

Creating bottom-up RNA transfer vehicles from synthetic protein assemblies

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Abstract

Evolution guides biological systems to populate ecological niches, with viruses being one of the most successful examples of that principle. Viruses evolved over billions of years for the efficient transfer of nucleic acids. Although highly diverse, most viruses converged toward a remarkable similarity in the size and shape of their capsids. In contrast, generative models for protein design enable the creation of protein architectures that are absent in nature. Here, we investigate whether AI-designed protein assemblies can be functionalized to construct nucleic acid transport vehicles that are independent of evolutionary trajectories. By combining natural protein domains with synthetic protein assemblies, we create more than a hundred bottom-up RNA transfer vehicles with unique sizes and shapes. These novel vehicles surpass the RNA transfer efficiency of widely used delivery vehicles by several orders of magnitude. Additionally, we demonstrate that their tropism can be programmed by incorporating computationally designed peptide binders and apply them to deliver various therapeutically relevant cargo RNAs, such as Gene Editors, into a wide range of cellular models. We show the *in vivo* biodistribution of one of these vehicles in a mouse with close to single-cell resolution and use it to perform a gene editing strategy for Duchenne muscular dystrophy in a pig. Our work demonstrates how proteins created by generative AI can be harnessed for the rational engineering of biological systems with desired properties by overcoming the limitations of natural protein diversity.

Introduction

Selective pressure is the driver of biological systems towards a local minimum on the evolutionary landscape, enabling them to occupy an ecological niche¹⁻³. Conceptualizing evolutionary trajectories as a vector, defined by the combination of selective pressures, raises the question of how these trajectories change if certain elements are altered. Homoplasy is one example of this concept in which similar selection pressure can lead to the convergence of biological features^{4,5}. Viruses, for instance, are highly optimized vehicles for gene transfer, but despite their diversity, they converged on similar features. Most viruses rely on large supramolecular protein capsids, composed of thousands of subunits, which self-assemble mostly into icosahedral or helical symmetries to enclose and protect their genome⁶⁻⁹. Viral capsids are selected for their resilience in harsh environmental conditions. However, when repurposed as vectors for genetic engineering, they are handled in controlled environments. This raises the question of whether features evolution selected for might become unnecessary or even disadvantageous when placing a biological system in a new context outside of its original ecological niche. Recently developed AI models for protein design could be harnessed to explore this question. These models create protein structures that are physically feasible but do not occur naturally¹⁰⁻¹⁴, enabling the manipulation of the evolutionary vector with non-natural protein architectures that are free of the constraints of natural proteins.

Here, we exemplify this idea by constructing bottom-up RNA transfer vehicles consisting of natural protein domains, combined with AI-designed synthetic protein assemblies. We name these novel RNA carriers *Synthetic Transfer Vehicles* (STV). STVs are distinct from known natural RNA transfer vehicles by

exhibiting unique characteristics, such as cyclic and dihedral symmetries, open structures, and low complexity of the assembled protein. We develop a multi-dimensional screening system that enables the testing of hundreds of designs and identify STV-C8, which is built from an unusual planar symmetry, as the most efficient structure for RNA delivery. We characterize the shape, content, and packaging capacity of STV-C8 and program its tropism by combining it with computationally designed peptide binders. Regardless of its distinct structure, STV-C8 is several orders of magnitude more efficient in RNA transfer compared to natural counterparts and clinically used lipid nanoparticles. We demonstrate the versatility of STV-C8 by delivering various cargo RNAs, such as reporter RNAs, gene editors, programmable antivirals, and transcription factors, into a wide variety of cellular models from multiple species. We perform a comprehensive *in vivo* biodistribution analysis of STV-C8 at near single-cell resolution in a mouse model. Finally, we evaluate the translational capacity of STV-C8 by delivering the CRISPR/Cas9 Gene Editor into the skeletal muscle of a pig to delete dystrophin exon 51 as a treatment strategy for Duchenne Muscular Dystrophy (DMD)¹⁵.

Results

Viral capsid-forming proteins consist of multiple domains that orchestrate the packaging of genetic material as well as the assembly and release of the capsid mostly at the plasma membrane of infected cells. The curved surface of assembled viral capsids induces the first step of particle release by membrane bending, and it has been proposed that not only the fully assembled capsid multimer induces membrane bending but also partially assembled protomers^{16,17}. This observation raises the question of whether the mechanism can be harnessed for creating RNA transfer vehicles from scratch by using simple, low-dimensional protein assemblies. To explore this possibility, we leverage AI-designed symmetric protein assemblies with diverse symmetries for building hundreds of vehicles in a bottom-up approach.

Screening of synthetic protein assemblies

Generative models for protein design, such as RFdiffusion¹⁰, can generate virtually infinite numbers of protein assemblies with various shapes, including icosahedral, dihedral, and cyclic symmetries that largely differ in size and architecture compared to natural capsids (Fig. 1a). To implement such synthetic protein assemblies as bottom-up RNA transfer vehicles, we fused them to three structural domains that are typically part of capsid-forming proteins: a membrane-binding domain, a late budding domain, and an RNA-binding domain (Fig. 1b)¹⁷. As the initial scaffold for such STV carriers, we employed the well-characterized protein assembly HE0902 (Extended Data Fig. 1a)¹⁰, and fused it to a membrane-binding domain derived from the pleckstrin homology domain of *Rattus norvegicus* phospholipase C delta (PHPLC δ). In addition, we created a synthetic budding domain composed of budding motifs from multiple viruses. This synthetic late-budding domain (SynL) exceeds the budding efficiency of the natural HIV p6 L-domain (Extended Data Fig. 1b-d). To enable RNA packaging, we added high-affinity RNA binding proteins to the construct^{18,19}. We transfected HEK293T cells with these initial STV constructs,

along with RNAs containing the corresponding packaging signal, and quantified the RNA release. All constructs successfully transferred their RNA cargo into the supernatant, with the STV construct built on tandem PCP (tdPCP) being the most efficient (Fig. 1c). Furthermore, we demonstrated that co-expression of VSV-G as fusogenic protein enabled these synthetic particles to deliver EGFP cargo RNA into target cells (Extended Data Fig. 1e-f).

HE0902 displays icosahedral symmetry as many viral capsids. Encouraged by the initial proof that STV-HE0902 enables efficient RNA release and transfer, we explored embedding non-natural symmetries into the STV scaffold. To comprehensively characterize these vehicles, we developed a screening method that allowed monitoring of three relevant dimensions: STV release (1), STV uptake (2), and RNA transfer efficiency (3). We created a reporter cell line expressing the N-split part (LgBiT²⁰) of NanoLuciferase (Nluc), as well as the C-split part (C-Fluc) of Firefly Luciferase (Fluc), fused to an N6 coil and the GP41-1 C-intein^{21,22}. The STV constructs contained a HiBiT tag that enabled quantification of STV release into the supernatant of producer cells (1). STV uptake into reporter cells was measured by reconstitution of Nluc from LgBiT, expressed in the reporter cell line, and the STV-delivered HiBiT tag (2). In producer cells, we co-expressed a PP7-tagged mRNA coding for the N-split part of Fluc, fused to the GP41-N-intein and an N5 coil. Reconstitution of wild-type Fluc reported the transfer efficiency of this mRNA from coiled-coil enhanced intein splicing in target reporter cells (Fig. 1d). To validate the screening method, we co-transfected producer cells with HiBiT-tagged STV-HE0902, PP7-tagged N-split Fluc cargo RNA along with VSV-G and successfully measured STV-HE0902 release into the supernatant, STV-HE0902 uptake into reporter cells as well as N-split Fluc mRNA transfer and expression (Extended Data Fig. 2a-c). Building on the established method, we screened 39 STV constructs consisting of synthetic assembly domains with icosahedral, dihedral, or cyclic symmetries (Fig. 1e). Many of these STV constructs were efficiently released from producer cells and delivered their cargo RNA into target cells. Strikingly, we observed that STVs built on non-natural dihedral and cyclic symmetries largely outperformed their icosahedral counterparts (Fig. 1f-h). We confirmed the high delivery efficiency for another cargo RNA, EGFP mRNA, with the four best-performing designs, HE0490, HE0499, HE0505 (all D3 symmetry), and HE0690 (C8 symmetry) into unmodified target cells (Extended Data Fig. 2d). These results suggest that AI-designed protein assemblies with non-natural symmetries can be harnessed for creating virtually infinite numbers of synthetic RNA delivery vehicles. To demonstrate this large potential for scaling, we designed an additional 30 assemblies with C8 symmetry, the symmetry of the best-performing structure HE0690 (Extended Data Fig. 3). Although being built on the same C8 symmetry, these newly created structures are highly diverse, allowing them to be screened for customized characteristics such as altered packaging density or low immunogenicity.

To further scale the design space of STVs, we combined the identified HE0690 assembly domain with a diverse panel of membrane-binding domains. We selected these membrane-binding domains by performing a structure-based search using FoldSeek²³, with the PHPLC δ domain from *Rattus norvegicus* as the template structure. We selected 29 domains from an unrestricted search across all species, a restricted search for human proteins, and a search for metagenomic proteins within the ESMAtlas

(Extended Data Fig. 4a-d)²⁴. We created a library of membrane binding domains fused to SynL-tdPCP-HE0690 and tested them using the established screening scheme for STV release, STV uptake, and RNA transfer efficiency (Extended Data Fig. 4e). This analysis revealed the membrane binding domain from *Ursus americanus* (Ua) PHPLC, a previously uncharacterized PH domain, as the most efficient domain for RNA packaging and transfer (Extended Data Fig. 4f-h). Additionally, we confirmed the robust membrane localization of UaPHPLC-STVs in producer cells and validated their ability to efficiently transfer EGFP mRNA into target cells (Extended Data Fig. 4i-k). The STV construct, consisting of UaPHPLC and SynL-tdPCP-HE0690, is subsequently named STV-C8 (Fig. 2a).

