Chemogenetic ON and OFF switches for RNA virus replication

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Therapeutic application of RNA viruses as oncolytic agents or gene vectors requires a tight control of virus activity if toxicity is a concern. Here we present a regulator switch for RNA viruses using a conditional protease approach, in which the function of at least one viral protein essential for transcription and replication is linked to autocatalytic, exogenous human immunodeficiency virus (HIV) protease activity. Virus activity can be en- or disabled by various HIV protease inhibitors. Incorporating the HIV protease dimer in the genome of vesicular stomatitis virus (VSV) into the open reading frame of either the P- or L-protein resulted in an ON switch. Here, virus activity depends on co-application of protease inhibitor in a dose-dependent manner. Conversely, an N-terminal VSV polymerase tag with the HIV protease dimer constitutes an OFF switch, as application of protease inhibitor stops virus activity. This technology may also be applicable to other potentially therapeutic RNA viruses.
**Viruses** have shown great potential as gene therapy vectors, as oncolytic viruses in cancer therapy and as vaccines in humans and animals. If replication of viruses or viral vectors could be externally regulated on demand, their individual and environmental safety would increase dramatically. While control of therapeutic DNA viruses has long been established using for example tetracycline-controlled transcriptional activation, effective external control of RNA viruses remains a challenge. In recent years, a small number of systems have been explored to provide some level of RNA regulation. Of those, RNA-aptazymes fused to a viral gene were shown to regulate virus replication over a range of <100-fold and viral transgene expression up to 30-fold. In addition, OFF-switch control of measles virus RNA replication was shown via small molecule-assisted shutoff (SMASh)–tags fused C-terminally to the viral P protein. An externally tunable ON switch for RNA viruses has so far only been shown with photo responsive elements which would be impractical for most clinical applications. None of these switches could completely block virus replication.

Vesicular stomatitis virus (VSV), a rapidly replicating negative strand RNA virus, has been widely studied as a vaccine vector, would be impractical for most clinical applications. None of these genes are separated by intergenic regions that enable the trans-splicing of several viral mRNAs from one template RNA. The human immunodeficiency virus HIV protease (PR) is comprised of 99 amino acids, is active as a homodimer and functions as an aspartyl protease. In HIV, it autocatalytically cleaves the polyprotein that is translated from the positive strand genome into functional proteins. HIV PR activity can be regulated by several clinically approved protease inhibitors (PIs) that are widely used in HIV therapy.

Here, we present a control mechanism for RNA viruses by fusing the autocatalytically active HIV protease dimer and its corresponding cleavage sites into or adjacent to essential viral proteins of VSV. Adding PI compounds either enabled or blocked virus replication, depending on the fusion design. The intramolecular insertion of the protease dimer in either the P or L protein of VSV constituted a functional ON-switch. In contrast, N-terminal fusion to L resulted in an OFF-switch when PI was applied. We performed in vitro and in vivo studies to confirm the dose-dependent and robust regulation of both, viral replication as well as viral transgene expression. The conditional proteolytic switch system allowed the control of RNA-virus replication from complete inhibition to nearly wild-type replication levels.

