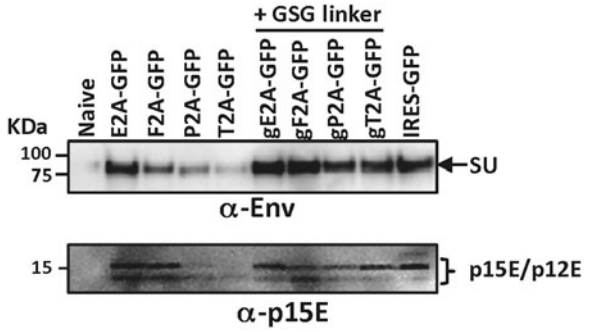
Supplementary Data

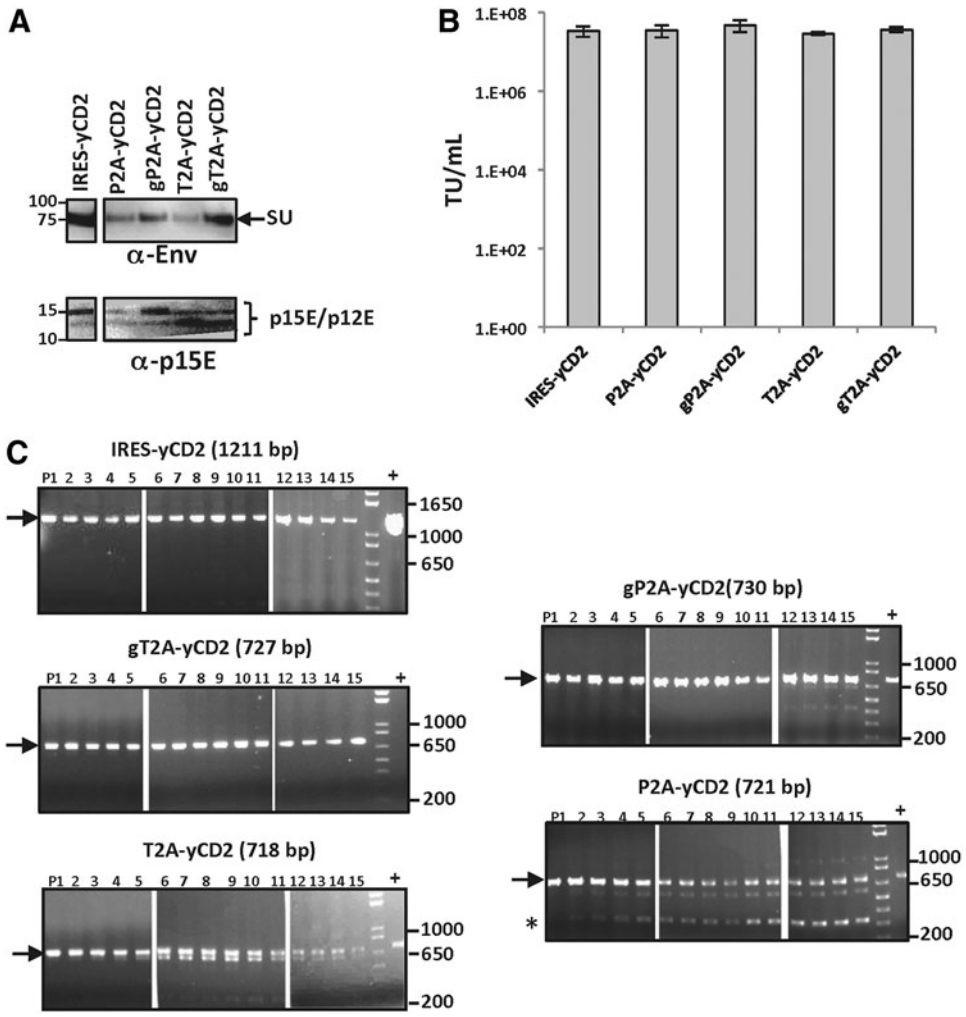
SUPPLEMENTARY MATERIALS AND METHODS

Cloning

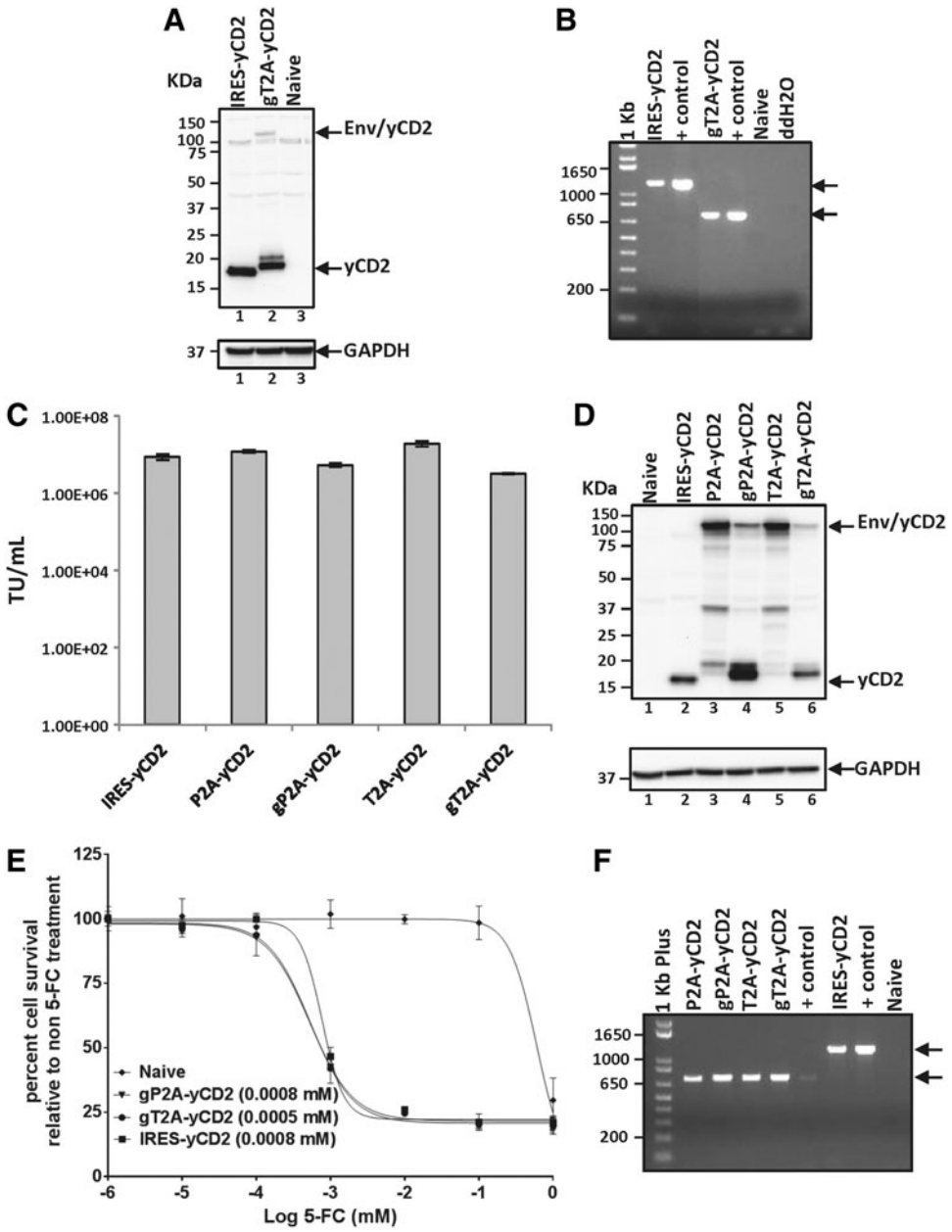
Coding sequences of each transgene were inserted into the AscI-NotI site in place of the transgene of the appropriate pAC3-2A vector backbone. The yCD2 transgene was PCR amplified from pAC3-yCD2 (*i.e.*, pAC3-yCD2 [Perez *et al*.]) using primers AscI-yCD2 (F: 5′-GATCGGCGCGCCTATGGTGACCGGCGGC AT-3′) and IRES (R: 5′-CCCCTTTTTCTGGAGA CTAAATAA-3′). HSV-1 TKO is derived from the HSV-1 thymidine kinase sr39tk gene (a mutant HSV-1 thymidine kinase with increased ganciclovir sensitivity (Black *et al*.) and human codon optimized. HSV-1 TKO was PCR amplified from pAZ.S1-tko (Inagaki *et al*., manuscript submitted) using primers AscI-TKO (F: 5′-CAGTGGCGCGCCTATGGCTTC ATATCCTTGCCAC-3′) and AscI-TKO (R: 5′-GTC AGCGGCCGCTCAGTTGGCTTC-3′). The double-transgene DNA fragments were synthesized (Genewiz, Inc.) to generate sequences of AscI-yCD2-GSG-P2A-hGMCSF-NotI and AscI-hGMCSF-GSG-P2A-yCD2-NotI, respectively, for subcloning into the pAC3-GSG-T2A backbone. The stop codons of the first transgene in each double-transgene constructs were removed to allow continuity of the open reading frame into the second 2A sequence and second transgene. Silent mutations were generated in regions of sequence homology of ≥6 bp between the two 2A peptide sequences and between two GSG linkers.



