

**Enhancing EV-AAV incorporation: Insights into natural loading and active
loading strategies for improved gene therapy vector delivery**



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Declaration

The work presented in this thesis was undertaken in Professor Matthew Wood's laboratory at the Department of Paediatrics, University of Oxford, United Kingdom, between 2021 and 2024. All work presented is my own with the following exceptions: (1) Fig 3.3 Electron Microscopy images were taken by Charlotte Melia from the Dunn School Bioimaging Facility, (2) Fig 3.6 Proteomics was performed by the Karolinska Core Facility, (4) Fig 4.1 Ratios of AAV to AAVR-EVs experiments were performed by Matthew Burton (Medical Student, Oxford University) and Nina Ashklog (Research Assistant, Department of Paediatrics), (5) Fig 4.4 Overexpression cell lines used in this study were generated by Claire Staton (DPhil student, Department of Paediatrics), (6) RepCap2/9-ITR plasmid designed by Dr. Hans Friedrichsen, and (7) Fig 4.15 Bioinformatic analysis was performed by Marina Luchner.

The experimental findings have not been submitted for any other degree at the University of Oxford or any other institution.

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Abstract

Gene therapy using adeno-associated viruses (AAVs) has emerged as a promising treatment for genetic disorders, with multiple FDA-approved therapies. However, challenges such as pre-existing immunity, the need for high doses, and manufacturing complexities limit their broader clinical application. This thesis investigates extracellular vesicle-associated AAVs (EV-AAVs) as a potential solution, focusing on their characterization and engineering for improved therapeutic delivery. Through systematic analysis using iodixanol density gradients and high-resolution size exclusion chromatography, we found that only 1.2-1.6% of EVs naturally incorporate AAVs, predominantly in larger vesicles enriched with phosphatidylserine. Multiple engineering strategies were explored to enhance EV-AAV yields, specifically surface loading, including using AAV receptor (AAVR), producer cell engineering through CD63 overexpression, and AAV9 capsid modification. CD63 overexpression resulted in a 10-fold increase in EV-AAV yields with 70% of associated AAVs protected within EVs. While surface-loaded AAVs showed promise *in vitro*, they demonstrated limited efficacy *in vivo*. Incorporation of the hepatitis A virus pX domain, and directed evolution of the AAV9 capsid, resulted in enhanced EV association. A comprehensive library screen revealed specific sequences, including LSGGLDILPKLC and LSDRKIERQGNVF, that significantly improved AAV incorporation into EVs. This work advances our understanding of EV-AAV biology and presents novel engineering approaches for enhancing their therapeutic potential, while highlighting critical challenges that must be addressed for clinical translation.

List of Abbreviations

AAP	Assembly-Activating Protein
AAV	Adeno-Associated Virus
AAVR	Adeno-Associated Virus Receptor
ADA-SCID	Adenosine Deaminase Severe Combined Immunodeficiency
ALIX	Apoptosis-Linked gene 2-Interacting Protein X
ARF6	ADP-Ribosylation Factor 6
ARRDC1	Arrestin Domain-Containing Protein 1
BASP1	Brain Abundant Membrane Attached Signal Protein 1
BLV	Bovine Leukemia Virus
BSA	Bovine Serum Albumin
BTV	Blue Tongue Virus
CAR-T	Chimeric Antigen Receptor T cell
CD	Cluster of Differentiation
CLIC	Clathrin-Independent Carrier
CMV	Cytomegalovirus
CNS	Central Nervous System
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EGFP	Enhanced Green Fluorescent Protein
ESCRT	Endosomal Sorting Complexes Required for Transport
EV	Extracellular Vesicle
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GPR108	G Protein-Coupled Receptor 108
GFP	Green Fluorescent Protein
HAV	Hepatitis A Virus
HEK293T	Human Embryonic Kidney 293T cells
HER2	Human Epidermal growth factor Receptor 2
HIV	Human Immunodeficiency Virus
HR-SEL	High-Resolution Size Exclusion Liquid Chromatography
HSPG	Heparan Sulfate Proteoglycan
HSV	Herpes Simplex Virus
IgG	Immunoglobulin G
ILV	Intraluminal Vesicle
IP	Intraperitoneal
ISEV	International Society for Extracellular Vesicles
ITR	Inverted Terminal Repeat

IV	Intravenous
LAMP2	Lysosomal Associated Membrane Protein 2
LamR	Laminin Receptor
LNP	Lipid Nanoparticle
MAAP	Membrane-Associated Accessory Protein
MHC	Major Histocompatibility Complex
MMTV	Mouse Mammary Tumor Virus
MSC	Mesenchymal Stem Cell
MVB	Multivesicular Body
NAb	Neutralizing Antibody
NDV	Newcastle Disease Virus
NLS	Nuclear Localization Signal
NPC	Nuclear Pore Complex
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PEI	Polyethylenimine
PLA2	Phospholipase A2
PTTG1IP	Pituitary Tumor-Transforming Gene 1 Protein-Interacting Protein
PTGFRN	Prostaglandin F2 Receptor Inhibitor
qPCR	Quantitative Polymerase Chain Reaction
rAAV	Recombinant Adeno-Associated Virus
RBE	Rep-Binding Element
RNA	Ribonucleic Acid
ROCK	Rho-associated protein kinase
scFv	Single-Chain Variable Fragment
SDS	Sodium Dodecyl Sulfate
SEC	Size Exclusion Chromatography
siRNA	Small Interfering RNA
SV40	Simian Virus 40
TBS	Tris-Buffered Saline
TFF	Tangential Flow Filtration
TSG101	Tumor Susceptibility Gene 101
VSVG	Vesicular Stomatitis Virus Glycoprotein
VPS4	Vacuolar Protein Sorting-Associated Protein 4
VR	Variable Region
VTa1	Vesicle Trafficking 1
WT	Wild Type

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1. Chapter 1 Introduction

1.1 Gene Therapy

Gene therapy is the delivery of genetic material to patient cells to prevent or treat genetic diseases by correcting defective genes, providing new genes, or altering gene expression patterns. In this way, gene therapy aims to address the root cause of genetic disorders rather than focusing on symptom management. The field of gene therapy traces its origins to the 1970s, marked by Paul Berg's pioneering work on recombinant DNA technology, in this study restriction enzymes were used to cut DNA from *E. coli* and bacteriophage λ , which was then ligated into SV40 viral DNA to produce a chimeric circular DNA molecule, demonstrating the ability to combine functional genes from different organisms into a molecular vector¹. A landmark moment came in 1990 when researchers treated a four-year-old girl with adenosine deaminase deficiency (ADA-SCID), demonstrating the therapeutic potential of gene transfer in clinical settings. However, progress was tempered by significant setbacks, such as the 1999 death of Jesse Gelsinger due to an adverse immune response to the viral vector used in his trial. These events prompted rigorous safety regulations and innovations, propelling the field forward².