**Results**

**Generation and in vitro functionality of ON-switch viruses.** We first incorporated the PR dimer (prot) into the viral P protein, flanked by corresponding cleavage sites and flexible linkers (Fig. 1a and Supplementary Figs. 1a and 2a). To initially test for control of P-protein function in a mini-genome assay, the PR dimer was cloned into a P-expression plasmid at amino acid position 196 of P, which was previously reported to tolerate intramolecular insertions (Pprot). Cells transfected with this plasmid and co-infected with a P-protein-deficient VSV variant encoding DsRed showed red fluorescence in the presence, but not absence of the PI amprenavir (APV) (Supplementary Fig. 1b). This result indicates that the P fusion protein was able to functionally engage in the N-P-L replication complex only when PR was inactivated (Supplementary Fig. 1C). Next, the Pprot ON-switch construct was cloned into the VSV genome, generating VSV-Pprot-GFP (Fig. 1a). GFP expression, cytopathic effects, and viral replication were only observed in the presence of APV with dose-dependent regulation of VSV replication in the range from 300 nM to 100 μM APV (Fig. 1g, h). Conversely, in the absence of APV, P would be cleaved and the replication complex disassembled (Fig. 1b). Other clinically used PIs including saquinavir (SQV, 10 μM), lopinavir (10 μM), and indinavir (10 μM) also regulated VSV-Pprot-GFP (Supplementary Fig. 1e). Western blotting against HIV protease confirmed the proteolytic cleavage in the absence of PI with a band marking the HIV protease dimer at ~22 kDa, compared to a band at 54 kDa corresponding to the Pprot fusion protein in the presence of PI (Fig. 1c). To address genetic stability, we performed in vitro serial virus passages in optimal (10 μM) and suboptimal (1 μM) amprenavir conditions. After each passage protease inhibitor dependency was assessed by GFP expression, in the presence or absence of PI (Supplementary Fig. 3a). After 20 passages (P20), no amprenavir escape virus variants could be observed and functional PI dependency was confirmed by GFP fluorescence and viral plaque assay (Supplementary Fig. 3b). A protease-insert-spanning PCR was used to confirm the presence of a single band matching the size for an intact PR dimer insert (Supplementary Fig. 3c). Subsequent sequencing of the P-prot insert (not the whole viral genome) and alignment comparison with the parental plasmid construct revealed one mutation (protease 2: nucleotide G23A; amino acid R8K) in the construct with no effect on the proteolytic control of virus activity (Supplementary Fig. 3d). To test if the regulatable proteolytic switch also works on another element of the viral replication complex, we inserted the PR dimer into the VSV L-protein at amino acid position 1620 previously identified by our group. The resulting recombinant virus VSV-Lprot-GFP (Fig. 1d and Supplementary Fig. 1d) showed similar characteristics as VSV-Pprot-GFP and responded to APV in vitro in a comparable dose-dependent manner (Fig. 1i). Consequently, western blot analysis revealed an intact large L protein HIV protease dimer fusion protein of ~266 kDa in the presence of PI and a separated HIV protease dimer band of ~22 kDa in the absence of PI (Fig. 1c, f). Sequencing of VSV-Lprot-GFP revealed secondary mutations in L (Supplementary Fig. 4), that had emerged during virus rescue, analogous to findings in our previous study suggesting that these mutations could be essential for the efficient replication of L-insertion VSV. We next compared the single step growth curve kinetics of the two regulatable virus variants with the parental VSV-GFP. In the presence of 10 μM APV VSV-Lprot-GFP showed no significant attenuation in replication kinetic while VSV-Pprot-GFP showed mild attenuation in the range of 1–1.5 logs compared to parental viral titers (Fig. 1j). Analogous to the Pprot variant above, 20-fold serial passage in the presence of both optimal (10 μM) and suboptimal concentrations (1 μM) of APV did not produce any viral progeny lacking dependency on protease inhibition (Supplementary Fig. 5). However, as a proof-of-concept to further minimize the risk for potential reversion to wild-type VSV, a tandem insertion was made combining the PR dimer insertions into P and L. We had confirmed feasibility of this approach by generating a recombinant VSV with two fluorescent protein insertions, one in P and one in L. Based on this observation, a recombinant VSV with PR dimer insertions in P and L (VSV-P-Lprot-GFP) was generated (Supplementary Fig. 6a, b). This virus also showed PI dependency, expressing GFP and generating small plaques only in the presence of APV (Supplementary Fig. 6d). However, the dual switch construct was significantly attenuated (>2 log reduction in...
virus titers) compared to single insertion variants and was not further investigated in this study.