Characterizing and programming of STV-C8

After having optimized the domain composition, we characterized the properties of STV-C8 as a novel bottom-up assembled RNA transfer vehicle. STV-C8 vesicles enriched their cargo RNA more than 10,000-fold in the supernatant (Fig. 2b). To further analyze the characteristics of STV-C8, we established a purification method by ultracentrifugation (Extended Data Fig. 5a), and analyzed the size distribution, particle number, and absolute protein/RNA content per particle (Extended Data Fig. 5b, c). Furthermore, we characterized the RNA content of purified STV-C8 particles and found strong enrichment of the EGFP cargo RNA (Fig. 2c). In line with previous reports²⁵, mitochondrial RNAs were depleted from STV-C8 particles, presumably because they are not accessible for packaging (Extended Data Fig. 6a). In addition to RNA, we characterized the protein content of STV-C8 particles and found a strong enrichment of ESCRT related proteins that are involved in budding (Fig. 2d and Extended Data Fig. 6b). Next we, imaged STV-C8 derived vesicles using cryo-ET (Fig. 2e). We found tens of STV-C8 assemblies with a typical diameter of 22 +/- 2 nm inside membrane-enclosed vesicles between 150 and 300 nm in diameter. Although STV-C8 assemblies are generally smaller than viral capsids, they induce the release of vesicles of similar size to enveloped viruses⁷. This finding points to a biophysical optimum of vesicle sizes and supports the initial hypothesis that non-natural synthetic protein assemblies act similarly as natural capsid protomers by initiating budding through membrane bending. Furthermore, these observations suggest that STV-C8 packages the cargo RNA on the surface of its oligomers, as the tdPCP RNA binding protein faces outside of the HE0690 structure, while the surrounding membrane provides protection for the cargo RNA.

Given its fundamentally different architecture, we next asked how efficiently STV-C8 delivers cargo RNAs into cells compared to its natural counterparts. Therefore, we benchmarked the efficiency of EGFP mRNA transfer into target cells against commonly used genetically encoded systems, *Virus-like particles* (VLP)²⁵, *Enveloped Protein Nanocages* (EPN24)^{17,25} and *Selective Endogenous Encapsidation For Cellular Delivery* (SEND)²⁶. For each of these vehicles, we added their corresponding packaging signal to an EGFP mRNA and compared their delivery efficiency in four cell lines from three species. Across all cell lines tested, we found the efficiency of STV-C8 to be at least an order of magnitude higher (Fig. 2f). Additionally, we benchmarked STV-C8 against clinically used Lipid Nanoparticles (LNP) and found a more than 100,000-fold lower RNA dose requirement for STV-C8 to induce the same expression level

(Fig. 2g). Unmodified mRNA is a potent trigger for the innate immune response. Therefore, we tested whether STV-C8-delivered RNA induces interferon signaling. Unlike plasmid DNA, which is a known trigger of the innate immune response, treatment with RNA-containing STV-C8 did not cause a detectable interferon response in A549-IFN reporter cells, suggesting that the biological production process of the cargo RNA alleviates the response (Fig. 2h).

In addition to efficiency, a critical aspect of delivery vehicles is their cell-type specificity. Recently, mutant versions of VSV-G have been created that retain endosomal escape activity but abolish binding to the target receptor LDLR. When combined with antibodies, such a blinded VSV-G can guide VLPs to preferentially target alternative receptors²⁷⁻²⁹. To overcome the limited availability of antibodies, we wondered whether the concept of using artificially designed proteins could be extended to the programming of cell-type specificity. We added computationally designed peptide binders against EGFR or IL7R α to STV-C8 particles³⁰, by fusing the binders to a signal peptide along with a transmembrane domain and expressed these constructs together with blinded VSV-G (K63Q/R370Q) in STV-C8(EGFP) producing cells. Upon budding of STV-C8(EGFP) from the plasma membrane, these synthetic binding modules were co-packaged and mediated cell-type specificity (Fig. 2i and Extended Data Fig. 7a). We added programmed STV-C8(EGFP) to EGFR or IL7R α target cells and measured their uptake into receptor-expressing cells using Flow Cytometry. We found a strong preference of programmed STV-C8 for their respective target cell line (Fig. 2j) and confirmed that cells expressing both receptors were permissive for STV-C8(EGFP) containing either EGFR or IL7R α binders (Extended Data Fig. 7b). Additionally, we recognized that the uptake of STV-C8(EGFP) containing both binders on their surface was further increased in target cells expressing both receptors, suggesting an additive effect that might be further explored for precise cell type-specific targeting (Extended Data Fig. 7c).

Viruses, such as AAVs and Lentiviruses, are widely used for delivering genetic material into cells, but they have limited packaging capacity. Therefore, we characterized the cargo size that can efficiently be packaged by STV-C8. We co-packaged an EGFP mRNA of constant length along with mRuby3 mRNAs of varying lengths. Across the mRNA sizes tested (1-10 kb), we detected similar expression levels of EGFP and mRuby3 in target cells, suggesting that STV-C8 does not have a packaging limit within the relevant length range (Extended Data Fig. 8a). We hypothesized that RNA packaging on the surface of HE0690 oligomers instead of inside a capsid shell relaxes the size constraints of STV-C8 compared to viruses. Additionally, we could not detect signs of STV-C8-induced apoptosis (Extended Data Fig. 8b), confirmed that STV-C8 are stable for at least one week under 4°C storage conditions, facilitating their practical use (Extended Data Fig. 8c), and characterized the optimal cargo RNA/STV-C8/fusogen ratio (Extended Data Fig. 8d-h).

Delivering RNAs into diverse cellular models

We showed that STV-C8 is a transport vehicle with unique characteristics, enabling highly efficient transfer of reporter RNAs into various cell lines. Building on these results, we delivered a panel of relevant cargo RNAs into complex cellular models. First, we tested the ability of STV-C8 to deliver the

EGFP cargo RNA into a multilayer cellular model. We transduced iPSC-derived retinal pigment epithelium (RPE) spheroids with STV-C8(EGFP) and detected EGFP expression in the inner layers of the spheroids, confirming their ability to penetrate deeper tissue (Fig. 3a,b and Extended Data Fig. 9a,b). We also demonstrated high delivery rates in primary human monocytes as a model for suspension cells (Fig. 3c). Next, we isolated primary cortical astrocytes from mouse brains and confirmed a high transduction rate of STV-C8(EGFP) also in this model (Fig. 3d,e). Additionally, we delivered mRNA encoding the pro-neuronal transcription factor *Ascl1* into cortical astrocytes and induced *Ascl1* expression in more than 30% of cells (Fig. 3f). One of the most relevant application areas of novel delivery vehicles is the efficient transfer of gene editors. We added the PP7 packaging signal to the 3'UTR of the Cas9 mRNA and cloned two sgRNAs containing the PP7 signal in the tetraloop of the sgRNA along with spacers targeting the intronic region up- and downstream of exon 51 of the dystrophin gene. Deletion of exon 51 of the dystrophin gene has been shown to be a viable strategy for rescuing a high proportion of DMD-related phenotypes^{15,31}. We treated primary porcine fibroblasts with STV-C8(Cas9/sgRNAs) particles (Fig. 3g). Amplification of the dystrophin locus from treated cells revealed a deletion frequency of approximately 40% (Fig. 3h). Recently, sequences of novel CRISPR proteins were created *in silico* by a protein language model. We demonstrated that one of these proteins, OpenCRISPR-1³², can efficiently be delivered with STV-C8 and induced similar editing rates as wild-type Cas9 in Traffic Light Reporter (TLR) cells (Extended Data Fig. 9c). These data illustrate that end-to-end programmable therapeutics could be within reach by combining bottom-up created delivery vehicles, equipped with synthetic receptor binders, and AI-designed cargo RNAs. To further demonstrate the flexibility of STV-C8, we packaged and delivered the programmable antiviral Cas13d-NCS³³. We added the PP7 signal to the 3'UTR of Cas13d-NCS mRNA and to the 3'part of a crRNA targeting the SARS-CoV-2 genome. We produced STV-C8(Cas13d-NCS/crRNA) particles, delivered them into iPSC-derived human lung cells, and infected these cells with SARS-CoV-2-GFP (Fig. 3i and Extended Data Fig. 9d). The viral replication was monitored in a live imaging setup, showing that STV-C8 delivered Cas13d-NCS almost entirely blocked the virus (Fig. 3j,k). To further validate the therapeutic potential of STV-C8, we tested their stability in human blood samples and found that they were unaffected by the blood treatment (Fig. 3l).

Delivering cargo RNAs into animal models

We demonstrated that STV-C8 effectively delivers a wide variety of cargo RNAs into diverse cellular models. Based on these results, we next tested the system in animal models. Notably, to our knowledge, no AI-designed proteins have been studied *in vivo* so far. We injected mice intravenously with STV-C8(EGFP) and applied an advanced whole-body clearing and imaging technique to comprehensively analyze the biodistribution of STV-C8 mediated EGFP expression at near single-cell resolution (Fig. 4a)³⁴. Surprisingly, we detected strong and highly specific expression in the lung but no expression in the liver, the common target tissue for most lipid-based vehicles (Fig. 4b,c and Extended Data Fig. 10a-c). The high resolution of the imaging method enabled us to detect a punctuated expression pattern in the lung, suggesting an additional layer of cell-type specificity within the tissue. To extend the *in vivo* proof-of-concept beyond a reporter RNA, we packaged the CRISPR/Cas9 system, deleting exon 51 from the

dystrophin gene into STV-C8 and injected them into the muscle of a pig (Fig. 4d). We amplified the dystrophin locus and confirmed the successful deletion of exon 51 in the STV-C8(Cas9/sgRNAs) treated muscle (Fig. 4e,f). To further characterize the deletion efficiency, we employed long-read nanopore sequencing and found a deletion frequency of up to 50% (Fig. 4g). Dystrophin exon 51 is a hotspot for mutations, and its deletion was shown to be a viable treatment strategy to rescue DMD-induced phenotypes^{15,31}. Therefore, these data in a large animal model emphasize the clinical potential of STV-C8.

Discussion

Here we demonstrated that protein assemblies designed by RFdiffusion¹⁰ can be harnessed to build synthetic RNA transfer vehicles from scratch. By establishing a multi-dimensional screening platform, we tested more than a hundred STV constructs with diverse structures. With STV-C8, we identified a novel vehicle architecture that outperformed the RNA transfer efficiency of biological and chemical vehicles and applied it to deliver a wide variety of cargo RNAs, such as transcription factors, Gene Editors, and programmable antivirals into various human, mouse, and pig derived cellular as well as animal models. Unlike previous studies that mimicked natural capsids^{17,25}, we leveraged AI-based protein design to create unusual but functional and flexible RNA transport vehicles. Viruses are highly diverse, yet most converged towards packaging their genomes in large, multimeric protein shells with icosahedral and helical symmetry⁶⁻⁹. We raised the question of whether these characteristics are essential for efficient RNA transport or whether it is possible to discover new paths outside of the evolutionary landscape by creating transport vehicles beyond natural characteristics. The high transport efficiency of STV-C8, combined with its novel characteristics, suggests that such aspects are less important when building RNA transport vehicles from scratch.