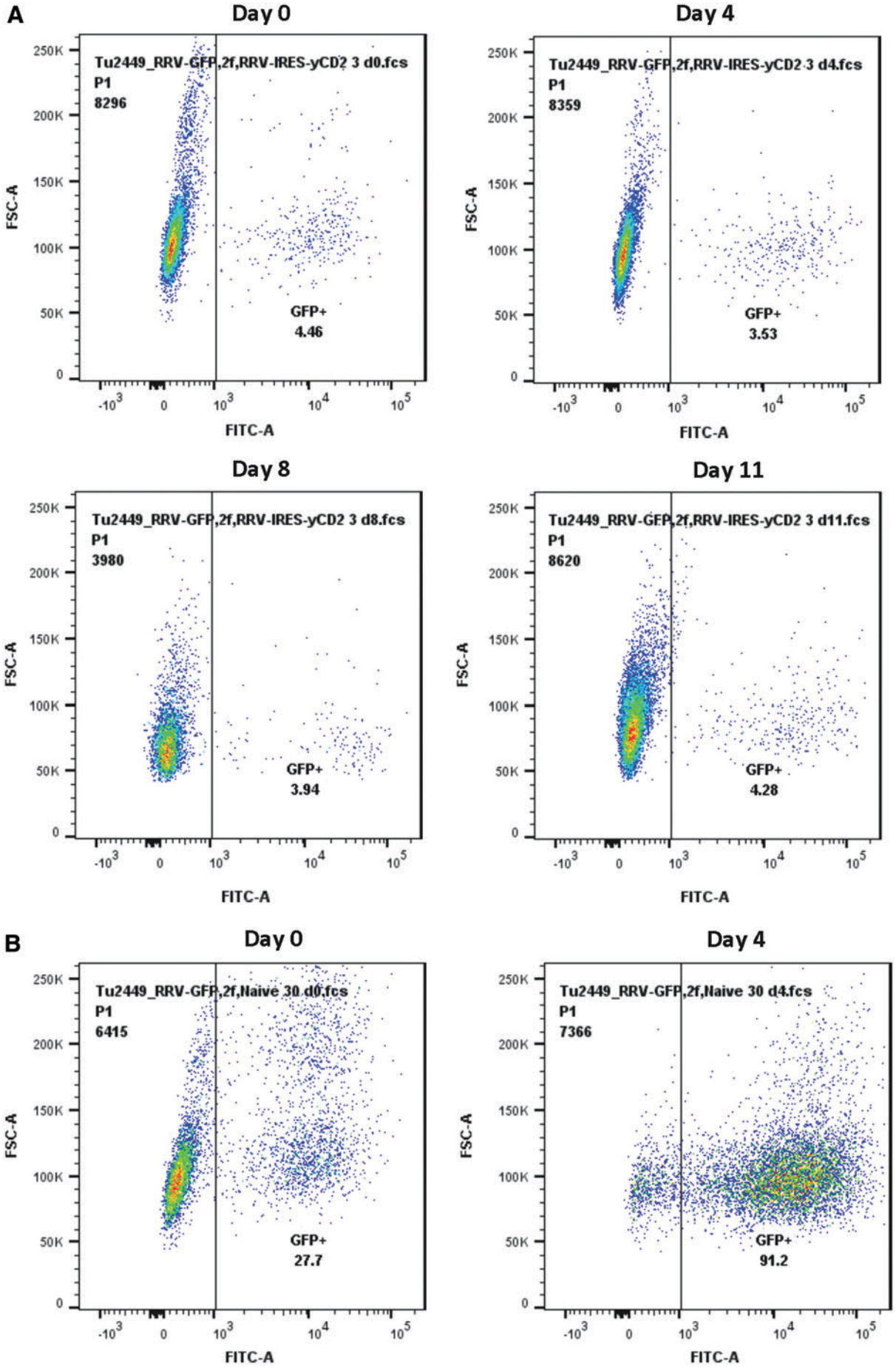
Supplementary Figure S1. Viral envelope protein expressed from RRV-2A-GFP configurations is properly processed and produce high titers. Western blot analysis of viral proteins produced from maximally infected U87-MG cells with the RRV-2A-GFP variants. One milliliter of 0.45 μm-filtered viral supernatants were centrifuged through a 20% sucrose cushion at 19,502 x g for 1 h. The viral pellets were re-suspended in 20 μL of XT loading buffer and resolved in a 4–12% gel. Membranes were incubated with anti-MLV Env antibody 83A25, which detects the SU domain of the viral envelope protein (top panel); anti-15E antibody 372 which detects unprocessed (p15E) and processed (p12E) TM domain of viral envelope protein (bottom panels); RRV-IRES-yCD2 was included as a positive control.