By 2017, the FDA approved the first gene therapy, and as of December 2024, 21 such therapies have been approved, showcasing the field's growing impact. Current gene therapy modalities include *ex vivo* methods, where genetic modifications are performed outside the body, as seen in CAR-T cell therapies, and *in vivo* approaches, where genetic material is delivered directly into patient tissues. Delivery methods play a critical role in the success of the therapy. Genetic material can be delivered by viral vectors, such as adenovirus, retrovirus, lentivirus, and adeno-associated virus, which are commonly used for their efficient delivery, or non-viral vectors, such as lipid nanoparticles, which are safer but often less efficient and stable. Among

these, adeno-associated viruses (AAVs) are particularly notable, serving as the delivery vector for seven out of ten FDA-approved *in vivo* therapies. This includes Luxturna, the first approved *in vivo* gene therapy, which uses AAVs to deliver a functional copy of the RPE65 gene aimed at preventing vision loss in patients suffering from inherited retinal dystrophy caused by RPE65 mutations³. Despite these advancements, challenges such as limited vector payload capacity, immunogenicity, complex manufacturing, and tissue-targeted delivery remain hurdles for AAV-based therapies^{4,5}. Another limitation of conventional AAV gene therapy is the development of neutralizing antibodies. These antibodies, often pre-existing due to natural exposure to AAVs or developed after initial treatment, can prevent effective therapy delivery in some patients and make repeated treatments difficult if not impossible⁶.

Extracellular vesicles (EVs) have emerged as a new non-viral delivery modality, which could also be complementary to conventional viral vector gene therapy. These naturally secreted particles facilitate intercellular communication by transferring biomolecules between cells. Their biocompatibility, low immunogenicity, and innate delivery potential position EVs as promising candidates for next-generation gene therapy vectors^{7,8}. Notably, EVs can shield their cargo from initial immune recognition, potentially reducing the impact of neutralizing antibodies and enabling safer and more effective delivery⁹⁻¹¹. Hybridizing AAVs with EVs to create EV-AAVs presents a novel strategy to harness the strengths of both platforms, potentially overcoming the limitations of conventional AAV therapy.

This chapter introduces AAVs and EVs, covering their biology and therapeutic applications. It culminates in a discussion of how hybrid nanoparticles composed of EVs and AAVs could address the limitations of conventional AAV gene therapy.

1.2 Adeno-Associated Virus

1.2.1 Structure and Genome

AAVs were first identified as contaminants of adenovirus cultures, which is how they received their name^{12,13}. Approximately 25 nm in diameter, these small, non-enveloped viruses belong to the genus *Dependoparvovirus* within the family *Parvoviridae*. This genus is unique among parvoviruses for their dependence on co-infection with helper viruses, such as adenoviruses or herpesviruses, to complete their replication cycle^{12,14}.

The AAV genome consists of a 4.7 kb single-stranded DNA flanked by 145 bp inverted terminal repeats (ITRs), which are critical for its replication and packaging. These palindromic sequences form secondary T-shaped structures that serve as substrates for the viral replication machinery, mediated by Rep proteins^{15,16}. The genome is divided into two main regions: the *rep* and *cap* genes, which encode proteins essential for replication and capsid formation, respectively.

The *rep* region of the genome encodes four non-structural proteins, Rep 78, Rep 68, Rep 52 and Rep 40, which are generated by alternative splicing and overlapping reading frames¹⁷. Rep 78 and Rep 68 are important for viral DNA replication and site-specific integration into the host genome, while Rep 52 and Rep 40 facilitate the packaging of the AAV genome into the capsid¹⁷⁻²⁰.

The *cap* gene encodes the structural capsid proteins VP1, 2 and 3 and three accessory proteins, Assembly-activating protein (AAP), Membrane-Associated Accessory Protein (MAAP) and the X protein. AAP is encoded by an alternative open reading frame in the *cap* gene and facilitates capsid assembly by stabilizing intermediate structures during capsid formation^{21,22}. MAAP is a small hydrophobic protein that contains membrane-binding domains and may facilitate viral egress through interaction with host cell membranes²³. The X protein is a 155-

amino acid product encoded by the X ORF, located at the 3 prime end of the *Cap* gene²⁴. It plays a role in enhancing AAV DNA replication and virus production, as its absence reduces replication levels and results in approximately a 33% decrease in recombinant AAV production; however, further studies are needed to fully understand its mechanism of action^{24,25}.

The AAV capsid is a highly organized 60-subunit structure with T=1 icosahedral symmetry, made of VP1, 2 and 3, in an approximate ratio of 1:1:10²⁶. An icosahedron is a three-dimensional shape with 20 equilateral triangular faces, 12 vertices, and 60 rotational symmetries. T=1 symmetry refers to the triangulation number which is a mathematical way of describing the arrangement of identical protein subunits and reflects how many smaller triangles each of the 20 equilateral triangular faces is subdivided into. A triangulation number of 1 indicates the simplest icosahedral shape, meaning each of the 20 equilateral triangles is made of 3 protein subunits which amounts to 60 total protein subunits²⁷. VP3, the smallest of the VP proteins, is 59-61kDa; VP1 and VP2 also share this sequence. This VP3 common region plays a major structural role and forms a beta-barrel core that defines the icosahedral symmetry of the capsid. VP2 is 64-67kDa and VP1 is even larger at 79-82 kDa, sharing a common region with VP2 and VP3²⁸. Both VP2 and 1 contain nuclear localization sequences. Only VP1 has a unique N-terminal domain with a phospholipase A2 (PLA2) domain²⁹, essential for endosomal escape by enabling membrane disruption under acidic conditions. VP1 and VP2 N-terminal extensions aid in nuclear trafficking of the AAV genome³⁰⁻³².

Each VP protein contains an 8-stranded beta-barrel core connected by flexible surface loop regions, which define the external architecture of the capsid. The AAV capsid surface features three distinct symmetry axes: three-, two-, and five-fold (Fig1.1)³³. The three-fold axis is characterized by dome-like protrusions that play critical roles in receptor binding and immune

A

ITR

p5 p19 p40

Rep

Cap

Variable Regions of interest

p81

pA

ITR

Rep 78

Rep 68

Rep 52

Rep 40

MAAP

AAP

X

VP1

VP2

VP3



8

VP1, VP2 and VP3, that form the viral capsid. Also shown are additional accessory proteins AAP, MAAP and X. Variable regions of interest (VR-III and VR-IV) within the Cap gene are highlighted as green boxes, as areas of interest for capsid engineering (A). Structural model of the AAV9 capsid (PDB, 3UX1) showing icosahedral symmetry with three axes of symmetry labelled: 5-fold, 3-fold, and 2-fold. The icosahedral triangular face is outlined to highlight these axes. The positions of VR-IV and VR-III, regions of structural variation within the capsid, are indicated on the surface of the capsid structure (B). Created in <https://BioRender.com>.

The assembly of the capsid is orchestrated by the AAP, which stabilizes intermediate structures and ensures proper folding of VP proteins³⁸. The surface variability in VP3 regions contributes to the distinct tropism of AAV serotypes, allowing interactions with specific proteoglycan receptors, such as heparan sulphate for AAV2³⁹, and enabling targeted delivery to different cell types. These variable regions also serve as key determinants of immune recognition, as they interact with neutralizing antibodies that influence the efficacy of therapeutic applications^{40,41}.

