	SC	SC	SC
662	31	ND	-	-	ND	ND	No	ND	ND	ND	ND	ND	ND
664	24	-	-	-	-	-	No	-	-	-	ND	-	-
667	31	ND	-	4.4E5	ND	ND	No	ND	ND	ND	ND	ND	ND
668	31	ND	-	3.1E6	ND	ND	No	ND	0.93E1	ND	ND	ND	ND
671	31	ND	ND	-	ND	ND	No	ND	ND	ND	ND	ND	ND
672	31	ND	ND	1.5E5	ND	ND	No	ND	1.0E1	ND	8.9E1	ND	ND
673	24	-	-	-	-	-	No	-	-	-	5.9E2	-	-
674	31	ND	ND	2.4E5	ND	ND	No	ND	ND	ND	1.0E1	ND	ND
676	14	-	-	-	-	-	No	-	-	-	6.0E1	-	-
677	31	ND	-	-	ND	ND	1.4E5	ND	2.6E1	ND	ND	ND	ND
678	16	-	-	-	-	-	No	-	-	-	5.3E2	-	-
679	61	-	-	-	-	-	No	-	ND	-	ND	-	-
681	61	-	-	8.4E4	-	-	No	-	ND	-	ND	-	-
684	10	-	-	-	-	-	No	-	-	-	6.4E3	-	-

688	5	ND	-	1.3E7	SC	1.7E1	No	SC	ND	ND	SC	4.9E2	SC

ND: undetected or less than the lower limit of quantitation (5 copies/ 5µL purified DNA),
SC: Samples lost to contamination,

(-) Not performed see protocol and amendments,

No= No: no scalp tumor. Mouse 677 had tumor extruding in scalp.

Supplementary Table 5. qPCR for rQNestin34.5 ICP22 gene (Group 7 animals)

Animal ID	Day	Bone Marrow	Blood	Brain	Heart	Kidney	Gonads	Liver	Lungs	Spinal Cord	Spleen	Lymph Node
751	4	ND	2.6E4	6.9E6	SC	ND	SC	ND	ND	SC	SC	SC
752	4	ND	ND	8.4E5	ND	ND	SC	SC	ND	SC	SC	SC
753	4	ND	-	3.4E5	SC	ND	SC	ND	ND	SC	SC	SC
754	4	ND	ND	-	ND	ND	SC	1.3E2	1.4E1	SC	SC	SC
755	4	ND	-	-	SC	ND	SC	SC	ND	SC	SC	SC
756	5	ND	-	-	SC	ND	SC	1.0E2	ND	SC	SC	SC
757	5	ND	ND	-	SC	ND	SC	ND	ND	SC	SC	SC
758	5	ND	-	-	SC	ND	SC	ND	ND	SC	SC	SC
759	4	ND	ND	2.9E5	SC	ND	SC	ND	ND	SC	SC	SC
760	4	ND	-	7.4E6	2.6E1	ND	SC	ND	0.9E1	SC	SC	SC
778	5	ND	-	2.8E6	SC	ND	SC	ND	3.0E1	SC	SC	SC
761	17	-	-	-	-	-	-	-	-	ND	-	-
763	20	-	-	-	-	-	-	-	-	4.7E1	-	-
764	31	ND	-	1.8E7	ND	ND	ND	ND	ND	ND	ND	ND
765	31	ND	-	1.3E8	ND	ND	ND	ND	ND	1.0E1	ND	ND
767	10	-	-	-	-	-	-	-	-	7.6E5	-	-
769	23	-	-	-	-	-	-	-	-	ND	-	-
770	20	-	-	-	-	-	-	-	-	2.8E1	-	-
771	31	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	ND
772	31	ND	ND	-	ND	ND	ND	1.6E1	ND	1.5E1	ND	1.4E1
774	10	-	-	-	-	-	-	-	-	ND	-	-
775	23	-	-	-	-	-	-	-	-	ND	-	-

777	35	-	-	-	-	-	-	-	-	ND	-	-
779	29	-	-	-	-	-	-	-	-	ND	-	-
781	17	-	-	-	-	-	-	-	-	ND	-	-
784	61	-	-	3.7E4	-	-	-	ND	-	ND	-	ND

ND: undetected or less than the lower limit of quantitation (5 copies/ 5µL purified DNA), SC: Samples lost to contamination, (-) Not performed see protocol and amendments.