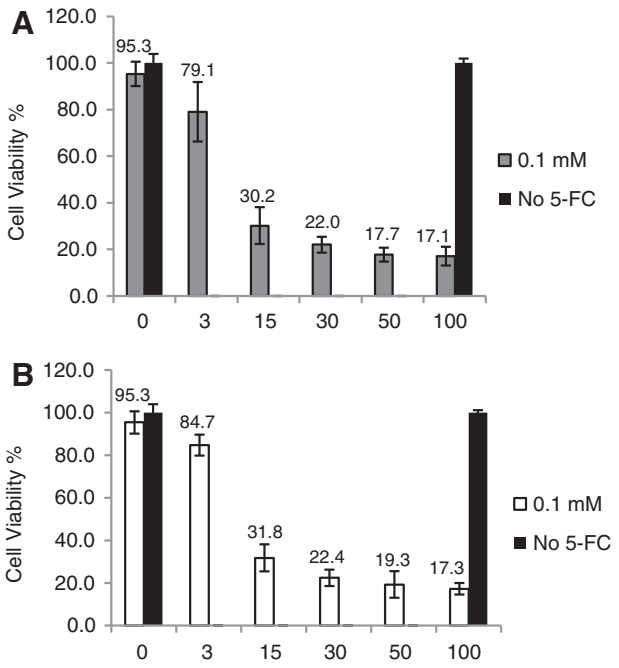
Supplementary Figure S2. Viral envelope protein expressed from RRV-2A-yCD2 configurations is properly processed and produce high titers. **(A)** Western blot analysis of viral proteins produced from maximally infected U87-MG cells with the RRV-2A-yCD2 variants. One milliliter of 0.45 μm-filtered viral supernatants were centrifuged through a 20% sucrose cushion at 19,502 x g for 1 h. The viral pellets were re-suspended in 20 μL of XT loading buffer and resolved in a 4–12% gel. Membranes were incubated with anti-MLV Env antibody 83A25, which detects the SU domain of the viral envelope protein (top panel); anti-p15E 372 antibody, which detects unprocessed (p15E) and processed (p12E) TM domain of viral envelope protein (bottom panels). RRV-IRES-yCD2 was included as a positive control. **(B)** Viral transduction titers of RRV-2A-yCD2 variants from RRVs collected from maximally infected U87-MG cells. RRV-IRES-yCD2 was included as a control and as a reference. **(C)** Genome stability of RRV-2A-yCD2 variants in U87-MG cells. Serial infection of RRV-2A-yCD2 variants in U87-MG cells over a total of 15 infection cycles: proviral DNA were harvested at the end of each infection cycle. Stability of the transgene cassette was determined by end-point PCR using primers (see Materials and Methods) spanning the transgene cassette. The numbers above each lane indicate infection cycle. Arrow indicates the size of the PCR product expected for the intact transgene cassette in each RRV-2A-yCD2 variants and asterisk indicates deletion in the transgene cassette. Positive controls are PCR products amplified from plasmid DNA corresponding to each RRV-2A-yCD2 variants. DNA molecular ladder (1 kb plus; Life Technologies) is shown in the first lane of the gel; numbers indicate the size of the ladders in base pair.