The vehicles we created here are an ideal use case for the abilities of current protein design methods, which are very effective in creating static, symmetric structures but limited in generating dynamic proteins or enzymatic activities^{10,11,35}. Future advancements, such as incorporating molecular dynamics data or developing multi-modal models that integrate structure, sequence, and functional annotations, could extend the abilities of these models toward challenging biological problems^{14,36}. Applying such tools to overcome the limitations of natural protein diversity will enable the programming of biology and, therefore, unlock new directions in various areas of biological and biomedical research.

Methods

Molecular cloning

All clonings were performed using standard molecular techniques. Fragments for cloning were generated by PCR using Platinum SuperFi II Master Mix (Thermo Fisher Scientific) and appropriate oligonucleotides (IDT DNA), by plasmid digests using standard restriction enzymes (NEB), or synthesized as Gene Fragments (TWIST Biosciences or IDT DNA). Fragment assemblies were performed

using the NEBuilder HiFi DNA Assembly Mix (NEB) or Instant Sticky-end Ligase Master Mix (NEB). Assembled fragments were transformed into self-made chemically competent *E. coli* DH5α cells. Correct clones were identified by plasmid preparation (Monarch Plasmid Miniprep Kit, NEB) and Sanger Sequencing (Azenta) or RCA directly on cells (Microsynth). Subsequently, plasmids were isolated using the Plasmid Maxiprep Kit (QIAGEN) and used for transfection. All cloned sequences of this study are listed in Supplementary Table X.

Plasmid transfection

One day prior transfection, cells were seeded at 3.0×10^4 cells per well for 96 well plates, 2.2×10^5 for 24 well plates, 7.5×10^5 for 6 well plates, and 4.0×10^6 for 10 cm dishes. Cells were transfected with JetOptimus DNA transfection reagent (Polyplus transfection). For 96 well 75 ng DNA per well were transfected, for 24 well plates 300 ng DNA, for 6 well plates 1 µg, and for 10 cm dishes 5 µg DNA.

Cell culture and cell lines

HEK293T cells were cultivated at 37°C, 5% CO₂ in an H₂O-saturated atmosphere, and maintained in DMEM (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin (Gibco). HEK293T Split-Luciferase reporter cell line was generated by Cas9 cleavage at the AAVS1 locus and homology-directed integration of a donor construct containing LgBiT, the C-terminal fragment of Firefly Luciferase, separated by a P2A sequence, and the puromycin resistance gene. Three days after transfection, the cells were selected for two weeks with 2 µg/ml puromycin (Thermo Fisher Scientific). HEK293T cells stably expressing EGFR or IL7Rα were generated by means of amplifying the EGFR sequence from AddGene plasmid #23935 (a gift from William Hahn & David Root), whereas IL7Rα was synthesized (TWIST Biosciences). Both coding sequences were cloned into the AAVS1 knock-in donor plasmid, transfected with AAVS1 targeting Cas9, and selected with 2 µg/ml puromycin. Dual-positive EGFR and IL7Rα receptor cells were generated by cloning IL7Rα into an AAVS1 donor plasmid containing a blasticidin resistance gene, and cells were transfected and selected in 10 µg/ml blasticidin medium (Thermo Fisher Scientific).

Quantification of STV-mediated target RNA release into the cell culture supernatant

Supernatants from the STV-releasing cells were collected and filtered through 0.45 µm PVDF filters (Merck Millipore) after 48 h. RNA was extracted with Monarch Total RNA Miniprep Kit (NEB), and isolated RNA was used as template for RT-qPCR with Luna Universal One-Step RT-qPCR Kit (NEB), along with a primer/FAM-probe set (custom design, Metabion), specific for EGFP mRNA. The reaction was analyzed on a QuantStudio 7 Flex device (Thermo Fisher Scientific).

Integration of diffusion-designed symmetric oligomers into STV design

Previously designed RFdiffusion symmetric oligomers were filtered for successfully assembled oligomers based on size exclusion data¹⁰. Additionally, all D2 symmetric oligomers were excluded. The resulting 39 sequences were synthesized (eBlocks, IDT DNA) and cloned as a C-terminal fusion to the additional STV components (PHPLC, SynL, and tdPCP).

Integrating structure-mined membrane binding domains into STV design

The pleckstrin homology domain PDB: 1MAI was used as input structure for structure-based homology search with FoldSeek^{23,37}. 10 sequences from three categories (other species, human, metagenome) were selected based on the highest homology to the input structure. Each sequence was synthesized (IDT DNA) and fused to the N-terminus of the previously identified ideal STV construct, containing SynL, tdPCP, and HE0690.

Sequence and structural alignments of the structure-mined membrane binding domains

The amino acid sequences of the structure-mined MBDs were aligned to that of *Rattus norvegicus* PHPLC δ , using the residues visible in the X-ray structure (PDB: 1MAI). The alignment was performed with the MAFFT version 7 --add tool³⁸, using default settings (strategy: auto, scoring matrix: BLOSUM62, gap opening penalty = 1.53, offset value = 0.0). For the structural alignment, the structure of the MBD region was extracted from the AlphaFold2³⁹ (human and other species MBDs) or ESMFold²⁴ (metagenomic MBDs) prediction of the respective structure-mined proteins containing these MBDs. These structures were aligned, and the RMSD to PDB: 1MAI was calculated using the PyMOL super alignment tool.

Screening of symmetric oligomer and membrane binding proteins for RNA release and uptake

Cells were seeded in 96 well format and transfected with each of the oligomer or membrane STV constructs along with plasmids coding for VSV-G and N-SplitLuc-PP7 (in a 2:1:7 ratio). 24 h post-transfection, 5 μ l of supernatant was collected from the transfected cells, mixed with 45 μ l PBS, and measured using Nano-Glo HiBiT Lytic Detection System (Promega) at a Centro LB960 device (Berthold Laboratories), using 0.5 sec integration time. 48 h post-transfection, 120 μ l of supernatant was collected and filtered through a 0.45 μ m PVDF 96 well filter plate (Sigma Aldrich) by centrifugation (1,500 g, 4°C, 20 min). Cleared supernatant was added to a seeded 96 well plate of C-split luciferase reporter cells. 24 h later Nano-Glo Dual-Luciferase Reporter Assay (Promega) was performed on the cells after complete removal of the supernatant. STV uptake was quantified by light emission from the NanoLuc substrate. N-splitLuc-PP7 mRNA uptake and expression were measured by the light emission from Firefly Luciferase substrate.

Validation of STV-mediated transfer of EGFP mRNA by Flow Cytometry

HEK293T producer cells were transfected in 24 well format with plasmids coding for STV constructs, VSV-G, and EGFP-PP7 (2:1:7 ratio). STV-containing supernatant was collected for two consecutive days, filtered through a 0.45 μ m PVDF membrane filter, and concentrated 5-10 fold with Lenti-X Concentrator

(Takara Bio) in fresh DMEM. 10-20 μ l of resuspended STVs were added to a 96 well plate of HEK293T cells. After 24 h, the treated cells were detached using StemPro Accutase (Thermo Fisher Scientific), mixed with FACS buffer (EDTA/BSA), and filtered through cell strainer-containing tubes. Subsequently, samples were gated for living, single cells and EGFP mRNA uptake and expression were analyzed by Flow Cytometry (BD FACSAria III, BD Biosciences). Data analysis was performed using the FlowJo software (BD Biosciences).

Design of additional oligomers with C8 symmetry

Additional oligomers featuring C8 symmetry were generated using the open-source version of RFdiffusion, along with the script provided for symmetric oligomers¹⁰. These computations were performed on a single A100 GPU.

Determination of subcellular STV localization

HEK293T cells were transfected with STV constructs containing different membrane-binding domains. 24 h later, cells were fixed with 10% Formalin (Sigma Aldrich) and permeabilized in 1% BSA/0.5% Triton X-100 containing PBS. Permeabilized cells were incubated with primary anti-HA antibody (Sigma Aldrich, cat. H3663), overnight at 4°C. Subsequently, the cells were washed and stained with an Alexa 488 coupled, secondary donkey anti-mouse antibody (Thermo Fisher Scientific, cat. A21202), overnight at 4°C. Stained cells were mounted with ProLong Diamond reagent (Thermo Fisher Scientific) and imaged at an Axio Imager M2 fluorescence microscope (Carl Zeiss).

Characterization of packaging capacity by Flow Cytometry

EGFP-PP7-STVs were produced in 24 well format as previously described. Additionally, producer cells were transfected with mRuby3-PP7 constructs containing random UTR sequences of variable lengths. Concentrated STVs were added to HEK293T target cells. After 24 h, EGFP and mRuby3 expression was quantified using Flow Cytometry, as described previously.

Concentration of STVs by ultracentrifugation for analytical and experimental purposes

Producer cells, seeded in Poly-L-Lysin (Sigma Aldrich) coated 10 cm dishes, were transfected with plasmids coding for STV-C8 components required for the respective experiment. If not specified otherwise, supernatants were collected for three consecutive days and stored until day 3 at 4°C. The collected supernatant was centrifuged for 5 min at 1,000 g and passed through a 0.45 μ m PVDF membrane filter. Filtered supernatant was added to a cushion of 20% (w/v) sucrose (Sigma Aldrich) in PBS. Subsequent ultracentrifugation was performed at 26,000 rpm for 2 h and 4°C using a SW28 rotor in an Optima L-60 ultracentrifuge (Beckman Coulter). After the centrifugation, the supernatant and the sucrose solution were removed, and the pellet was resuspended in 50 μ l ice-cold 1x PBS (Thermo Fisher Scientific) on an orbital shaker at 150 rpm for 45 min at 4°C. To remove debris, the resuspended pellet was centrifuged at 1,000 g for 5 min at 4°C and stored at -80°C. Following this process, samples were concentrated approximately 300-fold.