Supplemental Table 6 - Histologic Scoring Criteria

	Parameter	Score	Criteria
Brain	HSV Antigen Distribution	0	No detection of viral antigen
		1	Focal
		2	Multifocal within a plane of section
		3	Disseminated (involving two or more levels)
	Cytopathic effect (CPE)	0	No significant findings
		1	Early selective neuronal necrosis, limited in scope
		2	Selective neuronal necrosis
		3	Pannecrosis
	Inflammation (CD45 positive cells)	0	No CD45 immunoreactivity
		1	Focal
		2	Multifocal
		3	Disseminated
Glioma	Necrosis	0	No detectable necrosis on H&E stained sections
		1	Limited in scope (0-25%)
		2	25-50% necrosis (all tumor foci)
		3	50-75% necrosis
		4	75-100% necrosis
	HSV Antigen	0	No detectable viral antigen
		1	Antigen positive cells that are not necrotic
		2	Antigen positive necrotic cells
	Inflammation (CD45 positive cells)	0	No evidence of CD45 reactive cells associated with tumor.
		1	Mild increased in CD45 immunoreactivity
		2	Moderate increased in CD45 immunoreactivity
		3	Marked increased in CD45 immunoreactivity

Supplemental Materials and Methods

Engineering of rQNestin34.5v.2. Excision of the GFP-ICP6 fusion transcript from the initially engineered and published rQNestin34.5[1] to generate rQNestin34.5v.2 was not technically feasible. Therefore, we had to restart from different reagents and parental constructs. Overall, the step-by-step procedures and materials were published. [2] First, we had to re-engineer fHSVQuik-1, the bacterial artificial chromosome (BAC) that contains the F strain HSV1 sequence with the deleted diploid ICP34.5 genes. [3] **Supplemental Figure 1** depicts the re-engineering strategy: ET-cloning was utilized to recombine a *GmR* selection into the locus that contained the ICP6 promoter-EGFP-3' ICP6 cassette within the fHSVQuik1 bacterial artificial chromosome (BAC) maintained in E. coli. The PCR fragment that contains a gentamycin resistance (GmR) gene sandwiched between two FRT sites was amplified using the primer set; 5'-aataaagccactgaaacccgaaacgcgagtgttgtaacgtcctttgggcgggaggaagcc-3' and 5'-ggatcggccgctttacttgtacagctcgtccatgccgagagtgatcccggcggcggtcac-3' from the template pBSK-FRT-Gm DNA. This fragment was electroporated into R632KD cells which are stably transfected with the fHSVQuik-1 and pKD46 plasmids.[4] This led to the recombinatorial excision of the ICP6 promoter-EGFP-3' ICP6 cassette from hHSVQuik1. FLP-recombination then ensued to remove the selectable *GmR* marker, generating fHSVQuik-2. Next, a transfer plasmid encoding the Nestin/hsp68 promoter/enhancer-

ICP34.5 cassette[5] (marked as X in **Supplemental Figure 1**) was transfected in bacterial cells to recombine into fHSVQuik-2 (by Flp-recombination) to give rise to fHsvQ2-Nestin34.5. The fHSVQ2-Nestin34.5 BAC was then purified and transfected along with the pcnCre plasmid DNA into mammalian Vero cells by Lipofectoamin reagent (Invitrogen). Cre-Lox recombination then removed all BAC sequences in Vero cell. The new viral genomes generated viral plaques from infected Vero cells that were purified to generate rQNestin34.5v.2. Several single plaques were picked and purified. One of these was selected based on Southern blot analyses showing the expected genetic structure **(Data not shown)**. Sequencing confirmed removal of the ICP6-GFP fusion transcript and insertion of the Nestin promoter- ICP34.5 transcriptional cassette (**data not shown).** This new oHSV was thus designated as rQNestin34.5v.2.

In vitro studies. Western blots for the translation factor eiF2α and eiF2α–P^{ser51} were carried out in the following cells: human U251, human U87ΔEGFR, human U138 and human Gli36ΔEGFR glioma cells, human OG02 patient derived glioma cells[6], and primary human normal tissue cells: human astrocytes, human HUVEC umbilical vein endothelial cells, skeletal muscle cell, smooth muscle cells, and pulmonary fibroblast (Lonza, Switzerland). Media used for cell culture and assay are DMEM (Invitrogen) with 2% FBS for glioma cells and with 10% for HUVEC; Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen), 100 μg/ml penicillin/streptomycin, GlutaMAX (Invitrogen), and 50 μg/ml of both human EGF and FGF-2 (both from R&D Systems) for OG02 cells; and primary tissue cell were cultured using Lonza's media provided with each cell type. Cells were infected with F strain HSV1, r-HSVQ1, rQNestin34.5, or rQNestin34.5v.2 at a MOI