**ON-switch viruses are regulatable in vivo.** To validate the ON-switch viruses in vivo, we generated a luciferase-expressing variant VSV-Pprot-Luc. APV controlled bioluminescence activity of cells infected with this virus in a dose-dependent manner as well (Supplementary Fig. 7a). Intratumoral application of $2 \times 10^6$ infectious units (titrated via TCID$_{50}$) VSV-Pprot-Luc into subcutaneous U87 xenografts in nude mice resulted in an initial bioluminescence signal independent of PI application. This result...
can be explained by the presence of APV remnant from the virus preparations to block autoproteolysis during virus production and storage. Without further PI injections, the bioluminescence signal decreased significantly after 3 days (Fig. 2b) followed by loss of tumor control (Supplementary Fig. 7b). In contrast, in PI (APV + ritonavir (RTV)) -treated mice, luciferase signal plateaued for 17 days (Fig. 2a), and tumors were controlled in size (Supplementary Fig. 7b), confirming the in vivo functionality of the ON switch construct. Intratumoral treatment of U87 xenografts with the VSV-Lprot-GFP variant resulted in a significantly delayed tumor growth and survival benefit in the presence of PI (APV + RTV) compared to treatment without the compound mix (Fig. 2c, d). Western blotting against HIV protease displayed a large fusion protein, matching the combined size of the VSV polymerase, while only weak and spotty GFP fluorescence was observed within the injection site (Fig. 2j). In contrast, injection of VSV-Pprot-GFP was well tolerated with no signs of neurodegenerative abnormalities both in the presence or absence of PI (APV + RTV) co-treatment for an observation period of 9 days (Fig. 2e, f). Histological fluorescence analysis of brains harvested on the day of toxicity-related euthanasia after VSV-DsRed injection revealed extended spread of the virus through the brain parenchyma including midline crossing (Fig. 2h). In contrast, GFP expression from VSV-Pprot-GFP was restricted to the immediate lining of the injection needle track without any signs of further intracranial spread regardless of whether PI compound mix was systemically applied or not. As the CNS penetrability of APV is low, we performed a complementary neurotoxicity study with the VSV-Lprot-GFP virus applying an alternative PI treatment regimen with indinavir (combined with RTV; this combination has been shown to have a higher CNS penetration as well as providing 10 μM APV directly in the stereotactic injection volume. Despite the initial presence of PI in the injectate and the use of a PI regimen with higher expected CNS concentration, no signs of neurotoxicity or continued weight loss were observed (Fig. 2i and Supplementary Fig. 7c), and the virally induced GFP signal was still confined to the injection site (Fig. 2j).
neurotoxicity was reduced compared to parental VSV-GFP–treated mice (3 vs 6 out of 8 mice). The second group \((n = 8)\) was subsequently treated with a PI cocktail (SQV + RTV) 3x a day to initiate the OFF switch. No signs of neurotoxicity were observed in this group. After OFF switch activation tumor control was diminished and relapse occurred (Fig. 3g). In a parallel study, tumors treated with a single injection of VSV-GFP or VSV-Prot-OFF with or without PI co-treatment were harvested at 3, 7, and 10 days post virus inoculation. Histological analysis of VSV-Prot-OFF infected tumors revealed intratumoral spread, although diminished relative to wild-type VSV (Fig. 3h, i), suggesting that VSV-Prot-OFF is attenuated to some extent in vivo. In contrast,
PI treatment starting 3 days after virus inoculation abrogated spread of the virus, which was limited to a minor isolated region (Fig. 3i).

Discussion
We present here a regulatory switch putting the activity of a rhabdovirus under conditional control of an exogenously applied clinically approved small molecule. In the first configuration, the so-called ON-switch, virus replication is dependent on the presence of the compound. By changing the insertion site of the switch from an INTRA- to an INTER-molecular location, we can flip the effect of the compound from an ON- to an OFF-switch.

For optimal virus activity control, both ON and OFF switches were designed to interfere with an early stage of virus propagation by modulating proteins of the viral replication/transcription machinery. Hence our approach expands and complements the effectiveness and range of control of current methods of RNA virus regulation. In comparison to the aptazyme approach to expression of ON and OFF switches, our system focuses on viral replication control and therefore transgene expression levels are linked to viral replication. Conversely, the guanine aptazyme approach enables the control of transgenes independent from the virus, which might present an advantage over our system in certain applications. Hypothetically, the combination of RNA- and protein-based regulation approaches could hold potential for future developments of regulatable viruses, e.g., to regulate virus replication and transgene expression by different small molecules or to have multiple, completely different safety mechanisms in place as fail safe. Lastly, another advantage of RNA-based regulation via aptazymes is the independence from additional xenogenic proteins within the vector, which can be immunogenic or attenuate the virus.