Determination of STV purity for downstream analysis

STV-C8 samples were concentrated via ultracentrifugation, and the sample purity was determined by silver staining. Samples were prepared in 2x Laemmli buffer (Sigma Aldrich) for 10 min at 98°C. The SDS-PAGE was run on a 4-15% gradient TGX gel (BioRad) using a 1x Tris/Glycine/SDS running buffer (BioRad) for 60 min at 130 V. Subsequently, the gel was silver stained according to the manufacturer's description (Serva). A gel was run in parallel with the same samples and blotted onto a nitrocellulose membrane for 60 min, 100 V at 4°C in transfer buffer (Tris/Glycine-buffer, BioRad). The STV-C8 protein position on the membrane was determined by imaging with Nano-Glo HibiT Blotting system (Promega) in a Fusion SL Vilber machine (Peglab). The HibiT signal on the membrane was used as a reference to identify STV proteins on the corresponding silver-stained gel.

Visualization of STV-C8 assemblies by cryo-ET

In 10 cm dishes coated with Poly-L-Lysin (Sigma Aldrich), seeded producer cells were transfected with plasmids coding for STV-C8 and EGFP-PP7. 24 h after transfection, the cells were washed with PBS, and serum-free DMEM was added to the cells. After another 24 h, the supernatant was collected and concentrated via ultracentrifugation as described previously. For subsequent cryo-ET analysis, the purified STV-C8 vesicles were diluted to 10^9 particles/ μ l in PBS. The samples were applied to holey R 3.5/1 carbon 200 mesh copper grids (Quantifoil), covered with a homemade 3 nm thick continuous carbon film by flotation. The grids were treated by glow discharge (at 4 mA for 10 s), then blotted and cryo-cooled into liquid ethane using a Vitrobot IV (Thermo Fisher Scientific) with the chamber operating at 95% humidity and at 10°C. For each tomogram, tilt-series were automatically acquired using Tomo5 software on a Krios G4 equipped with a cold-FEG operated at 300 kV and equipped with a Falcon IVi camera and a Selectris X energy filter (Thermo Fisher Scientific). A magnification of 81,000x was applied at a pixel size of 1.63 Å. Each tilt was acquired in the EER format and fractionated into 270 frames for a total dose of 2 e/ $\text{\AA}^2$. Each tomogram was composed of 61 tilts, acquired according to a dose symmetric scheme with angles between 60° and -60° and a 2° increment between tilts. Processing was performed using Relion 5 beta3 software⁴⁰. MotionCor2 was used to apply the gain reference and to align the EER fractionation by groups of 45 frames⁴¹. CTF estimation was performed using CTFFIND 4.1, tilt-series alignment was performed using AreTomo2^{42,43}. The tomogram was reconstructed using Relion 5 beta3, and denoising was performed using CryoCARE in Relion 5 beta3. Data segmentation was performed with MemBrain-seg for membranes⁴⁴ and manually in Amira for C8 particles.

Characterization of STV-C8 RNA content

STV-C8 particles were produced and purified as described in the previous section. RNA was isolated from the particles, as well as from corresponding producer cells, using the Monarch Total RNA Miniprep Kit (NEB). Subsequently, Illumina RNA-Seq Library Prep and sequencing with 20 million paired-end reads per sample were performed on a NovaSeq device. Sequencing reads were mapped to the human

reference transcriptome using the STAR aligner and differential expression analysis was performed using DESeq2. Library preparation, sequencing, and data analysis were performed by Azenta (Leipzig).

Characterization of STV-C8 protein content

STV-C8 were produced and purified as described in the previous section. Total protein was extracted by lysing the sample with lysis buffer (Preomics, Martinsried) supplemented with *cOmplete* Protease Inhibitor (Roche). The released protein was quantified using a BCA assay (Thermo Fisher Scientific). 10 µg of protein per sample was further processed by Filter Aided Sample Preparation (FASP⁴⁵) and subsequently measured on a QExactive HFX mass spectrometer online coupled to a Ultimate 3000 RSLC (Thermo Fisher Scientific). Data analysis was performed by label-free quantification in MaxQuant 2.4.9.0 (MPI, Martinsried⁴⁶) using a merged database of SwissProt human protein database and the sequences of exogenously expressed proteins. Statistical analysis was performed in Perseus (MPI, Martinsried⁴⁷).

RNA and protein Gene Set Enrichment Analysis

Significantly enriched or depleted genes (p adjusted < 0.005 , $\log_2FC +3/-3$) or proteins ($-\log q < 0.05$, $\log_2FC +3/-3$) were selected, and a gene set enrichment analysis was performed using gProfiler2 with default options (e111_eg58_p18_30541362).

Benchmarking of EGFP mRNA delivery efficiency of STVs compared to SEND, EPN, and VLP

SEND/MmPeg10 was ordered from Addgene (#174858, a gift from Feng Zhang), and EPN-MCP, VLP-MCP, EGFP-MS2, SEND-EGFP constructs were ordered as synthesis (Twist Bioscience) and cloned into a CAG promoter expression backbone. For each system, the corresponding capsid scaffold and cargo RNA plasmids were co-transfected with VSV-G plasmid in 24 well format. Supernatants were produced for 48 h and concentrated as previously described. Concentrated vehicles were added to a 96 well plate of HEK293T, Vero E6, N2a, and HepG2 cells. 24 h later EGFP expression was analyzed by Flow Cytometry as described earlier.

Benchmarking of STV against LNP characteristics

A plasmid encoding EGFP under the control of the T7 promoter was cloned. The plasmid was linearized via digesting downstream of the stop codon, leaving a 3'UTR of similar length as in the STV cargo plasmid. The reaction was purified (Monarch DNA Cleanup Kit, NEB) and used as template for *in vitro* transcription (HiScribe T7 Quick High Yield RNA Synthesis Kit, NEB). Subsequently, the RNA was purified (Monarch RNA Cleanup Kit, NEB) and capped with the Vaccinia Capping System (NEB). The reaction was purified again and polyadenylated with *E. coli* Poly(A) Polymerase (NEB). After a final purification step, the EGFP coding mRNA was diluted to 150 ng/µl in 20 mM citrate buffer (pH 4.0). LNPs were composed of ALC-0315 (Cayman Chemical, cat. 34337), DOPE (Avanti Polar Lipids, cat. 850725), Cholesterol (ChemCruz, cat. sc-202539), and DMG-PEG 2000 (Avanti Polar Lipids, 880151) in the respective ratio

(50:10:38.5:1.5). The lipid and RNA solutions were quickly mixed at a 1:3 volume ratio, resulting in a final weight ratio of 40:1. 1 µl of the prepared sample was diluted in 3 ml of PBS in a cuvette (Sarstedt) and analyzed by DLS using a Zetasizer Pro (Malvern Panalytical). EGFP mRNA-containing STV-C8 particles were prepared via ultracentrifugation, as described previously. The absolute STV-C8 protein content was determined by extrapolation from a HiBiT Control Protein (Promega) standard curve. EGFP-mRNA content in STV-C8 was determined by absolute RT-qPCR quantification (Luna Universal One-Step RT-qPCR Kit, NEB) with an *in vitro* transcribed EGFP mRNA standard and STV-C8 particle number was determined by DLS. Different concentrations of STV-C8(EGFP) and EGFP mRNA containing LNPs were added to HEK293T cells and EGFP expression was analyzed by Flow Cytometry. For the concentration of each vehicle, in which approx. 50% of cells turned EGFP positive, the required amount of EGFP mRNA for both vehicles was calculated and compared.

Analysis of STV-C8-induced interferon signaling

A549-IFN-GFP cells (a gift from Ralf Bartenschlager) that report interferon signaling by GFP expression were transfected with luciferase plasmid DNA as a positive control for interferon stimulation and treated with STV-C8 particles, containing a luciferase mRNA. GFP expression upon treatment was monitored after 24 h at an EVOS imaging device (Thermo Fisher Scientific).

Comparison of LNP and STV-C8-induced cytotoxicity

HEK293T cells were seeded in 96 well plate format and transfected with 50 ng EGFP mRNA containing LNPs and transduced with purified STV-C8 (EGFP) particles. Both particles were used at a concentration that induced EGFP expression in approx. 50% of cells. After 24 h, cells were detached with Trypsin 0.05% (Thermo Fisher Scientific), resuspended in Annexin V binding assay buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl₂, pH 7.4), and labeled 1:100 with Annexin V-iFluor 680 (Abcam). Subsequently Annexin V staining intensity was quantified using Flow Cytometry.

Establishing cell-type specific STV-C8 by peptide binder engineering

Previously designed EGFR_n, EGFR_c, and IL-7R_α minibinders³⁰ were exposed on the STV-C8 surface by expressing them as a fusion construct, consisting of a signal peptide, minibinder sequences, and a transmembrane domain, along with STV-C8 components and an LDLR-binding deficient mutant of VSV-G (K63Q, R370Q²⁹). Transfections were performed in 6 well plates with EGFP mRNA cargo, the supernatant was collected for 48 h and concentrated with LentiX concentrator (TakaraBio). 30 µl of concentrated supernatant was transferred to either WT-HEK293T cells or HEK293T cells stably expressing the EGFR or IL-7R_α receptors. 24 h later, the EGFP expression was analyzed by Flow Cytometry as described earlier.