of 1. For western blot assay, cell lysates were prepared in lysis buffer, consisting of 50 mmol/L Tris-Cl (pH 7.4), 150 mmol/L NaCl, 2.5 mmol/L EDTA,0.5 % TritonX-100,40 µmol/L MG132, 5 mmol/L DTT, PhosSTOP (Sigma) and protease inhibitor cocktail (Roche), sonicated before centrifugation at 20,000 g for 10 minutes at 4ºC. Supernatants were used for immunoblot analyses using antibodies against eIF2α and phosphor-eIF2α at serine-51 (Cell Signaling Technology). The sample amount was adjusted based on Pierce BCA protein assay kit (Pierce, now Thermo). For cytotoxicity assays, human U87, U87ΔEGFR, U251 glioma cell lines and OG02 patient derived glioma cells as well as normal human astrocytes, fibroblasts, smooth muscle cells, skeletal muscle cells and mouse astrocytes were plated on dishes at 80% confluence without growth factors or FBS. rQNestin34.5v.2 was then added at a MOI of 0.1 the following day. The percentage of surviving cells was then assayed 72 hours later by Coulter counting. For replication assays, human U251, Gli36, T98G, U87ΔEGFR glioma cell lines and G97, OG02, and X12 patient derived glioma cells, as well as normal human umbilical vein endothelial HUVEC cells, skeletal muscle cells, smooth muscle cells and pulmonary fibroblasts were plated at 80% confluence. rQNestin34.5v.2, rHSVQ1 and F strain HSV1 were then added the following day at a MOI or 0.1. 72 hours later, supernatants and cell lysates were harvested, progeny viruses were purified by gradient centrifugation and then viral yields were assayed by plaque counting on Vero cells.

Non-GLP preliminary mouse studies. All experimental procedures using animals were carried out under an animal protocol reviewed and approved by the Ohio State University CCC's IACUCs and performed in accordance with relevant guidelines and regulations. Athymic (nu/nu) (6-8 weeks) and Balb/C (8 weeks or older than 6 months) were utilized

for pilot toxicity assays. Mice were anesthesized by mixed solution (90-100 µL per 20g body weight) of Ketamine (12mg/ml) and Xylazine (5mg/ml) in 0.9% Saline solution. For intracranial (IC) injections, rQNestin34.5v.2, F strain HSV1, or vehicle were injected intracerebrally at stereotactic coordinates (ventral 3.5-mm, rostral 0.5-mm and right lateral 2.0-mm from the bregma) using a stereotaxic apparatus. The injected doses in total 5µl PBS are shown in **Supplemental Table 1.** For intrathecal (IT) subventricular injections we stereotactically targeted the 1.0-mm postal, 1.0-mm lateral, 2.5-mm vertical position. . For intrahepatic (IH) injections, the needle was advanced into the peritoneal cavity before entering into the hepatic space and a total of 30µL was injected in the right hepatic lobe. Finally, for intravenous (IV) injections, tail veins were cannulated to inject the indicated amount of virus in a total of 100µL PBS. At the conclusion of the procedure, mice were awaken from anesthesia and then followed. When mice met early removal criteria (neurologic deficits or weight loss greater than 20%) they were euthanized with an overdose of Ketamine and perfused with 10% neutral buffer formalin following PBS. Brains were then removed for eventual histologic analyses.

Histological scoring- Tissues were scored according to criteria outlined in **Supplemental Table 6**. Inflammation is scored separately for tumor, which includes CD45 immunoreactivity in immediate proximity to the tumor, and brain regions distant from tumor and associated with foci of HSV antigen. Note that tumor volume was an estimate based upon serial cross-sectional areas of tumor and brain.

References

1. Kambara, H, Okano, H, Chiocca, EA, and Saeki, Y (2005). An oncolytic HSV-1 mutant expressing ICP34.5 under control of a nestin promoter increases survival of animals even when symptomatic from a brain tumor. *Cancer Res* **65**: 2832-2839.

2. Nakashima, H, and Chiocca, EA (2014). Modification of HSV-1 to an oncolytic virus. *Methods Mol Biol* **1144**: 117-127.

3. Terada, K, Wakimoto, H, Tyminski, E, Chiocca, EA, and Saeki, Y (2006). Development of a rapid method to generate multiple oncolytic HSV vectors and their in vivo evaluation using syngeneic mouse tumor models. *Gene therapy* **13**: 705-714.

4. Saeki, Y, Fraefel, C, Ichikawa, T, Breakefield, XO, and Chiocca, EA (2001). Improved helper virus-free packaging system for HSV amplicon vectors using an ICP27-deleted, oversized HSV-1 DNA in a bacterial artificial chromosome. *Mol Ther* **3**: 591-601.

5. Ali, M, Roback, L, and Mocarski, ES (2019). Herpes simplex virus 1 ICP6 impedes TNF receptor 1-induced necrosome assembly during compartmentalization to detergent-resistant membrane vesicles. *J Biol Chem* **294**: 991-1004.

6. Okemoto, K, Kasai, K, Wagner, B, Haseley, A, Meisen, H, Bolyard, C, *et al.* (2013). DNA demethylating agents synergize with oncolytic HSV1 against malignant gliomas. *Clin Cancer Res* **19**: 5952-5959.