In theory, the ON-switch system could also present as an additional environmental safety element, although not tested in this study. As virus progeny depend on the presence of PI, potentially shed virus is not active for productive infection of a new host. This could be of importance, when therapeutic or vaccine RNA viruses can cause or mimic notifiable animal diseases, as was recently discussed in a quantitative risk assessment estimate for oncolytic treatments with Seneca Valley virus (SVV)35. Of note, while not being directly a serious pathogen for livestock, wild-type VSV infection clinically mimics foot-and-mouth disease, and therefore exposure to animals should be limited36,37.

Neurotoxicity of wild-type based VSV has been abrogated in recent years by genetic engineering for chimeric variants38,39 or by naturally occurring relatives within the rhabdovirus family40 with some of these variants having already entered clinical testing41. Therefore, to address potential toxicities of replication-competent viral therapeutics in principle and to test our regulatable ON and OFF switch constructs in vivo, we had to focus on the wild-type VSV backbone, which reproducibly elicits neurotoxicity. However, application of wild-type-based VSV in the athymic nude mouse model did not elicit neurotoxicity, which can be immunogenic or attenuate the virus. We therefore employed an alternative xenograft model for testing the ability to reduce neurotoxicity after activating the OFF switch in VSV-Prot-Off-GFP: this made use of the highly immune deficient NOD-SCID host38. In this model, we observed clear neurotoxicity, which could be successfully prevented when triggering the OFF switch as late as 15 days after the oncolytic treatment start. The most consistent way to trigger neurotoxicity in mice, is the direct intracranial instillation of VSV. With our conditional proteolysis ON-switch approach, another factor comes into play, however.
Most, if not all, HIV protease inhibitors show only limited penetration into the CNS, thus not allowing PI-dependent activation of viral replication in the CNS. Even applying a PI with higher CNS availability, and adding PI to the injectate, no neurotoxicity and viral spread could be observed in our experiments. We believe this actually adds an additional layer of safety to the system, as the PIs required to maintain activity of the ON switch constructs and thus mediate neurotoxicity only reach a fraction of their serum levels in the central nervous system. This might be of importance for attenuated VSV variants, that are safe with peripheral application but have shown toxicity potential once entered into the brain.
Supernatants were collected at indicated time points and virus progeny titer was determined via TCID₅₀ assay (mice bearing subcutaneous human glioma G62 xenografts were treated intratumorally twice with 7 day interval (dotted line) with either VSV-GFP or OFF-GFP. Shows individual tumor growth kinetics. Right panel depicts the Kaplan-Meier survival curves (Log-rank (Mantel-Cox) test, p values from comparison to PBS control). In a parallel study, PI (SQV + RTV) treatment was initiated 3 days after single virus injection and tumors treated with VSV-GFP or VSV-Prot-OFF-GFP (n = 3) were harvested 1 week later and analyzed for virus spread using anti-VSV immunofluorescence staining. Representative images show wide intratumoral spread of VSV-GFP (h), disseminated spread of VSV-Prot-OFF-GFP without SQV (i) and isolated reduced virus staining of VSV-Prot-OFF-GFP under SQV treatment (j). Western blot experiments in panel (c) were performed three times. High-resolution fluorescence microscopy experiments in panel (d) were repeated twice with 2 wells per condition. 10 positions of each well were monitored automatically for up to 18 h post infection. Viral growth curve studies in panels (e, f) were performed two times times. Tumor growth and survival study in panel (g) were performed once. Source data are provided in the Source Data File.