STV-C8 mediated EGFP mRNA delivery into RPE spheroids

Human retinal organoids were differentiated from the hiPSCs-F49B7 cell line, derived from healthy donors, and tested for pluripotency markers as well as germ layer differentiation potential. hiPSC were

seeded on 6 well plates coated with Matrigel (Corning) and cultured in mTeSR plus medium (STEMCELL Technologies). The medium was changed every two days. At 70% confluency, iPSCs were passaged in small clumps using 0.5 mM EDTA (0.5 M, pH 8.6, Thermo Fisher Scientific). On day 0, hiPSCs were dissociated as small aggregates using 0.5 mM EDTA. The aggregates were suspended in cold Matrigel (GFR, Corning) and incubated at 37°C for 20 min to allow gelling. hiPSCs/Matrigel aggregates were gently dispersed in the Neural Induction Medium (DMEM/F12+GlutaMax, 1% B27 with Vit A supplement, 0.5% N2 supplement, 0.1 mM 2-Mercaptoethanol, 2 mM GlutaMAX, and 1% penicillin/streptomycin, all from Thermo Fisher Scientific). The aggregates were cultivated in Ultra-Low Adherent 6 well culture plates (Costar, Corning). On day 5, the floating cysts were seeded on Matrigel-coated 6 well plates. On Day 15, the cysts were detached by adding Dispase (0.5 mg/mL in DMEM/F12, STEMCELL Technologies) for 3-4 mins at 37°C, followed by washing with DMEM/F12 medium and growing in the Retinal Differentiation Medium (DMEM/F12 +GlutaMax, 2% B27 without vitamin A, 1% NEAA, and 1% penicillin/streptomycin, all from Thermo Fisher Scientific). On Day 25, the immature retinal organoids were transferred to Retinal Maturation Medium (DMEM/F12 +GlutaMax, 8% FBS, 2% B27 without vitamin A, 1% NEAA, 1% A/A, all from Thermo Fisher Scientific, and 1% 100 mM taurine from Sigma-Aldrich). Half of the medium was changed every 2-3 days, and all organoids were cultured in a humidified incubator at 37°C and 5% CO₂ until the end of the experiment. The retinal pigment epithelium (RPE) was developed during the retinal organoid generation as a patch attached to it. On day 200, RPE spheroids were dissected from human retinal organoids. Then, they were sorted into 96-well U Bottom Ultra-Low Attachment plate (Nucleon Sphera, Thermo Scientific). Each well consisted of 3-4 RPE spheroids. RPE spheroids were transduced with 10 µl of STV-C8(EGFP)/VSV-G or STV-C8(EGFP). The RPE spheroids were fixed two days after the treatment and then gradually dehydrated in 10% sucrose at RT, 30% at RT, and 50% overnight at 4°C. The spheroids were embedded in O.C.T (Tissue-Tek O.C.T. compound, Sakura) and immediately frozen at -80°C until solidification. RPE spheroids were sectioned into 10 µm thickness using a cryostat (Leica CM3050 S, Leica Biosystems). Cryosections were rehydrated and incubated in a 5% chemo-blocker solution (Merck) for 30 min, followed by 30 min incubation in 0.3% triton-X. Anti-RPE65 (Proteintech, cat: 17939-1-AP) and anti-GFP (Santa Cruz, cat. sc-101536) primary antibodies were diluted in 5% chemo-blocking solution and incubated overnight at 4°C. The cells were washed three times in PBS. Goat anti-rat Alexa Fluor 488 (Thermo Fisher Scientific) and donkey anti-rabbit Alexa Fluor 555 (Thermo Fisher Scientific) secondary antibodies were diluted in 5% chemo-blocking solution and incubated for 1 h at room temperature. Finally, the sections were washed with PBS and mounted using Fluoroshield with DAPI (Sigma Aldrich). Immunolabeled RPE spheroids were imaged using a Leica TCS SP8 spectral confocal laser scanning microscope (Leica Microsystems).

EGFP delivery into human monocytes

Primary human monocytes (ATCC, cat. CRL-3622) were seeded in 96 well format. 5 µl of concentrated EGFP mRNA containing STVs were added to the cells, and EGFP expression was analyzed by Flow Cytometry 24 h later.

Isolation of primary astroglia from mouse postnatal cortex and Ascl1 mRNA delivery

Primary astrocytes were isolated from the cerebral cortex of postnatal day 5 C57BL/6N mice. The cortex was isolated, cut into small pieces, and mechanically dissociated by vigorous pipetting. Subsequently, the cell suspension was centrifuged for 7 min at 1,300 rpm, the cell pellet was plated in a T25 flask and cultivated for 7-13 days in DMEM/F-12 GlutaMAX, supplemented with 10% FBS, 10% penicillin/streptomycin, 5% horse serum, 4.5% D-(+)-glucose, 2% B27, 10 ng/ml bFGF, 10 ng/ml EGF (all from Thermo Fisher Scientific). Upon reaching 90% confluency, the cells were passaged using 0.05% Trypsin/EDTA (Thermo Fisher Scientific) and approx. 75,000 cells were seeded onto Poly-D-Lysin (Sigma Aldrich) coated glass coverslips. 24 h later, 15 μ l of concentrated EGFP or Ascl1-P2A-EGFP containing STV-C8 was added to the cells. After 48 h, cells were fixed in 10% formalin (Sigma Aldrich) and incubated with anti-GFP (Abcam, cat. ab13970) or anti-Mash1 (Abcam, ab211327) primary antibody in PBS containing 1% BSA (Sigma Aldrich) and 0.3% Triton X-100 (Sigma Aldrich) overnight at 4°C. After washing, the cells were stained with Alexa488 coupled donkey anti-chicken (Dianova, cat. 703-546-155) or Alexa594 coupled donkey anti-rabbit (Thermo Fisher Scientific, cat. A21207) secondary antibody for 1-2 h in darkness at room temperature. Subsequently, cells were DAPI stained, coverslips were mounted using Aqua Poly/Mount (Polyscience), and samples were imaged using an Axio Imager M2 fluorescence microscope (Carl Zeiss).

Exon 51 deletion of dystrophin gene in primary porcine fibroblasts

Two Cas9 sgRNA plasmids containing a PP7 motif in the stem-loop of the sgRNA, along with porcine dystrophin targeting spacers, were cloned. The sgRNAs target intron 50 (AGAGTTCCTAAGGTAGAGAG) and intron 51 (ATAAAGATAAGAGCTGGCAG) to delete exon 51¹⁵. Additionally, a plasmid coding for NLS and NES fused Cas9, along with a 3'UTR PP7 motif, was cloned. HEK293T producer cells were seeded in poly-Lysin coated 10 cm dishes and co-transfected with Cas9 mRNA and the two sgRNA plasmids (1:1:1 ratio), along with STV-C8 and VSV-G coding plasmids. STV-C8 particles were collected and concentrated via ultracentrifugation, as described before. Pig primary fibroblasts were seeded in a collagen-coated 48 well plate in DMEM medium⁴⁸, supplemented with 1% NEAA, 10 mM HEPES, 15% FBS (all from Thermo Fisher Scientific) and 2-Mercaptoethanol (Merck). Seeded cells were treated with 20 μ l STVs for 72 h. Subsequently, genomic DNA was extracted (Monarch Genomic DNA Purification Kit, NEB), and a 2 kb fragment covering the deleted region was amplified (Primer: CCCATGACATTTACCCTATTATTATCCC and GCTAATGTTTCATTTTAAAAAGGAATCTGTC) using Platinum SuperFi II Master Mix (Thermo Fisher Scientific). The PCR product was run on a 1.5% agarose gel and imaged.

Treatment of SARS-CoV-2 infected iPSC-derived human lung cells with STV-delivered Cas13d-NCS

For lung cell differentiation, hiPSCs (ISFi001-A - RRID: CVCL_YT30) were cultured in StemMACS medium (Miltenyi Biotec) on plates coated with Geltrex Reduced Growth Factor (Thermo Fisher Scientific). 70% confluent iPSC colonies were isolated as single cell suspension with Accutase (Thermo Fisher Scientific), 5 min at 37°C, neutralized with StemMACS medium, centrifuged for 3 min at 200 g, room temperature, and $1.0-1.2 \times 10^6$ cells were seeded onto non-adherent 6-well plates (Corning, 3471) in StemMACS medium supplemented with 10 μ M Y2763 (Enzo Life Sciences). Differentiation basal

medium (DBM) was prepared with DMEM/F12 1:1 GlutaMAX (Thermo Fisher Scientific) supplemented with 1x NEAA (Thermo Fisher Scientific), 0.1% Albumax (Thermo Fisher Scientific), 1× B27 (Thermo Fisher Scientific). Formation of embryonic bodies (EB) was induced by changing the medium to 50% StemMACS medium /50% DBM with 20 ng/ml Activin A (Bio-Techne). Medium was replaced entirely to DBM with 20 ng/ml Activin for 48 h. Definitive endoderm (DE; Days 0 to 5) was induced by plating EBs onto Geltrex Reduced Growth Factor-coated plates at 7 EBs per cm² of culture surface in DBM supplemented with 150 ng/ml Activin A and 25 ng/ml bone morphogenic protein 4 (BMP4) (Thermo Fisher Scientific) for 5 days with daily medium changes. Anteriorization of DE (Days 6 to 10) was elicited by changing DBM supplements to 50 ng/ml EGF (Invitrogen) and 50 ng/ml bFGF (Thermo Fisher Scientific), 3 μM SB431542 (Milenyi Biotec) and 10 ng/ml Noggin (Sigma Aldrich) for 5 days with medium changes every day. Lung progenitors giving rise to alveolar epithelial cells type II (Days 10 to 17) were generated by changing the medium to DBM containing 50 ng/ml BMP2 (Thermo Fisher Scientific), 50 ng/ml FGF10 (Peprotech), 50 ng/ml BMP4, 50 ng/ml bFGF, and 50 ng/ml WNT3A (Bio-Techne) for 7 days. Successful differentiation into alveolar epithelial cells was confirmed by expression analysis of ACE2 and SLC34A2 by RT-qPCR (Luna Universal One-Step RT-qPCR, NEB). Additionally, NLS and NES containing Cas13d-NCS³³ was cloned into a PP7 motif containing backbone in the 3'UTR, and a PP7 motif was attached 3' to a crRNA, targeting the SARS-CoV-2 3'UTR region (GUCAUCCAAUUUGAUGGCACCUG). Subsequently, lung progenitor cells were seeded into Geltrex-coated 96-well plates at a density of 2×10⁴ cells/well (Merck) and differentiated for 7 days in differentiation medium. Differentiated lung cells were transduced with 40 μl concentrated STV-C8, containing Cas13d-NCS/SARS-CoV-2 or non-target crRNA. 24 h later, the cells were infected with SARS-CoV-2-GFP (MOI10), and viral replication was monitored for 72 h in an Incucyte S3 live imaging system (Sartorius).