1.2.2 Life cycle

The lifecycle of wild-type adeno-associated virus (AAV) is biphasic, consisting of productive infection in the presence of a helper virus and latent infection in its absence. AAV replication requires a helper virus, such as adenovirus (Ad) or herpes simplex virus (HSV), to supply essential factors that the virus lacks. In the absence of a helper virus, AAV can establish latency by integrating its genome into the host cell DNA at a specific locus on chromosome 19 (AAVS1)^{42–44}. The AAV lifecycle consists of five key stages: attachment and entry, intracellular trafficking, nuclear entry, replication/integration and assembly and release⁴⁵.

The first step to entering the cell for AAV is attachment to the cell surface. AAV serotypes bind to specific receptors, determining their tropism and entry efficiency. For example, AAV2 primarily binds to heparan sulfate proteoglycan (HSPG) as a primary receptor, with integrins serving as secondary co-receptors, enabling broad tropism. In contrast, AAV9 utilizes

the 67 kDa laminin receptor (LamR), enabling efficient transduction of the central nervous system, including crossing the blood-brain barrier^{39,46}. There are two necessary entry factors which can be important for cell attachment but are required for internalization for AAV serotypes that infect primates. Once attached to the cell surface, AAV engages the adeno-associated virus receptor (AAVR) and G Protein-Coupled Receptor 108 (GPR108), a Golgi-associated protein critical for transduction to facilitate internalization⁴⁶⁻⁴⁸. AAV2, AAV3B, AAV5, AAV6, AAV8, AAV9 and AAVrh10 require AAVR for transduction. GPR108 is required for the transduction of AAV2, AAV5, AAV8 and AAV9 serotypes. Internalization occurs through various endocytic pathways, including clathrin-mediated and caveolin-mediated endocytosis, depending on the host cell type and conditions⁴⁹⁻⁵¹. Interaction between AAV and clathrin adaptor proteins drives the formation of vesicles, which are then pinched off from the plasma membrane by dynamin, thus internalizing the virus⁵². There is also a clathrin-independent route that is facilitated by uncoated tubulovesicular carriers known as clathrin-independent carriers (CLICs)⁵³. Once internalized, the virus is enclosed within an endosome, which must be transported to the perinuclear space, where it subsequently must escape the endosome to gain access to the nucleus.

The early endosome containing the virus is trafficked towards the perinuclear region with the capsid remaining intact. To avoid lysosomal degradation, the virus must undergo the critical step of escaping the endosome before the endosome matures into a late endosome or lysosome⁵⁴. The acidic environment of the endosome induces a conformational change in the AAV capsid, exposing the N-terminal PLA2 domain of VP1, which then disrupts the endosomal membrane, releasing the AAV into the cytoplasm²⁹, while failure to escape results in lysosomal degradation or proteasomal targeting.

The small size of the virus facilitates its interaction with the nuclear pore complex (NPC); however, nuclear entry is primarily mediated by active transport. This process is enhanced by nuclear localization signals (NLS) within the viral capsid proteins VP1 and VP2, which interact with importins, such as importin β 1, to enable translocation through the NPC^{38,55}. This NLS is on the N-terminus and becomes exposed during the conformational change of the capsid at low pH. This step is often rate-limiting, with only 17% of docked virions successfully entering the nucleus³⁰. During mitosis, the nuclear envelope temporarily disassembles, allowing AAV particles to enter the nucleus directly, which greatly enhances transduction efficiency in dividing cells. AAV must rely entirely on NPC-mediated transport in non-dividing cells, where the nuclear envelope remains intact. This is a slower process, making AAV transduction more efficient in proliferative tissues^{55,56}.

Once inside the nucleus, the ssDNA genome of AAV is converted into a dsDNA form by host cell DNA polymerases, which utilize the 3' hydroxyl group exposed by the self-annealing nature of the ITRs at each end of the genome. The ITRs form T-shaped secondary structures that act as primers for host DNA polymerase to synthesize the complementary strand, resulting in a dsDNA molecule. Next, the dsDNA viral genome can take one of two pathways: integrating into the host genome or remaining as an extrachromosomal circular episome⁵⁷. These pathways are tightly regulated by AAV-encoded non-structural Rep proteins, which orchestrate replication, packaging, and integration¹⁸. Rep 78 and Rep 68 bind to the Rep-binding element (RBE) within the ITRs⁵⁸. Their ATP-dependent helicase activity unwinds the DNA to facilitate replication and site-specific integration into the AAVS1 locus on human chromosome 19^{44,59}. This integration can occur in the absence of a helper virus because the low-level expression of the rep genes can provide sufficient levels to facilitate genome recombination. However, the presence of a helper

virus, such as adenovirus, can trigger a productive infection that leads to the production of new viral particles⁶⁰. In the absence of such a helper virus, AAV can persist in a latent state, residing within the host genome until conditions change, prompting the viral genome to be rescued and replicated⁶¹.

When adenovirus infection is present, several adenoviral proteins play a role in AAV replication. E1A activates transcription of AAV rep and cap genes by promoting cellular RNA polymerase II activity. E1B55K and E4orf6 enable efficient replication of the AAV genome by suppressing cellular defences and enhancing replication processes. E2A encodes the DNA-binding protein that stabilizes ssDNA during replication. Viral-associated RNAs inhibit host cell protein kinase R, which is activated in response to double-stranded RNA produced during adenoviral infection. Activation of PKR leads to suppression of protein translation by phosphorylating eIF2 α , which could inhibit viral protein synthesis, including that required for AAV replication^{14,62}. ITRs act as the origin of replication for the AAV genome, providing the 3' hydroxyl group to initiate second-strand synthesis and facilitate the conversion of the ssDNA genome into a double-stranded form. ITRs also function as packaging signals during the AAV capsid assembly. Rep 52 and Rep 40 bind to the ITRs acting as helicases to unwind the DNA and enable efficient encapsidation of the genome^{17,56,63}. Newly synthesized capsid proteins assemble in the nucleus, facilitated by AAP, which stabilizes intermediate structures^{22,38}. Mature virions are released from the cell, possibly with the assistance of MAAP, which interacts with host membranes to promote egress²³.

1.2.3 Serotypes and tropism

Variable regions in the VP3 common region result in interaction with different cell surface receptors, and as a result, different serotypes have distinct tropisms. For example, AAV1

efficiently transduces skeletal muscle, cardiac muscle and the central nervous system (CNS), while AAV2 has a strong affinity for the liver, CNS and retinal tissues. AAV5 and AAV8 are known for high liver tropism, with AAV8 being particularly efficient for hepatocyte transduction following systemic delivery. AAV9 uniquely crosses the blood-brain barrier, making it suitable for CNS applications while also targeting cardiac and skeletal muscle^{40,64}.