Using the same autopoietic system but in a functionally different genome location, we also developed the reversal mechanism of protease-dependent OFF regulation, which works by the replacement of an intergenic region with the HIV protease dimer. In this construct the protease must be active to separate two viral proteins, similar as it does in HIV. Adding protease inhibitor in this construct leads to non-functional fusion proteins, which inhibit viral activity. The wild-type like characteristics both in vitro and in vivo suggest that this OFF Switch is attractive for therapeutic viruses, as in oncolytic virus therapy or in gene therapy with persistent viral vectors or replicons. Here the drug would only be given in the case of viral or transgene toxicity. Thus, the presented control mechanisms might prove particularly valuable for viruses that have residual toxicity or are armed with therapeutic cargos with a small therapeutic window, such as bispecific T-cell engagers (BiTEs), IL-12, TNF-α and others. Resembling in principle the SMASH-tag®, an advantage over this system is that the transgene (in this case GFP) is built into the switch, meaning a potential loss of the switch would necessarily entail the deletion of the potentially toxic transgene as well, i.e., would be self-disarming.

Although not the focus of the current study, we hypothesize that the presented regulatory principles can generalize to viruses other than VSV if certain conditions are met. As a central precondition to generate Prot-ON RNA viruses, target genes should be an essential part of the viral replication complex and be permissive for intramolecular insertions of transgenes. Such insertion sites have been described for several viruses, e.g., measles virus13, rinderpest virus14, canine distemper virus15, Ebola virus16, or rabies virus.10 Insertion herein of the HIV protease, or other proteases amenable to autopoietic activity, should generate a regulatable Prot-ON virus similar to the presented VSV-Pprot. Conversely, the Prot-OFF principle of virus activity control should also work for other viruses when two stipulations are met. For one, replication-essential genes need to tolerate minor N- or C-terminal tags comprised of the residual amino acids of the protease recognition sites after cleavage. However, without such autopoietic cleavage these gene products would have to remain non-functional fusion constructs in the presence of large tags such as fluorescence proteins.47,48. While numerous fluorescent protein fusion tags have been described on viral structural proteins48,49, studies on terminal fusions to proteins involved in viral replication are limited.50. Here, additional studies might be necessary to evaluate a virus’ suitability for the OFF switch.

Taken together, the Prot-OFF and Prot-ON systems presented here allow effective control of the oncolytic virus VSV in the host by several drugs that have a long-term clinical safety record. Our chemoenetic proteolysis approach might be used for other therapeutic or vaccine viruses as well. Thus, this technology can potentially support a broad range of applications and provides the basis for future developments of safer vaccines and virotreatments.

Methods

Plasmids and viruses. The coding DNA sequence for the phosphoprotein with a linked-dimer protease in position aa196 and the flanking sequences of the VSV N and M (N-Pprot-M) were synthesized by GeneArt/Thermo Fisher Scientific (Regensburg, Germany). The protease linked-dimer construct was flanked by (GGSG)₃ linker sequences to allow spatial separation between the P and the protease dimer (Supplementary Fig. 2a). P expression plasmid was cloned by digestion of a standard pUC19 expression vector (GenBank # AJ318514) and the insert-containing vector (GeneArt) with XbaI and Bst1107I followed by ligation with a T4 ligase (NEB, Frankfurt, Germany). In all, 293T cells were transfected with this pPro construct using TransIT®-LT1 transfection kit (Mirus Bio LLC, Madison, WI, USA) and infected with a VSV-AP variant.53. The VSV-AP was equipped with a red fluorescent protein as reporter gene.