Analysis of STV-C8 inactivation in human blood samples

Peripheral blood mononuclear cells (PBMCs) were isolated by diluting blood 2-4 times the volume of PBS. 35 ml of the diluted blood suspension was carefully layered onto 15 ml of Ficoll (density = 1.077 g/mL) in a Falcon tube and centrifuged without brake at 400 g for 30 min at 20°C. After centrifugation, the upper layer was aspirated, leaving PBMCs at the interphase. The PBMC layer was transferred to a fresh Falcon tube, filled with PBS, and centrifuged again at 300 g for 10 min at 20°C. The resulting cell pellet was resuspended in PBS, and cell counting was performed using Trypan blue staining. For long-term storage, PBMCs were frozen at the density of 1×10⁷ cells/ml in FBS supplemented with 20% DMSO. Blood samples were collected in EDTA-free tubes for the isolation of blood serum. The tubes were gently inverted several times to mix the blood and then allowed to clot at 4°C for 3-4 h. After clotting, the samples were centrifuged at 2500 g for 10 min at room temperature (RT). Using a sterile pipette, the top clear layer (serum) was carefully transferred to new sterile microcentrifuge tubes or storage vials. For long-term storage, aliquoted serum was stored at -80°C. STV-C8(N-Split-Luc) were produced in 24 well plates and collected for 48 h. The collected supernatant was concentrated, using LentiX (TakaraBio) and concentrated as described. 30 μl of concentrated STV-C8 particles were mixed with 30 μl of 1:10 diluted

serum, 30 μ l of resuspended PBMCs (approximately 3.0×10^5 cells) or PBS and incubated at 37°C for 60 min. After the incubation period, 50 μ l of the STV-C8 with PBMCs or serum mixes were transferred to a 96 well plate of Split-luc reporter cells. The next day, N-Split-Luc RNA expression was analyzed using ONE-GloEX Luciferase (Promega) assay.

Testing of STV-C8 storage conditions

STV-C8(N-Split-Luc) were produced in 6 well format for 2 days, concentrated, using LentiX (TakaraBio), and stored for 7 days at 4°C or -80°C. Subsequently, 50 μ l of stored samples were added to Split-Luc reporter cells. The next day, N-Split-Luc RNA expression was analyzed using ONE-GloEX Luciferase (Promega) assay.

Delivering of OpenCRISPR-1 with STV-C8

The coding sequence of OpenCRISPR-1 was ordered (Twist Bioscience) and cloned into a CAG promoter containing expression plasmid. The coding sequence was fused to two NLS and one NES signal, and the PP7 aptamer was added to the 3'UTR. Additionally, a sgRNA containing the PP7 aptamer in the stem-loop region and a spacer targeting the stop codon in eTLR cells (GCUCCACAAACGAAGACUGAC) was cloned⁴⁹. STV-C8 particles, containing OpenCRISPR-1 or Cas9 and the sgRNA, were produced in 6 well format for 3 days, concentrated, using LentiX (TakaraBio), and 20 μ l concentrated particles were added to a 96 well plate of eTLR cells. 3 days later, the cells were imaged at an EVOS imaging device (Thermo Fisher Scientific).

Analysis of mouse whole-body biodistribution of STV-C8 mediated EGFP expression

STV-C8(EGFP) particles were produced in coated 10 cm dishes for 3 days and concentrated by ultracentrifugation, as described before. 50 μ l of concentrated STV-C8(EGFP) samples were injected intravenously into four-week-old female Balb/c WT mice. The mice were sacrificed 24 or 72 h after injection and intracardially perfused with heparinized PBS (10 U/ml heparin) and 4% paraformaldehyde (PFA). The skin was removed, and the bodies were fixed in 4% PFA overnight at 4°C. As previously described³⁴, for vDISCO whole-body staining and clearing the following steps were performed: In brief decolorization (25% CUBIC reagent in PBS), decalcification (10% (wt/vol) EDTA in PBS), signal-enhancement with anti-GFP nanobodies (Chromotek, anti-GFP-AF647), dehydration (with tetrahydrofuran), delipidation (with dichloromethane) and refractive-index matching with a mixture of benzyl alcohol and benzyl benzoate (BABB). A Blaze light sheet system (LaVision BioTec) with an axial resolution of 4 μ m was used for light sheet imaging. Full-scale mouse body imaging was performed using a 4x magnification objective (Olympus XFLUOR 4x corrected/0.28 NA [WD=10 mm]). High-magnification tile scans were obtained with 22% overlap, and the light-sheet width was reduced to 80%. For the z-step, the size was set to 6 μ m, with a time exposure of 40 ms in the background channel (488 nm) and 60 ms in the signal channel (640 nm, 647-boosted GFP signal). A Fiji plugin was used to stitch the raw TIFF files to a full plane. The individual planes were merged into a 3D file format with Imaris converter and visualized by Imaris³⁴.

***In vivo* treatment of porcine muscle cells to delete exon 51 from the dystrophin gene**

Large animal work was approved and ethically monitored by the Bavarian local authority (ROB-55.2-2532.Vet_02-19-39). STV-C8(Cas9/sgRNAs) were produced in coated 10 cm dishes and concentrated by ultracentrifugation as described before. The pig was sedated by intramuscular injection of ketamine and azaperone. For analgesia, fentanyl was applied intravenously. Subsequently, the injection site was shaved and disinfected, and 1 ml of concentrated STV-C8 sample was injected at 1.75 cm depth, using a 22G safety needle, into the right hind leg (M. biceps femoris). The animal was clinically monitored post-injection. After 3 days, the animal was sedated and euthanized by i.v. injection of pentobarbital. Several muscle samples around the injection site, as well as samples from the uninjected back (latissimus dorsi), were prepared, and genomic DNA (Monarch Genomic DNA Purification Kit, NEB) was extracted. A PCR, using Platinum SuperFi II Master Mix (Thermo Fisher Scientific), was performed (Primer: CCCATGACATTTACCCTATTATTATCCC and GCTAATGTTCATTTTAAAAAGGAATCTGTC) to assess the deletion efficiency on an agarose gel by comparing band intensities. The resulting bands at 2 kb (wild type) and 1 kb (genomic deletion) were extracted from the gel and verified by Sanger sequencing (Microsynth). Additionally, the PCR product was sequenced using Oxford Nanopore sequencing (Eurofins Genomics), and the deletion frequency was analyzed using Geneious Prime (Dotmatics).

Statistical Analysis

Statistical tests and graphical representations of the numerical data were performed using GraphPad Prism.

Declarations

Data availability

All data are available in the manuscript or supplementary information. RNA-Seq data will be deposited in a public repository upon publication.

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Contributions

C.G., W.W., and F.G. conceived the study, acquired funding, and jointly supervised the study. A.K., K.R., F.R., L.M., R.M.W.R., M.K.S., C.M.R.L., B.T., L.K., M.L., A.Em., N.W., N.A., J.G., D.-J.J.T., G.S., G.G.W., C.G., and D.V.W. generated the DNA constructs used. L.M. quantified RNA release into the supernatant. F.R., R.M.W.R., and C.G. established the split-luciferase reporter cell line. M.K.S. performed and analyzed all screen-related experiments. F.R. performed sequence and structure alignments of MBD. B.T. and M.K.S. performed validation experiments of screen results. L.H., F.J.T., and C.G. designed the additional C8 assemblies using RFDiffusion. L.M., M.K.S., and F.G. conducted the subcellular STV localization experiments. N.W. and R.M.W.R. performed STV packaging capacity experiments. M.K.S. and B.T. established and performed all STV purification and purity characterization experiments. B.B., M.J., and C.M.R.L. conducted and analyzed the cryo-ET experiments. M. K. S., C.G., and Azenta performed and analyzed the RNA-seq experiments. J.M.-P. and C.M.R.L. conducted experiments to characterize the STV protein content. M.K.S. analyzed enriched gene sets. L.M., C.M.R.L., C.G. and B.T. performed LNP/EPN/VLP benchmarking experiments. B.T. and C.G. performed immunogenicity analyses. C.M.R.L. performed cytotoxicity and monocyte-related experiments. E.Y., N.W., L.M., and R.M.W.R. performed and analyzed experiments related to minibinder-based cell targeting. L.M. D.Y.O., E.B., and M.B. conducted RPE spheroid experiments. I.B. and L.M. were responsible for astrocyte-related experiments. L.C., N.K., F.G., L.M., B.T., and C.G. performed primary porcine fibroblast experiments. C.M.R.L. and L.M. generated lung epithelial cells. Z.M., C.M.R.L., C.G., and G.E. conducted experiments related to SARS-CoV-2. R.M.W.R., R.I., and L.M. performed blood inactivation assays. A.K. performed storage and OpenCRISPR-related experiments. K.K., A.E., M.K.S., C.G., and F.G. were responsible for whole-body mouse experiments. A.B., L.C., N.K., F.G., M.K.S., B.T., and C.G. performed all pig-related experiments. L.M., F.R., and C.G. created figures. All authors edited the manuscript.

Competing Interests

C.G., F.G., W.W., F.R., M.K.S., R.M.W.R., and C.M.R.L. are co-inventors of a related patent application.