1.2.4 AAV gene therapy

The discovery of AAV in 1965 marked the beginning of an important journey in gene therapy research^{12,13}. The AAV2 genome was subsequently cloned and sequenced in 1982, which laid the groundwork for further exploration into its therapeutic potential⁶⁵. Recombinant AAVs (rAAVs) were engineered by replacing the wild-type AAV genome with therapeutic transgene cassettes, retaining only the inverted terminal repeats (ITRs) needed for replication and packaging^{14,64,66}. This design not only incorporates the therapeutic transgene but has the additional benefit that rAAVs remain replication-incompetent, even in the presence of helper viruses, as they lack the necessary Rep and Cap genes. By 1995, AAVs were applied in human gene therapy for the first time, demonstrating their utility as vectors for delivering genetic material⁶⁷. The first AAV-based gene therapy to receive marketing authorization from the European Medicines Agency (EMA) was Glybera (alipogene tiparvovec) in 2012, aimed at treating lipoprotein lipase deficiency⁶⁸. Following this, Luxturna (voretigene neparvovec) became the first AAV gene therapy approved by the U.S. Food and Drug Administration (FDA) in 2017, targeting inherited retinal diseases³. Since the approval of these initial therapies, the landscape of AAV gene therapy has expanded significantly. As of October 2023, six additional AAV gene therapies have been approved by the FDA, with Kebilidi being the most recent. Kebilidi is designed for the treatment of aromatic L-amino acid decarboxylase (AADC) deficiency, a rare genetic disorder that impacts

neurotransmitter synthesis⁶⁹. This rapid progression underscores AAV's position as a leading viral vector in gene therapy and a focal point in research, with numerous clinical trials exploring their efficacy across various genetic disorders⁷⁰.

1.2.4.1 Advantages of AAV-based gene therapy

AAV-based gene therapy offers a range of compelling advantages that make it a preferred choice for gene replacement therapeutic applications. One of the key benefits is that AAV has no known pathogenicity in humans, significantly reducing the risks associated with its use as a vector^{64,66,71}. This safety profile has been supported by extensive research, indicating that AAV vectors are not pathogenic, making them suitable for a variety of therapeutic applications^{72,73}. Additionally, AAV primarily facilitates episomal expression, meaning that the delivered genetic material remains separate from the host genome, which lowers the risk of insertional mutagenesis^{74,75}. This episomal nature not only enhances safety but also supports long-term transgene expression in postmitotic cells, making AAV an effective tool for treating chronic and genetic diseases^{76,77}.

1.2.4.2 AAV engineering

Advancements in gene therapy have focused on the engineering of the AAV vector to enhance its properties, particularly in terms of tropism, transduction efficiency and immune evasion. AAV capsid engineering can be employed to change the surface of the capsid for improved targeting and gene delivery. One of the earliest approaches was the creation of mosaic and chimeric capsids through the swapping of capsid protein regions between different AAV serotypes, a method known as capsid shuffling⁷⁸. This technique allowed the generation of novel vectors with improved tissue specificity and reduced immunogenicity. Directed evolution further

advanced this field by subjecting libraries of mutated capsids to selective pressures to isolate variants with desired properties^{79,80}.

In terms of specific capsid modifications, two key regions have been targeted for engineering: the two prominent loops on the capsid surface (VR-IV and VR-VIII) and the N-termini of the viral proteins (VPs)^{81,82}. The N-terminus of VP2 or VP1 can accommodate large peptide insertions, but since these VPs are present in lower quantities compared to VP3, the resulting capsids are mosaic in nature, with only some subunits displaying the insertion.

Insertions into the surface-exposed loops, particularly the VR-IV and VR-VIII loops, have the advantage of the option of modifying all three VP proteins, ensuring a uniform display of the inserted peptide across the capsid surface. For example, inserting an integrin-binding peptide into the VR-VIII loop of AAV2 disrupts the heparin binding motif responsible for the native tropism of AAV2 successfully retargeting the virus to cells expressing integrin receptors, enhancing its ability to transduce desired cell types while avoiding off-target interactions^{83,84}. This alteration can redirect viral tropism and reduce off-target effects. Another example of insertion into a loop is the incorporation of single-chain variable fragments (scFvs) or nanobodies into the capsid loops to retarget the virus to specific receptors⁸⁰. Modifications at the VR-VIII loop not only allow for the insertion of targeting motifs but also disrupt heparan sulfate proteoglycan (HSPG) binding, which is the primary receptor for AAV2⁸⁵. This alteration can redirect viral tropism and reduce off-target effects. However, large insertions into these loops can interfere with capsid assembly and reduce viral yields, leading to limitations on which modifications can be made⁸¹. The C-terminal regions of VP proteins are crucial for proper capsid assembly and stability. Modifying this region risks disrupting essential inter-subunit interactions required to form the icosahedral capsid. Additionally, the C-terminal regions of VP proteins are buried inside the

capsid, making them less accessible for external interactions. Engineering of the N-terminal PLA2 domain in VP1 could improve endosomal escape.

Although much research has focused on engineering the AAV capsid, engineering the ITRs can also improve AAV transduction by stabilizing the AAV genome and reducing its susceptibility to degradation. ITRs have a high GC content and a palindromic structure which can lead to mutations and truncations during plasmid amplification, which in turn have been shown to impact AAV yield and potency⁸⁶. Additionally, CpG rich DNA has been shown to activate the innate immune response⁸⁷. It has been shown that it is possible to genetically remove CpG sites within the ITRs without affecting AAV *in vivo* gene delivery⁸⁸. Furthermore, in human embryonic stem cells, it has been shown that ITR-induced p53-dependent apoptosis could be prevented by using an AAV with a synthetic ITR where putative p53 binding sites were disrupted by substitution mutations⁸⁹. Thus, engineering the ITRs can enhance the persistence of the vector genome^{86,90}.

Techniques such as directed evolution and rational design have been employed to create novel capsids or modify specific amino acid residues, enhancing tropism and facilitating the delivery of therapeutic genes to challenging tissues like the muscle, brain, and retina^{91–94}. Capsid and ITR engineering can improve targeting and transduction of AAV vectors. These innovations have significantly expanded the potential applications of AAV vectors in gene therapy, particularly for diseases that require precise targeting and sustained expression of therapeutic genes.

1.2.4.3 Disadvantages of AAV-based gene therapy

Despite its significant advantages, AAV-based gene therapy faces notable limitations and challenges. One major constraint is the virus's small packaging capacity, limited to

approximately 4.7 kb, which restricts its use for larger transgenes^{5,82}. This constraint makes it challenging to deliver large or complex transgenes, particularly those requiring additional regulatory elements such as promoters, enhancers and polyadenylation signals. For example, therapeutic applications targeting conditions like Duchenne muscular dystrophy (DMD), which require large transgenes, often necessitate alternative strategies such as multi-vector systems. These approaches attempt to split the therapeutic gene across two vectors, which then recombine within the host cell, but this method is inherently inefficient and further complicates delivery⁹⁵.

High doses required for systemic delivery often trigger immune responses, including the production of neutralizing antibodies, which can diminish therapeutic efficacy and make repeat administrations impractical^{6,96}. This inability to redose is particularly problematic because AAV is a non-integrating vector; its delivered transgene remains episomal rather than integrating into the host genome. Consequently, the therapeutic gene becomes progressively diluted in dividing cells over time, especially in rapidly proliferating tissues, reducing long-term efficacy⁹⁷. Adverse effects, such as hepatotoxicity, genotoxicity, thrombotic microangiopathy and neurotoxicity, have further raised concerns about safety, particularly for high-dose applications⁹⁷. Furthermore, administration of high doses of AAVs ($>1 \times 10^{14}$ vg/kg) has resulted in multiple fatalities during clinical trials due to innate immune response to the vector. For example, Pfizer's Duchenne's Muscular Dystrophy trial reported a 27-year-old patient died after administration of 1×10^{14} vg/kg dose of rAAV9^{98–100}.