Supplementary Figure S3. Characterization of RRV-2A-yCD2 variants in Tu2449SC and Tu2449 cells. **(A)** Twenty micrograms of total protein lysates of infected Tu2449SC cells were loaded per well. Membranes were incubated with anti-yCD2 antibody. Detection of GAPDH using the anti-GAPDH antibody was included as a loading control. **(B)** Stability of 2A-yCD2 transgene cassettes from proviral DNA in maximally infected Tu2449SC cells (17 days post infection). Arrows indicate size of the PCR product expected for the intact 2A-yCD2 transgene cassette. Positive controls are PCR product amplified from plasmid DNA corresponding to each RRV-2A-yCD2 variants. DNA molecular ladder (1 kb plus; Life Technologies) is shown in the first lane of the gel; numbers indicate the size of the ladders in the base pair. **(C)** Transduction titer of RRV-2A-yCD2 variants produced from maximally infected Tu2449 cells. RRV-IRES-yCD2 was included as a control. **(D)** Twenty micrograms of total protein lysates were loaded per well. Membranes were incubated with anti-yCD2 antibody. Detection of GAPDH using the anti-GAPDH antibody was included as a loading control. **(E)** LD50 of 5-FC-mediated killing of maximally infected Tu2449 cells with RRV-g2A-yCD2 variants. Cells were cultured in the presence of 5 FC in different concentrations for 7 days. Naïve cells were included as a control for 5-FC cytotoxicity at each concentration. Cell viability was quantified by using MTS assay at 7 days post 5-FC addition and the percentage of cell survival was calculated relative to RRV-infected cells not treated with 5-FC. The data set represents one of the three independent experiments. Error bars indicate the standard deviation of the data set. **(F)** Stability of 2A-yCD2 transgene cassettes from proviral DNA in maximally infected Tu2449 cells (34 days post infection). Arrow indicates size of the PCR product expected for the intact 2A-yCD2 transgene cassette. Positive controls are PCR product amplified from plasmid DNA corresponding to each RRV-g2A-yCD2 variants. DNA molecular ladder (1 kb plus; Life Technologies) is shown in the first lane of the gel; numbers indicate the size of the ladders in the base pair.



Supplementary Figure S4. Tu2449 cells maximally infected by RRVs are resistant to super infection. **(A)** Tu2449 cells maximally infected with RRV-IRES-GFP or RRV-IRES-yCD2 were co-cultured at a fixed ratio of 3% Tu2449/RRV-IRES-GFP to 97% Tu2449/RRV-IRES-yCD2 cells and monitored for percentage GFP+ cells over a time course of 11 days. The measurement of GFP+ cells were conducted at day 0, 4, 8, and 11 by flow cytometric analysis. **(B)** As a control for viral replication competency of RRV-IRES-GFP, Tu2449/RRV-IRES-GFP infected cells were mixed with non-infected Tu2449 cells at 30/70 ratio and monitored for RRV-IRES-GFP spread as indicates by increased percentage of GFP+ cells 4 days post co-culturing.



Supplementary Figure S5. *In vitro* 5-FU bystander cytotoxicity assays. 5-FU-mediated cell killing was assessed by mixing different ratios of Tu2449 cells infected with **(A)** RRV-IRES-yCD2 + RRV-IRES-GFP or **(B)** RRV-gT2A-yCD2 + RRV-gT2A-GFP and cultured in the presence or absence of the prodrug 5-FC. 5-FC (0.1 mM) was added 24 h post cell seeding and replenished on day 3 and day 5. Numbers along the *x*-axis indicate the percent of yCD2 infected cells in the mixture. Cell viability was determined by MTS assay on days 3, 5, and 7 post 5-FC treatment. The data set shown represents cell viability from day 7 post 5-FC, and error bars indicate the standard deviation of the data set.

Supplementary Table S1. *Viral titer of RRV-2A-GFP configurations produced from transiently transfected 293T cells*

Vector	*Titer (TU/mL)*
IRES-yCD2	4.8 ± 0.5E6
IRES-GFP	1.7 ± 0.2E6
E2A-GFP	1.2 ± 0.3E6
F2A-GFP	1.6 ± 0.3E6
P2A-GFP	1.8 ± 0.3E6
T2A-GFP	3.3 ± 0.1E6
gE2A-GFP	1.7 ± 0.3E6
gF2A-GFP	1.3 ± 0.1E6
gP2A-GFP	1.3 ± 0.1E6
gT2A-GFP	2.7 ± 0.2E6

Titers were performed in triplicate, and the titer values represent means ± *SD* from one of two independent experiments.

Supplementary Table S2. *Viral titers of RRV-2A-TKO configuration produced from transiently transfected 293T cells*

Vector	*Titer (TU/mL)*
IRES-GFP	0.8 ± 0.1E6
P2A-TKO	1.1 ± 0.2E6
gP2A-TKO	1.1 ± 0.01E6
T2A-TKO	1.2 ± 0.1E6
gT2A-TKO	1.5 ± 0.2E6

Titers were performed in triplicate, and the titer values represent means ± *SD* from one of two independent experiments.

Supplementary Table S3. *Viral titers of RRV containing the different GSG-linked 2A peptides for multiple transgene expression*

Vector	*Titer (TU/mL)*
IRES-yCD2	3.4 ± 0.8E6
IRES-hGMCSF	4.1 ± 1.1E6
gT2A-hGMCSF-gP2A-yCD2	4.0 ± 0.3E6
gT2A-yCD2-gP2A-hGMCSF	4.0 ± 0.2E6

Titers were performed in triplicate, and the titer values represent means ± *SD* from one of two independent experiments.