Wildtype-based recombinant VSV Indiana strain and VSV-GFP were described previously.42,54. To generate regulatable VSV variants, the N-P-M genome part of a VSV strain P52W was replaced by N-Pprot-M (synthesized by GeneArt). GPF at position 5 was used as marker gene. All PCR primers are listed in detail with sequence and target region in Supplementary Table 1. N-Pro-M was amplified by PCR (primers N-35nt-before-BstZ17I and P-35nt-after-XbaI) containing sequences overlapping 2 restriction enzyme sites (XbaI and Bst1107I) to the full-length VSV genome. The construct was then generated by Gibson assembly cloning, resulting in the ON switch VSV-Prot-GFP. VSV-luciferase vector was generated by cloning a pltinus pyralis firefly luciferase cassette, amplified via PCR (primer pair Xhol-luc-for and -rev) into pGL4.51 (Promega #E1230) via XhoI/Nhel restriction sites. VSV-P-Prot-luciferase was cloned analogously to the VSV-Prot-GFP variant using the VSV-luciferase vector. To generate regulatable VSV-Lpro, the linked protease dimer construct was inserted at L protein position 1620 by four-fragment Gibson assembly as previously described.42. In brief, the large vector fragment was provided by restriction enzyme digestion with enzymes SfiI and FseI of VSV-GFP (fragment 4). The linked protease dimer (fragment 1) was PCR amplified by primers GS/GSGG-prot-for and -rev. Linker sequences surrounding fragment 1 that were lost by vector digestion (fragments 2 and 3), were generated via PCR on a VSV vector. Fragments 2 and 3 received overhangs to the (GGSG)₃ linker at the 5′ end with
prot-OFF viruses. Viruses were rescued using a helper virus-free calcium phosphate method (TD50) of restriction enzyme BstZ17I. P-Lprot was cloned without (GSSG), linkers in L to restrict spatial movement of the protease dimer and avoid potential interaction with the linked-dimer in P. Fragments 2 and 3 in this variant therefore received overlaps to the protease cleavage site at the 5′ end with primer L-1620-prot-rev and 3′ end with primer L-1620-prot-for. The protease dimer with primer pair prot-for/prot-rev. The OFF switch constructs were generated as follows. The VSV-GFP plasmid was digested with Msel (inside GFP) and Hpal (inside L) to remove the intergenic region between GFP and L. Three overlapping fragments were generated by PCR amplification of the two new terminal parts of GFP (Fragment 1: primers GFP-for/BstZ17I-forward before Msel-for to GFP-GSSG-rev or GFP-cut2-rev), to insert the HIV protease dimer (Fragment 2: primers (GSSG)-prot-for to (GSSG)-prot-rev and prot-for to prot-rev) and to replace the N-terminal part of L (Fragment 3: GSSG-L-for or cut2-L-for to N-term-rev). Fragment 1 had an overlap to the vector VSV-GFP and an overlap to the N-terminal of the protease insert, either with a (GSSG), linker or without a linker sequence. Fragment 2 was either an HIV protease dimer with or without (GSSG), linker. Fragment 3 had an overlap with the C-terminus of the protease insert and a 30 bp overlap to L. The three fragments were first joined by a fusion PCR. The resulting fusion fragment was cloned into the digested vector by Gibson assembly. In vitro comparison of both flexible and rigid Prot-OFF viruses revealed that the construct without linker was more sensitive to SQV control and hence was chosen for subsequent in vivo experiments. For sequence confirmation, viral RNA was purified by Viral DNA/RNA Kit, qeQGold (Peqlab/VWR, Darmstadt, Germany). Subsequently, cDNA synthesis was performed with RevertAid First Strand cDNA Synthesis Kit (ThermoFisher, Vienna, Austria). PCR was performed with the following Hot Start High-Fidelity DNA Polymerase (NEB).