Additionally, pre-existing neutralizing antibodies against common AAV serotypes in the human population can significantly hinder treatment outcomes. A study revealed that among 552 human serum samples, the highest prevalence of neutralizing antibodies (NAbs) against AAV serotypes, excluding AAV2, were observed for AAV1 (74.9%), followed by AAV6 (70.1%),

AAV5 (63.9%), AAV8 (60.4%) and AAV9 (57.8%)¹⁰¹. In addition to the NAbs, other immune responses exist. When administered, AAV capsid proteins can be processed by antigen-presenting cells (APCs) and presented on MHC II molecules, leading to the activation of CD4+ T helper cells¹⁰². These cells can stimulate cell-mediated immunity by activating CD8+ cytotoxic T cells, which may target and destroy transduced cells expressing AAV-delivered transgenes^{102,103}. Additionally, the transgene product itself can be recognized as foreign, triggering an immune response that may limit the duration and efficacy of transgene expression¹⁰⁴.

Manufacturing challenges also present a critical bottleneck, as scaling up the production of high-quality rAAV vectors remains resource-intensive and costly^{4,105}. AAV production requires co-infection of cells with a helper virus or transfection with multiple plasmids encoding helper functions, complicating the manufacturing process. Downstream purification techniques can be complex and costly. HEK293 adherent cells are often used for production and require large surface areas for growth, limiting scalability and increasing costs for large-scale vector production. Suspension HEK293 cells allow for more efficient scalability in bioreactors, enabling high-density cell growth and facilitating large-scale production, but have the drawback of being more difficult to transfect in comparison to adherent cells¹⁰⁶. To overcome this, stable producer cell lines have been developed that express one or more of the required genes for AAV production¹⁰⁷.

While AAV-based gene therapy offers potential for treating genetic diseases, its current limitations highlight the need for continued research and innovation. Addressing challenges such as limited packaging capacity, immune responses and safety concerns will be critical to unlocking its full therapeutic potential.

1.3 Extracellular vesicles

1.3.1 Classification and Composition

Extracellular vesicles (EVs) are endogenous lipid nanoparticles released by cells. EVs were first observed as precipitable factors in platelet-free plasma in 1946, and then later in 1967 were described as “Platelet dust” using electron microscopy^{108,109}. Two decades later, the first evidence for the biological function of EVs was found in studies showing vesicles shedding transferrin receptors in maturing reticulocytes^{110,111}. Further research demonstrated that rather than merely serving as a cellular mechanism for discarding unnecessary receptors, EVs were demonstrated to carry cargo involved in intercellular signalling^{112–114}. The field has since evolved with technological advancements in isolation and characterization methods, including nanoparticle tracking analysis, flow cytometry and advanced microscopy techniques, leading to the establishment of the International Society for Extracellular Vesicles (ISEV) in 2012. EVs are classified into subtypes based on their size, biogenesis and release mechanisms¹¹⁵. EVs can be subdivided into three primary groups of overlapping size ranges based on their cellular origin: exosomes (30-150 nm), ectosomes (50 nm-10 µm) and apoptotic bodies (50-2000 nm)¹¹⁶.

Exosomes are formed through the endocytic pathway, where invagination of the plasma membrane leads to the formation of early endosomes¹¹⁶. Early endosomes mature into late endosomes or multivesicular bodies (MVBs) with intraluminal vesicles (ILVs), which are formed by inward budding of the endosomal membrane. A central role is played by the Endosomal Sorting Complexes Required for Transport (ESCRT) machinery, which contains four different protein complexes: ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III, as well as associated proteins such as vacuolar protein sorting-associated protein 4 (VPS4), vesicle trafficking 1 (VTA1), and Programmed cell death 6-interacting protein (PDCD6IP or ALIX)¹¹⁷.

ESCRT proteins can facilitate the sorting of ubiquitinated proteins into ILVs during MVB formation, where ESCRT-0 recognizes and clusters ubiquitinated cargo, ESCRT-I and ESCRT-II promote membrane deformation and budding and finally, ESCRT-III and VPS4 finalize vesicle scission^{118,119}. Parallel to this canonical pathway, several ESCRT-independent mechanisms have been identified, including a ceramide-dependent pathway where neutral sphingomyelinase 2 generates ceramide, creating membrane curvature through its cone-shaped structure¹²⁰. Tetraspanin-enriched microdomains play crucial roles in organizing cargo and facilitating vesicle formation, with CD63, CD81 and CD9 being particularly important in this process. Once MVBs are formed, they fuse with the plasma membrane to release the ILVs into the extracellular space, thus becoming exosomes. Alternatively, the MVBs can fuse with lysosomes to be degraded, a process mediated by Rab GTPases. Rab31 influences the early endosome to MVB transition and cargo selection to control an ESCRT-independent exosome secretion pathway¹²¹. Rab5 regulates the maturation of early endosomes into late endosomes, where ILVs are formed¹²². Rab7 controls MVB maturation by directing MVBs either to fusion with lysosomes, degradation or trafficking to the plasma membrane⁵¹. Rab35 regulates actin to support MVB trafficking and exosome release. Rab27a and Rab27b regulate the docking of MVBs to the plasma membrane and their subsequent fusion, facilitating exosome release¹²³. Finally, Rab11 participates in the recycling endosome pathway, promoting exosome secretion in a calcium-dependent manner¹²⁴. Together, these mechanisms highlight the intricate interplay of molecular components that regulate exosome formation, enabling their pivotal role in intercellular communication and signalling.

Ectosomes are formed through the outward budding of the plasma membrane, a process that is initiated by specific stimuli such as an increase in intracellular calcium levels. This rise in calcium disrupts the cytoskeleton and induces actin-myosin contractions, leading to membrane

deformation and vesicle formation¹²⁵. Concurrently, there is a loss of lipid asymmetry in the plasma membrane, with phosphatidylserine flipping to the outer leaflet, which promotes membrane curvature and facilitates the budding process¹²⁶. During this biogenesis, specific cargo, including proteins, lipids and nucleic acids, is selectively incorporated into the ectosomes, reflecting the physiological state of the parent cell. The mechanisms underlying cargo sorting into ectosomes involve higher-order oligomerization of membrane proteins, anchoring to lipid rafts and interactions with tetraspanin-enriched microdomains, which contribute to the selective enrichment of cargo¹²⁷.

The molecular machinery that governs ectosome formation includes small GTPases, particularly ADP-ribosylation factor 6 (ARF6) and RhoA, which play crucial roles in regulating cytoskeleton reorganization and membrane budding^{125,128}. Activation of ARF6 leads to the activation of phospholipase D and subsequent phosphorylation of ERK, which promotes myosin light chain kinase activity and cytoskeletal contraction¹²⁸. RhoA, through its effector ROCK, further modulates actomyosin contractility, enhancing the budding process.¹²⁹ The final membrane fission is facilitated by the ESCRT-I protein Tumor susceptibility gene 101 (TSG101) and the ESCRT-III protein Arrestin domain-containing protein 1 (ARRDC1), which are recruited to the plasma membrane through interactions with specific lipid domains¹³⁰. This intricate interplay of molecular signals and structural changes culminates in the release of ectosomes into the extracellular environment, where they play significant roles in intercellular signaling and immune response modulation¹³¹.