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Figures

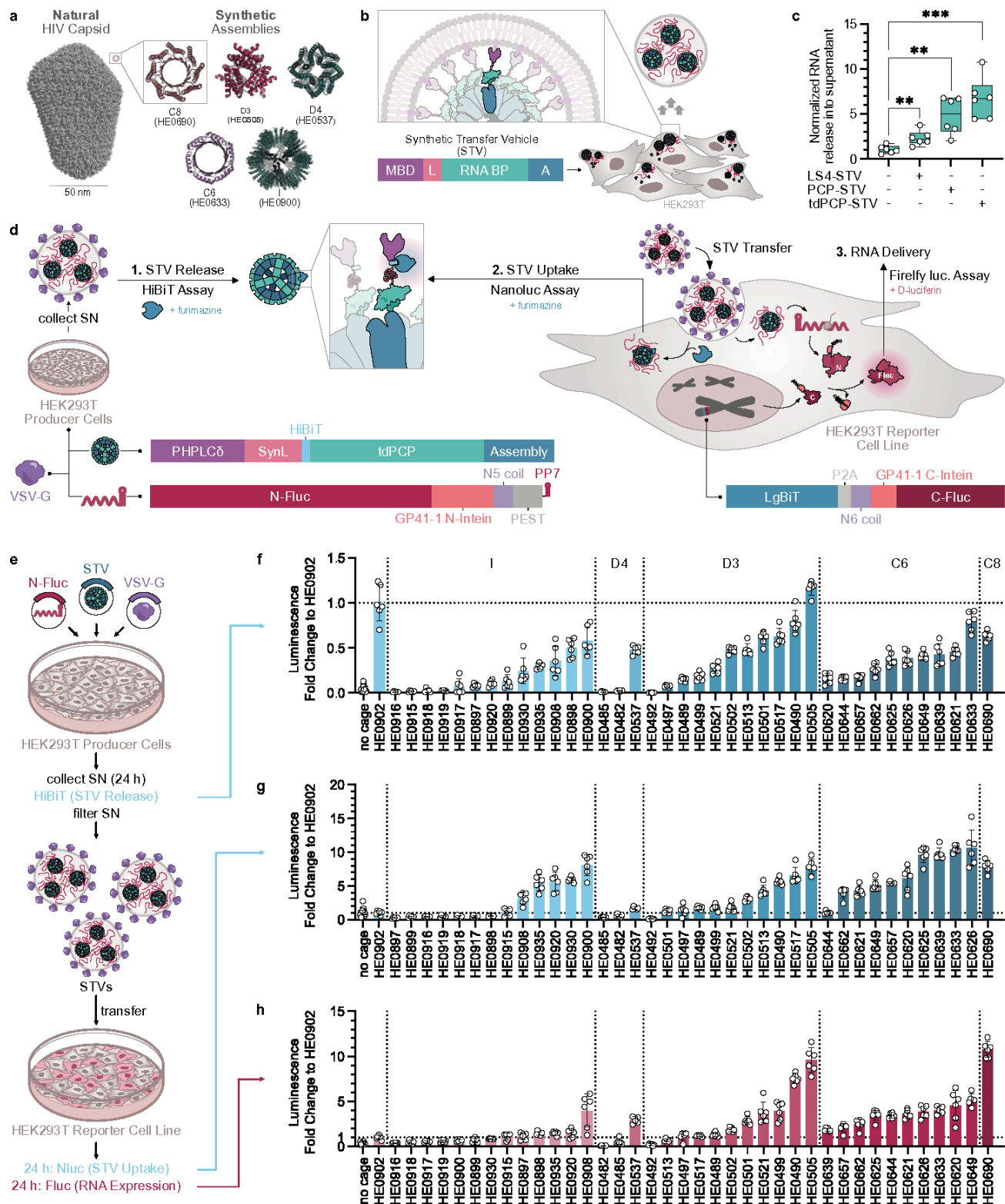


Figure 1

Design and screening of bottom-up assembled STV RNA carrier. **a**, Size and shape comparison of viral and AI-designed protein assemblies. **b**, Principle of mimicking viral release and RNA packaging by expressing synthetic protein assemblies in cells (MBD: Membrane binding domain, L: Late budding domain, RNA BP: RNA binding protein, A: Assembly domain). **c**, RT-qPCR quantification of RNA release by HE0902-based STV constructs, consisting of different RNA binding proteins (Unpaired Student's t-

test, $**P < 0.01$, $***P < 0.001$, mean $\pm$ s.d. for $n = 6$ biological replicates). **d**, Screening scheme for STV release, uptake, and RNA delivery efficiency based on reconstitution of split luciferases in supernatant and cells. **e**, Workflow for testing STV release, uptake, and RNA expression over the course of 72 h. **f**, Luminescence measurement of HiBiT signal in the supernatant of producer cells to quantify the release of STV constructs containing different AI-designed assembly domains by LgBit/HiBiT binding (mean $\pm$ s.d. for $n = 6$ biological replicates). **g**, NanoLuc measurement in the lysate of LgBit expressing Split-Luc reporter cells to test for uptake of different STV constructs by reconstituting NanoLuc from LgBit and STV containing HiBiT (mean $\pm$ s.d. for $n = 6$ biological replicates). **h**, Firefly measurement in the lysate of C-split Fluc expressing Split-Luc reporter cells to quantify N-split Fluc RNA delivery efficiency from different STV constructs (mean $\pm$ s.d. for $n = 6$ biological replicates).

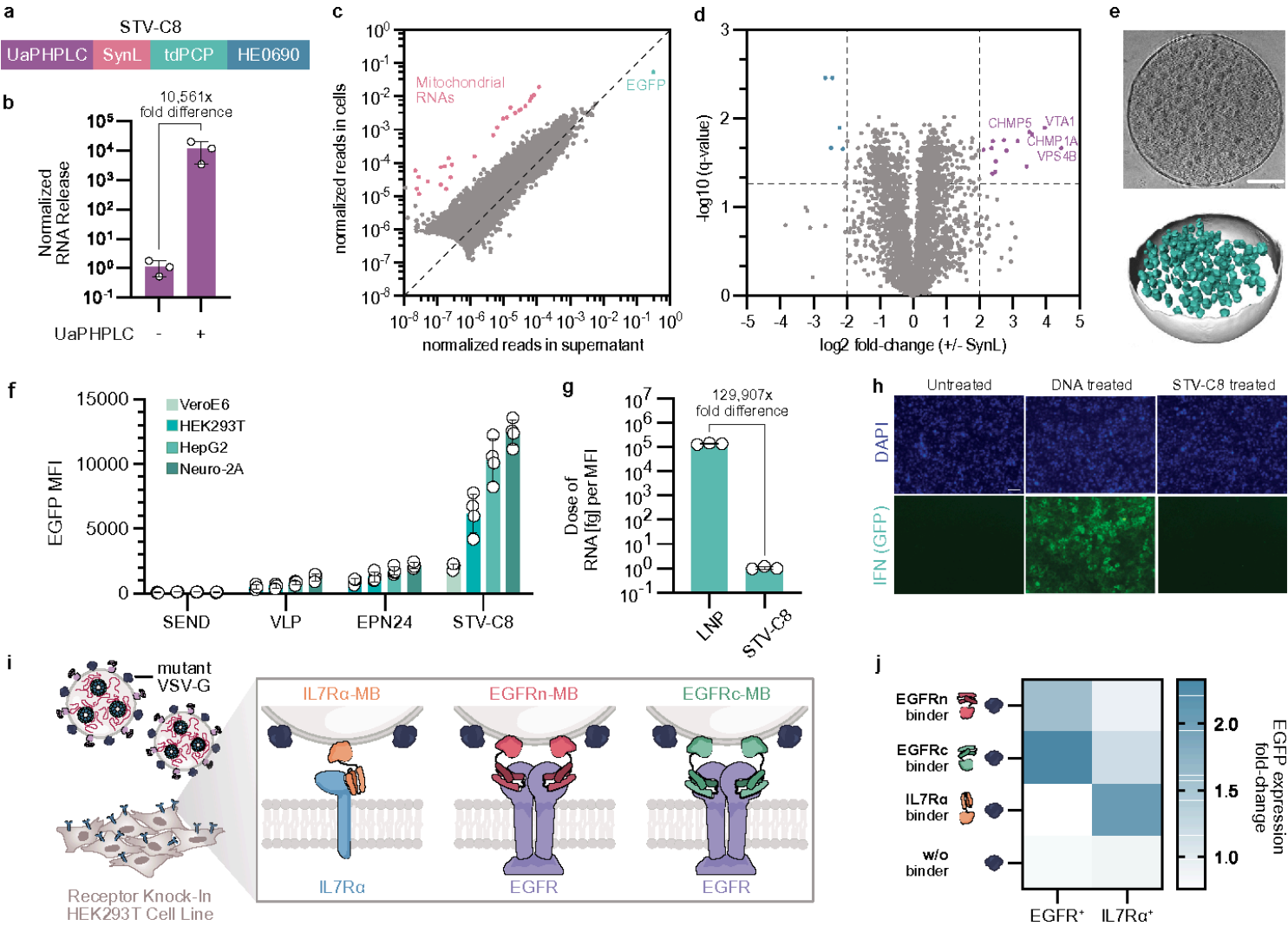


Figure 2

Characterization and programming of the STV-C8 RNA carrier. **a**, Schematic representation of the optimal STV-C8 construct, consisting of the pleckstrin homology domain from *Ursus americanus* phospholipase C (UaPHPLC), a synthetic budding domain derived from viral ESCRT recruiting motifs (SynL), a tandem coat protein from Pseudomonas phage PP7 (tdPCP), and an AI-designed assembly

domain with C8 symmetry (HE0690). **b**, RT-qPCR quantification of target RNA release into the supernatant by STV-C8 (mean $\pm$ s.d. for $n = 3$ biological replicates). **c**, RNA-seq based correlation analysis of producer cell and STV-C8 RNA content ($n = 4$ biological replicates). Mitochondrial RNAs are colored in red and the EGFP cargo transcript in green. **d**, Proteomic analysis of STV-C8 protein content by comparing protein content in purified supernatant of budding (+SynL) or non-budding (-SynL) STV-C8 constructs ($n = 3$ biological replicates). ESCRT-related proteins are colored in purple. **e**, 1 nm-thick slice from a tomographic volume of a STV-C8 assembly (top) and corresponding 3D segmentation showing the STV membrane in grey and the C8 particles in cyan (scale bar: 100 nm). **f**, Comparison of EGFP mRNA delivery efficiency for STV-C8 and other genetically encoded delivery vehicles into different target cell lines, measured by Flow Cytometry (mean $\pm$ s.d. for $n = 4$ biological replicates). Each mRNA was tagged with its corresponding packaging signal. **g**, Comparison of the required amount of EGFP mRNA to induce expression of one MFI, quantified by Flow Cytometry, in target cells by LNP or STV-C8 dependent delivery. STV-C8 RNA content was quantified by RT-qPCR by comparing it to an *in vitro* transcribed reference EGFP RNA (mean $\pm$ s.d. for $n = 3$ biological replicates). **h**, Induction of GFP expression under the control of the IFN- β promoter in A549 reporter cells upon treatment with STV-C8 or plasmid transfection (scale bar: 50 μ m). **i**, Concept for programming STV-C8 cell-type specificity by incorporating AI-designed minibinders. **j**, Flow Cytometry-based quantification of EGFP mRNA delivery into HEK293T cells with EGFR or IL7R α receptor knock-ins by expressing mutant VSV-G together with receptor targeting minibinders ($n = 3$ biological replicates).