For in vitro testing, the ON switch systems, serial virus passage on BHK-21 cells was performed in optimal (10 µM) and suboptimal (1 µM) amprenavir conditions. After each passage protease inhibitor dependency was confirmed by performing a sample on 293-VSV cells and assessing whether the plaque was visible. For testing genetic stability of the ON switch systems, serial virus passage on BHK-21 cells was performed in optimal (10 µM) and suboptimal (1 µM) amprenavir conditions. After each passage protease inhibitor dependency was confirmed by performing a sample on 293-VSV cells and assessing whether the plaque was visible. For testing genetic stability of the ON switch systems, serial virus passage on BHK-21 cells was performed in optimal (10 µM) and suboptimal (1 µM) amprenavir conditions. After each passage protease inhibitor dependency was confirmed by performing a sample on 293-VSV cells and assessing whether the plaque was visible.
or control buffer. PI treatment (APV + RTV) was initiated 1 h before virus application. To test the OFF-switch system, G62 xenografts in NOD/SCID mice with a median volume of 0.07 cm³ were intratumorally injected with 20 µl containing the maximum feasible dose of 2 x 10³ TCID50 of the indicated VSV variants or control buffer. Virus treatment was repeated seven days later. PI treatment (SQV + RTV) was initiated either 8 days post second virus injection for studies on tumor control or 3 days post single virus treatment for histological studies. Bioluminescence in vivo imaging of luciferase-expressing VSV variants was performed with an IVIS® Lumina II (Perkin Elmer, Waltham, MA) system as previously described39. In brief, NMRI nude mice (Janvier) bearing subcutaneous U87 tumors were treated intratumorally with 2 x 10⁶ TCID50 of luciferase-expressing VSV-PpoL-Luc. In all, 1.5 mg o-aminophenyl (Promega, Madison, WI, USA) was administered intraperitoneally 15 min prior to measurements. Luminescence data acquisition and analysis was performed using Caliper Live Sciences-Image software (4.3.1.). Tumors were measured with a caliper and volume was calculated using the formula: length x width² x 0.4. Human endpoints were defined by either tumor size >1.5 cm³, weight loss >20% or clinical signs of neurotoxicity. Mice were euthanized via cervical dislocation after isoflurane anesthesia. For histological analysis, VSV variant-treated tumors were harvested 10 days post inoculation and fixed in 4% PFA at 4 °C over night, dehydrated in 30% sucrose, and sliced at –20 °C into 7-µm-thick sections (Microm HM560, ThermoFisher Scientific, Austria). 2% BSA (Roth, Karlsruhe, Germany) and 0.01% Triton-X. Primary antibody a-VSV-N (10G4, E8B009, raised in mouse, Kerafast, Inc., Boston, USA) was diluted 1:250 and incubated over night. Secondary antibody goat-a-mouse IgG2a Alexa 594 (A-21135, ThermoFisher, Vienna, Austria) was diluted 1:750 and incubated for 30 min. Histological sections were analyzed using a fluorescence microscope (Eclipse Ti, Nikon CEE GmbH, Vienna, Austria).

**Visual presentation**. Contrast and color of the photomicrographs were linearly adjusted with Adobe Photoshop. Cartoons and schematic illustrations were generated with BioRender (biorender.com), Adobe Photoshop, and Inkscape 0.92.3.

**Statistical analysis.** GraphPad prism software (GraphPad Software, Inc., La Jolla, CA) was used for statistical analysis and data presentation. Statistical significance was determined by Student’s t test and analysis of variance (ANOVA) with Turkey’s multiple comparison correction. P values of <0.05 were considered statistically significant. Kaplan–Meier survival curves were compared using the Log-rank (Mantel-Cox) test. Statistically significant differences were indicated as follows: *p < 0.05; **p < 0.01; ***p < 0.001.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

All pertinent data to support this study are included in the manuscript and supplementary files. Source data are provided with this paper. Further data supporting the findings are available upon request. The sequences of the prot constructs have been deposited at NCBI's GenBank with the accession codes MW316665 (not codon optimized) and MW316666 (codon optimized). Recombinant virus variants described herein can be made available with a Material Transfer Agreement (MTA). Requests require the review and approval of ViralTheapeutics GmbH to confirm compliance with intellectual property and confidentiality obligations. Source data are provided with this paper.

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Author contributions

D.v.L. conceived the initial concept. E.H., G.W., and D.v.L. designed the experiments. E.H., J.K., L.E., and T.N. conceived cloning strategies. H.G.K. assisted in prototype construct design and PI application strategy. E.H. and B.H. generated recombinant viruses. E.H., B.H., A.R., I.B., C.U., and G.W. performed experiments. E.H., H.G.K., G.W., and D.v.L. wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

A patent application relating to all aspects of the manuscript has been filed under the application number 19181717 by Boehringer Ingelheim International GmbH (application date 21 June 2019). E.H., J.K., L.E., and T.N. conceived cloning strategies. H.G.K. assisted in prototype construct design and PI application strategy. E.H. and B.H. generated recombinant viruses. E.H., B.H., A.R., I.B., C.U., and G.W. performed experiments. E.H., H.G.K., G.W., and D.v.L. wrote the manuscript. All authors declare no competing interests.

Additional information

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