Apoptotic bodies are formed by the blebbing of the plasma membrane of cells undergoing programmed cell death. Unlike other Evs, apoptotic bodies contain various cellular organelles, including fragmented mitochondria, nuclear fragments, and condensed chromatin.

They are uniquely packed with genomic DNA fragments, histones and cellular proteins that have been modified by caspase-mediated proteolysis^{113,116,132}. Apoptotic bodies can play significant roles in immunostimulation by recruiting phagocytic cells and presenting antigens to activate immune responses, thereby contributing to increased inflammation¹³².

The molecular composition of EVs is intricately linked to their cellular origin and biological function, encompassing a diverse array of bioactive molecules. The lipid bilayer membrane of EVs is notably enriched in cholesterol, sphingomyelin and phosphatidylserine, which not only contribute to their stability but also facilitate interactions with recipient cells^{127,133,134}. This lipid composition influences the mechanical properties of the membrane, including its rigidity and permeability, thereby affecting the vesicles' functionality and cellular uptake mechanisms^{134,135}. In addition to lipids, the protein cargo of EVs includes a variety of membrane trafficking proteins such as Rab GTPases, tetraspanins (e.g., CD63, CD9, CD81) and heat shock proteins (HSP60, HSP70, HSP90), alongside cell-type-specific proteins^{127,136}. These proteins play essential roles in the biogenesis, sorting and uptake of EVs, highlighting the complexity of their molecular architecture. The presence of specific lipid species, including ceramide and phosphatidylserine, is particularly noteworthy as these lipids enhance membrane rigidity and serve as recognition signals for recipient cells, thereby influencing the functions of EVs¹³⁶. Moreover, the asymmetric distribution of phosphatidylserine in ectosomes acts as a crucial recognition signal for recipient cells, facilitating their uptake and contributing to various functions such as apoptosis and intercellular communication¹²⁷. This lipid asymmetry is a hallmark of EVs and is vital for their role in mediating cellular interactions and signalling pathways. Since the size ranges of these three types of EVs overlap, isolating a particular group

based on size and density alone is unfeasible. Therefore, they are collectively referred to as extracellular vesicles unless their origin is specifically identified.

1.3.2 EVs as therapeutic tools

The therapeutic potential of EVs was first recognized in the early 2000s, when researchers discovered their role in intercellular communication and biomolecule transfer^{112,114}. Initial studies focused on their ability to carry RNA and proteins, sparking interest in their use as natural delivery vehicles¹¹⁴. By the 2010s, advancements in isolation and engineering techniques allowed EVs to be explored for targeted drug delivery and regenerative medicine, including the use of mesenchymal stem cell-derived EVs for wound healing and immune modulation^{137,138}. Additionally, EVs reflect their cell of origin and as such can serve as biomarkers of disease and have been applied as biomarkers for several cancers¹³⁹. EVs are being investigated as an effective delivery vehicle for multiple therapeutic cargos^{140,141}.

1.3.2.1 Advantages of EVs as therapeutics

EV therapy offers several advantages that make it a promising tool for modern medicine. EVs can be used as a therapeutic option either for their innate qualities or can be engineered to carry diverse cargos. For example, EVs derived from MSCs have been shown in clinical trials to be a beneficial treatment for graft-versus-host disease, as well as to improve wound healing in clinical settings^{137,142}. In contrast to traditional MSC cell therapy, EVs are not living and, therefore, have more stability and can be lyophilized; additionally, they cannot replicate as cells can¹⁴³.

EVs offer several advantages over synthetic particles such as lipid nanoparticles (LNPs) as therapeutic delivery vehicles. Unlike LNPs, which often induce inflammatory responses and

have limited biocompatibility, EVs are naturally derived from cells and exhibit superior biocompatibility with reduced immunogenicity^{144,145}. For example, EVs loaded with siRNA demonstrated significantly lower systemic toxicity and immune activation compared to LNP-based delivery in preclinical models^{144,145}. EVs exhibit greater stability in physiological conditions, with studies showing reduced off-target accumulation and longer circulation times compared to LNPs, which are rapidly cleared by the liver and spleen^{146,147}. This difference is partly due to the physical and biochemical properties of EVs, which are typically smaller, have a near-neutral surface charge, and present endogenous membrane proteins such as CD47 that help them evade clearance by the liver and spleen^{148,149}. In contrast, LNPs are more readily opsonized upon systemic administration, as serum proteins—particularly apolipoprotein E (ApoE)—adsorb onto their surface, directing them to Kupffer cells in the liver via LDL receptor-mediated uptake¹⁵⁰. Their synthetic lipid composition and lack of self-markers further promote rapid clearance by the mononuclear phagocyte system^{150,151}.

EVs can protect therapeutic cargos such as RNA, proteins, or small molecules from degradation and immune detection, enabling efficient delivery to target cells. For instance, siRNA-loaded EVs demonstrated stability and effective gene silencing in breast cancer models¹⁵². Furthermore, EVs' natural ability to cross biological barriers, including the blood-brain barrier, makes them ideal for delivering therapeutics to the central nervous system, as evidenced by studies targeting neurodegenerative diseases such as Alzheimer's^{153,154}. EVs are thought to cross the blood-brain barrier via mechanisms such as receptor-mediated transcytosis, leveraging surface ligands like integrins or transferrin that interact with endothelial receptors, as well as through endogenous uptake and vesicular trafficking pathways. In contrast, rAAVs cross the BBB through specific capsid-receptor interactions, often requiring serotype selection or

capsid engineering for example AAV9 or PHP.B to enhance CNS delivery, with efficacy varying significantly across serotypes^{91,148,155–158}.

EVs and lentiviral vectors share several similarities that have made both attractive platforms for therapeutic delivery. Both are enveloped nanoparticles capable of packaging and delivering biologically active cargo into recipient cells¹⁵⁹. Interestingly the biogenesis pathways of EVs and lentiviruses intersect. As previously discussed, exosomes are formed via the endosomal pathways, budding into MVBs before being released into the extracellular space through fusion with the plasma membrane¹¹⁶. Lentiviral assembly can take place in the MVB or at the plasma membrane¹⁶⁰. This overlap in biogenesis contributes to the physical and compositional similarity between EVs and retroviral particles, including shared membrane lipids and incorporation of host-derived proteins. There are several key differences between EVs and lentiviruses which impact their therapeutic applications. Lentiviral vectors carry an RNA genome and encode the necessary machinery for reverse transcription and genomic integration, which allows for long-term expression of transgenes but raises safety concerns related to insertional mutagenesis and oncogenic potential¹⁶¹. EVs on the other hand are non-replicative and do not direct the integration of DNA into the host genome, conferring a superior biosafety profile, particularly for clinical applications requiring transient gene expression in non-replicating cells. Furthermore, EVs naturally express immunomodulatory proteins such as CD47 and can escape neutralizing antibody responses, making them more amenable to repeated systemic administration, whereas lentiviral vectors face significant limitations due to anti-vector immunity^{149,162}.