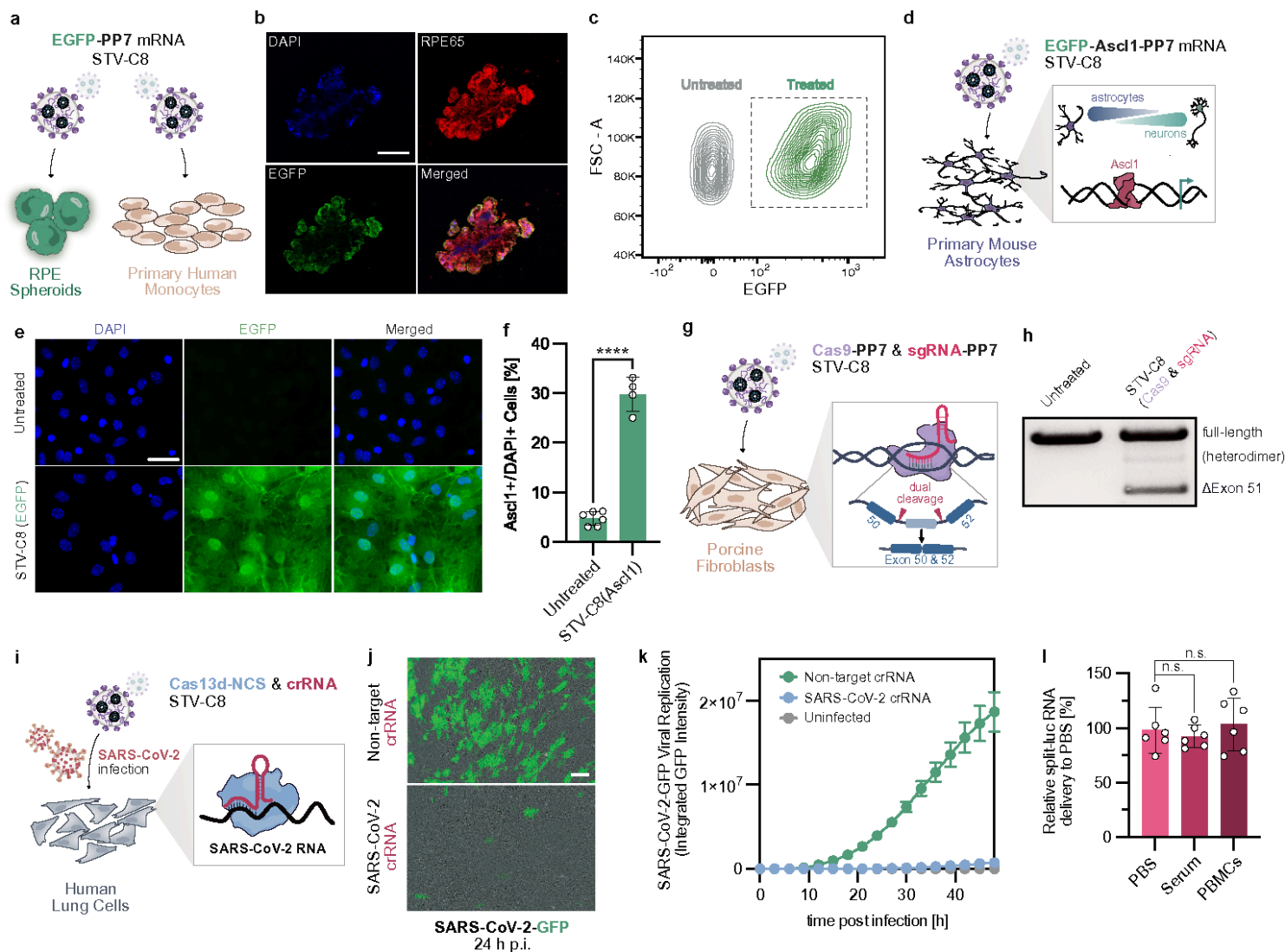


Figure 3

STV-C8 dependent cargo RNA delivery into diverse cellular models. **a**, Packaging of EGFP mRNA into STV-C8 and delivering into monocyte suspension cells and RPE spheroids. **b**, Fluorescence imaging of RPE spheroids, 2 days after transduction with STV-C8, containing EGFP mRNA, and co-stained with RPE65 as a specific marker protein for the RPE cells (scale bar: 100 μ m). **c**, Flow Cytometry analysis of human monocytes, untransduced or transduced with STV-C8 containing EGFP mRNA, 24 h after the treatment. **d**, Packaging of polycistronic mRNA coding for the Ascl1 transcription factor along with EGFP into STV-C8 and transduction of primary mouse astrocytes. **e**, Fluorescence imaging of mouse astrocytes, 3 days after transduction with STV-C8 packaging EGFP mRNA (scale bar: 50 μ m). **f**, Quantification of Ascl1 positive astrocytes, transduced with Ascl1 containing STV-C8 and stained for Ascl1 (Unpaired Student's t-test, ****P < 0.0001, mean $\pm$ s.d. for n = 6 control and n = 4 treated, independent astrocyte cultures). **g**, Schematic illustration of STV-C8 mediated Cas9/sgrRNA delivery into porcine fibroblasts, resulting in the deletion of dystrophin exon 51 by sgrRNAs cleaving in the flanking introns. **h**, PCR amplification of the dystrophin gene from Cas9/sgrRNA STV-C8 treated porcine fibroblasts 3 days after the treatment. **i**, STV-C8 mediated delivery of SARS-CoV-2 targeting Cas13d-NCS

into virus infected human lung cells. **j**, Fluorescence imaging of iPSC-derived human lung cells, infected with SARS-CoV-2-GFP (MOI10) and treated with STV-C8(Cas13d-NCS/crRNA), 24 h post-infection (scale bar: 100 μ m). **k**, Live imaging of SARS-CoV-2-GFP replication in STV-C8(Cas13d-NCS/crRNA) treated iPSC-derived human lung cells for 48 h (n = 3 biological replicates). **l**, Measurement of N-Split-Luc mRNA transfer into Split-Luc reporter cells upon pretreatment of STV-C8 delivery vehicles with human whole blood and serum samples (Unpaired Student's t-test, mean $\pm$ s.d. for n = 6 biological replicates).

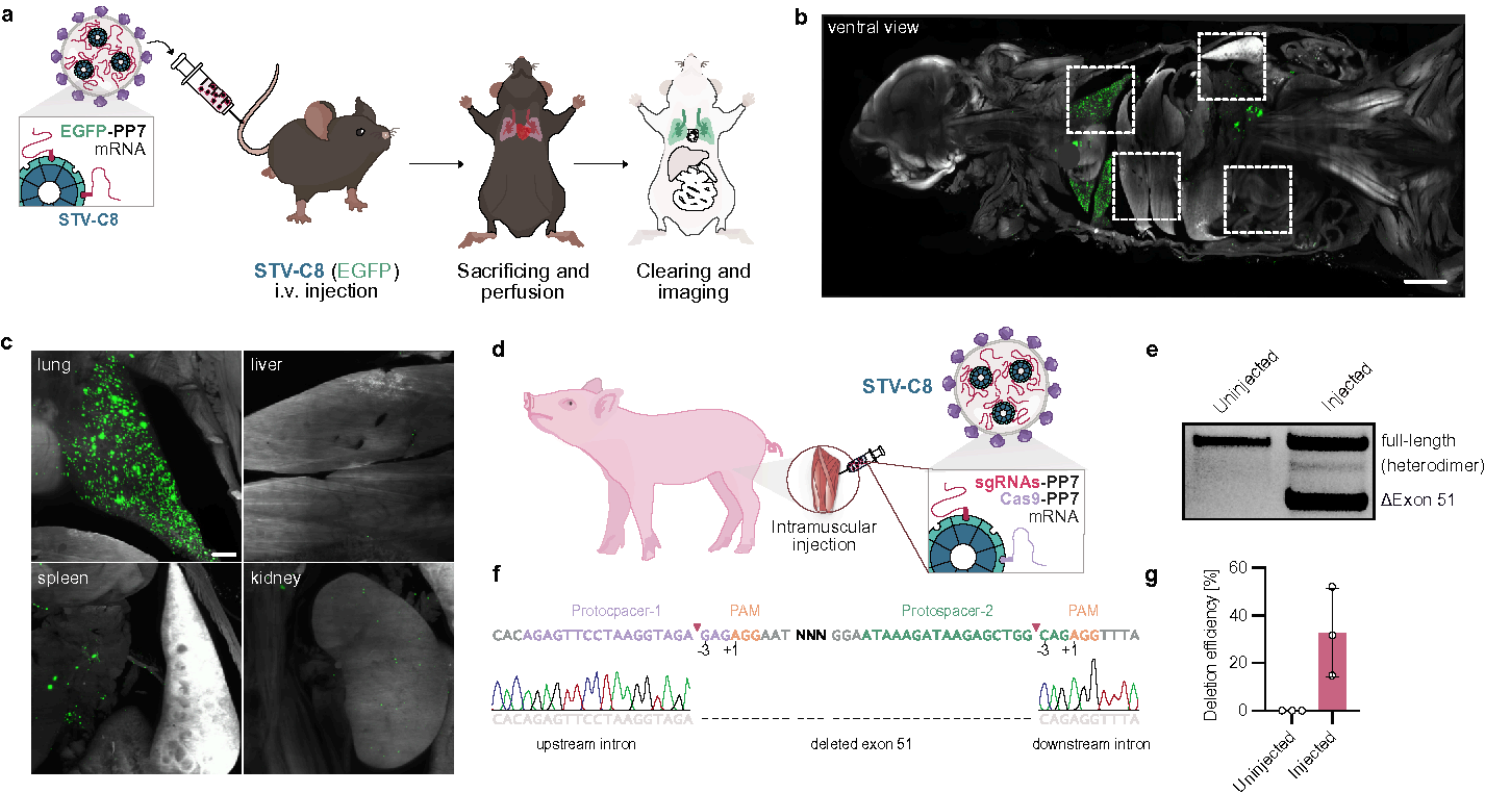


Figure 4

STV-C8 biodistribution and delivery of gene editing cargos in mouse and pig models. **a**, Schematic illustration of *in vivo* biodistribution analysis of STV-C8 mediated EGFP expression by mouse whole-body clearing and imaging. **b**, Ventral view of amplified EGFP expression in cleared mouse body, imaged by light-sheet microscopy, 72 h post i.v. injection (scale bar: 5 mm). Depicted rectangles mark magnified regions in panel c. **c**, Imaging of EGFP signal in cleared lung, liver, spleen, and kidney tissues (scale bar: 500 μ m, kidney tissue was imaged from a different plane). **d**, Schematic illustration of Cas9/sgrNA delivery into pig muscle by local injection of STV-C8 vehicles. **e**, PCR analysis of the edited dystrophin gene 72 h post i.m. injection. Upon treatment with STV-C8(Cas9/sgrNAs), exon 51 is deleted from the gene. **f**, Sanger sequencing of the PCR band, corresponding to deleted exon 51 in treated pig muscle cells. **g**, Long-read nanopore sequencing quantification of deletion efficiency in pig muscle (mean $\pm$ s.d. for n = 3 technical replicates of the same injection site).

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