1.3.2.2 Disadvantages of EVs as therapeutics

EV therapeutics still face challenges. EVs are a heterogeneous population and are highly dependent on the cell of origin's condition, and even from the same cell, different EV populations can have different effects¹⁴⁶. EV subtypes, as discussed above, have overlapping size ranges, making isolation of specific subtypes difficult. Additionally, different EV isolation techniques—such as ultracentrifugation, size-exclusion chromatography, precipitation, and immunoaffinity capture—yield EVs with varying purity, size distributions, and surface compositions, which can strongly influence downstream functionality. For example, the high speeds used during ultracentrifugation can result in co-isolation of aggregates or lipoproteins that can affect EV function¹⁶³. The loading capacity of EVs can be limited. Although small molecules can be loaded into EVs through sonication, larger molecules need to be actively loaded either through the engineering of the producer cells or through surface modification of the EV to bind therapeutic cargo at the surface. EVs can be engineered to load specific cargo, however it cannot be guaranteed that other molecules are not also loaded into the EV. Therefore, cell sources should be carefully considered; for example EVs from tumour cells have been observed to promote metastasis¹⁶⁴. Additionally, EVs get cleared very quickly from the bloodstream after intravenous injection, which can impact therapeutic delivery¹⁴⁹. This can be mitigated in engineered EVs through addition of CD47, B2M or PDL1 to protect EVs from phagocytosis^{149,165}. Once the EV arrives at the target cell, the therapeutic cargo must be released, thus endosomal escape can become a limiting factor^{8,131}.

1.3.2.3 EV engineering

One of the key advantages of EVs is their amenability to engineering, allowing researchers to overcome many of the challenges associated with therapeutic delivery.

Engineering strategies for EVs include surface modification, which can significantly improve targeting and uptake by specific cells and, consequently, therapeutic efficacy by incorporation of ligands or antibodies onto EV surfaces. Loading strategies can be split into two categories: endogenous loading and exogenous loading. Endogenous loading of EVs involves the natural incorporation of biomolecules, such as proteins, lipids and RNAs, during the vesicle formation process within the parent cell. Exogenous loading involves the direct introduction of therapeutic or diagnostic agents into pre-isolated EVs through methods such as electroporation, sonication or chemical treatment. For example, endogenously engineering EVs to have RGD peptides has been used to enhance tumour targeting in cancer therapy¹⁶⁶. In some cases, EV scaffold proteins have been fused with therapeutic cargo to actively load proteins of interest, broadening their therapeutic potential. A screen of such scaffold proteins identified several top candidates¹⁶⁷. For example, one such scaffold protein called Pituitary Tumor-Transforming Gene 1 Protein-Interacting Protein (PTTG1IP) was used as a scaffold to load gene editing machinery¹⁶⁸. Exogenous loading has been demonstrated through electroporation to load siRNA into EVs for gene silencing in breast cancer cells¹⁵². Other methods, such as sonication and chemical treatments, have been developed to encapsulate nucleic acids or small molecules into EVs effectively. For instance, Haraszti et al. (2017) demonstrated that chemically stabilized hydrophobic siRNAs could be effectively loaded into EVs, providing a viable method for delivering RNA-based therapeutics to the central nervous system¹⁶⁹. Aptamer-functionalized EVs allow for selective interaction with specific receptors on target cells, enhancing therapeutic efficacy while minimizing off-target effects¹⁷⁰. These two loading strategies can be combined. For example, EVs can be endogenously engineered to display a fragment crystallizable (Fc) binding region on the EV surface and then be exogenously combined with an antibody of choice

to target specific tissues or tumours. For example, anti-human epidermal receptor 2 (HER2) antibodies can bind Fc-EVs to deliver cargo specifically to cells overexpressing HER2¹⁴⁶.

One benefit of EVs is their ability to carry multiple cargos simultaneously, with different types of therapeutic agents localized to the surface and interior of the EVs. For example, EVs can be engineered to display targeting ligands, such as antibodies or peptides, on their surface while encapsulating therapeutic cargo within their lumen. A study demonstrated EVs could be loaded with an anti-EGFR antibody on the surface and doxorubicin, a chemotherapeutic agent, within the EV lumen, which allowed for targeted cancer therapy with enhanced tumor suppression¹⁷¹. This dual-loading approach highlights the versatility of EVs for combination therapies, enabling synergistic effects between surface-targeting agents and internal therapeutic cargos.

Stability and circulation time are critical for EV efficacy in therapeutic applications. Engineering strategies such as hydrogels or polymeric coatings can protect EVs from premature degradation and enhance bioavailability. For example, the incorporation of silicates into endothelial progenitor cell-derived EVs has been shown to significantly improve therapeutic effects in myocardial infarction models by enhancing angiogenesis and revascularization⁹. Many EVs are rapidly cleared from circulation by macrophages; however, the addition of “don’t eat me” signals, such as CD47, has been shown to significantly extend EV circulation time¹⁴⁹.

Hybrid EV systems have been developed by combining EVs with synthetic delivery platforms, such as liposomes, to improve stability and drug delivery efficiency. For example, the creation of hybrid EVs has shown improved stability and drug delivery efficiency¹⁷². These innovative strategies demonstrate the versatility of EV engineering in addressing current limitations and enhancing therapeutic outcomes.

In conclusion, EV engineering encompasses diverse approaches, from surface modifications and cargo loading to enhancing stability and biological targeting. Their ability to carry multiple therapeutic agents simultaneously, combined with engineering innovations, continues to expand the therapeutic potential of EVs, offering significant advantages over traditional drug delivery systems and paving the way for their use in precision medicine.

1.4 EV-AAVs

EVs are being investigated as an effective delivery vehicle for multiple therapeutic cargos^{8,140}. Interestingly, several non-enveloped viruses have been shown to take advantage of the EV sorting machinery to become quasi-enveloped particles¹⁷³. For example, hepatitis A virus (HAV), a non-enveloped picornavirus, has a pX domain which associates with the ESCRT Pathway component ALIX (Apoptosis-linked gene 2–interacting protein X), moving HAVs into EVs and shielding them from NABs^{174,175}. Similarly, enteroviruses like poliovirus have been documented to spread as clusters within extracellular vesicles, enabling simultaneous transmission of multiple viral particles to target cells and potentially enhancing infection efficiency¹⁷⁶. This phenomenon has also been observed with hepatitis E virus and rotavirus, suggesting that vesicle-mediated transmission may be a more common strategy among non-enveloped viruses than previously recognized¹⁷³.

A proportion of the AAVs naturally incorporate into EVs, forming EV-AAVs¹⁷⁷. EV-AAVs have been shown to more efficiently transduce in the liver, retina, nervous system and inner ear in comparison to AAVs alone^{178–181}. They also have the added benefit of being able to co-package additional molecules for improved targeting or immunomodulation. Multiple AAV particles can be encapsulated within EVs due to their size difference, which can enhance delivery efficiency (Fig1.2)¹⁷⁷. The vesicle membrane provides natural protection against immune

recognition, which is theorized to allow for repeated administration in gene therapy applications – a significant advantage given that pre-existing immunity to AAV capsids remains a major challenge in the field¹¹. However, EV-AAVs' efficacy as a candidate for clinical therapeutics is limited by low levels of endogenous incorporation of AAVs into EVs and the lack of understanding of EV-AAV biogenesis, which complicates engineering approaches. Ongoing advancements in production techniques and a deeper understanding of their biology are crucial to fully harnessing the capabilities of EV-AAVs in clinical settings.

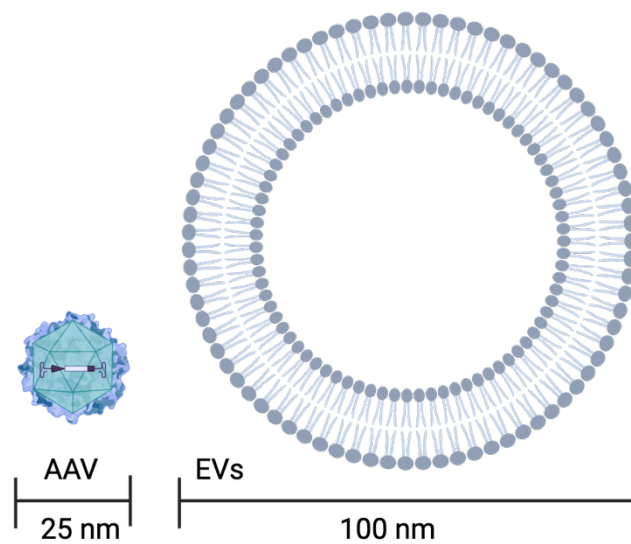


Figure 1.2 Size comparison between AAVs and EVs

A size comparison illustrating the relative dimensions of an AAV and EVs. The AAV particle has a diameter of approximately 25 nm, while EVs, represented by a lipid bilayer structure, range in size from 50 to 150 nm. A 100 nm EV is depicted as an example. The theoretical maximum loading capacity assuming a spherical shape and a packaging efficiency of 50-60% results in 32-39 AAVs per 100nm particle. Assuming both particles are spherical and applying a packing efficiency of 50–60%, the theoretical internal volume of a 100 nm EV could accommodate approximately 32–39 AAV particles. However, in practice, EVs rarely encapsulate this number, most likely due to structural and biological constraints¹⁷⁷.

1.5 Thesis Aims

EV-AAVs have the potential to overcome some of the limitations of conventional AAV-based gene therapies. However, despite these promising features, the biological characteristics and mechanisms governing EV-AAV formation remain poorly understood. This thesis aims to address critical gaps in understanding of EV-AAV biology and to explore innovative engineering strategies for enhancing their therapeutic utility.

The following aims will be addressed in this thesis:

1. Characterization of EV-AAVs:

Although EVs have been shown to naturally incorporate AAVs, the specific vesicle characteristics and mechanisms underlying this incorporation remain unclear. In this thesis, I aim to characterize EVs that incorporate AAVs, focusing on their molecular composition, size and functional properties. This will provide foundational knowledge essential for understanding the biology of EV-AAVs and inform future engineering strategies.

2. Development of Engineering Strategies to Enhance EV-AAV Yield:

Current levels of AAV incorporation into EVs are insufficient for therapeutic applications. To address this, several engineering strategies to increase EV-AAV yield were explored. These include:

- **Aim 2A:** Loading AAVs at the surface of EVs to improve their functional delivery.
- **Aim 2B:** Engineering the producer cells to enhance AAV incorporation into EVs.
- **Aim 2C:** Modifying the AAV capsid to increase its association with EVs.

Together, these approaches will contribute to the establishment of a robust framework for the efficient production and therapeutic application of EV-AAVs.

2 Chapter 2. Materials and Methods

2.1 Materials

Table 2.1 List of Antibodies

<u>Western Blot primary antibody</u>	<u>Source</u>	<u>ID/ Manufacturer</u>	<u>Dilution</u>
CD9	Mouse monoclonal	#sc-13118 Santa Cruz Biotechnology	1:500
AAV-VP1-3	Mouse monoclonal	#65158 Progen	1:500
Laminin Receptor	Rabbit polyclonal	#ABC934 Merk Millipore	1:1000
AAVR, KIAA0319L	Mouse polyclonal	#ab105385 Abcam	1:1000
Vinculin	Mouse monoclonal	#V9131 Sigma Aldrich	1:100000
β -Actin	Rabbit polyclonal	#622101 BioLegend	1:1000
Alix	Mouse monoclonal	#634502 BioLegend	1:1000
CD63	Mouse monoclonal	#10628D Thermo Fisher	1:1000
Syntenin	Mouse monoclonal	#TA504796 OriGene	1:1000
CD81	Mouse monoclonal	#ab79559 Abcam	1:500
Flotillin-1/2	Rabbit Polyclonal	#TA321128 Origine	1:1000
Calnexin	Rabbit Polyclonal	#GTX101676 Genetex	1:1000
GFP	Mouse monoclonal	#ab291 Abcam	1:1000
TSG101	Mouse monoclonal	#612696 BD Biosciences	1:1000
AnnexinA1	Rabbit monoclonal	#ab214486 Abcam	1:1000
<u>WB Secondary antibody</u>	<u>Source</u>	<u>ID/ Manufacturer</u>	<u>Dilution</u>
Anti-mouse IgG, HRP-linked	Horse	#7076 Cell Signalling Technology	1:10000
Anti-rabbit IgG, HRP-linked	Goat	#7077 Cell Signalling Technology	1:10000

<u>Immunoassay Antibody</u>	<u>Source</u>	<u>ID/ Manufacturer</u>	
TIM-4	CHO cells	# AG-40B-0180B-C010 Adipogen	
CaptureSelect Biotin Anti-AAV9 Conjugate	<i>S. cerevisiae</i>	#7103332100 Thermo Fisher	
Anti-AAV9 (intact particle) mouse recombinant, ADK9-1R	Mouse	#610178 Progen	
MSD SULFO-TAG Labelled anti-mouse antibody	Goat	#R32AC-1 Mesoscale Discovery	

Table 2.2 List of Primers

Transgene	Forward sequence (5'-3')	Reverse sequence (5'-3')
<i>EGFP</i>	GAACCGCATCGAGCTGAA G	TGCTTGTCGGCCATGATATA G
<i>iRFP</i>	CTGGAATTCATGCGCAACATA G	GTGATGGCAAACGATCAAT CC
<i>Nanoluciferase</i>	GGATTGTCCTGAGCGGTGAA	CAGTGTGCCATAGTGCAGG A
<i>mScarlet</i>	CGCGTGATGAACTTCGAGGA	GCGGAGCTTCACCTTGTAGA
AAV9Capsid Library Sequencing (~200bp)	CGACTAATGAATCCACTCATC G	CGTTGTTGTCGGTAGCTG
CAP primers for titration (OHF345 and 346)	CGGTAGCAACGGAGTCCTAT G	AAGTTGCCGTCCGTGTGAGG
GAPDH	TGTAGTTGAGGTCAATGAAG GG	TGGGTGTGAACCATGAGAA G

2.2 Methods

2.2.1 Cell Culture

HEK293T (Millipore Sigma), Huh7 AAVR knockout, Huh7 wildtype and SH-SY5Y-Traffic light cells were cultured in DMEM (Life Technologies) supplemented with 10% heat-inactivated FBS (Life Technologies) and 1x antibiotic/antimycotic solution (Sigma). Cells were

cultivated at 37 °C in 5% CO₂, 90% humidity. Cells were routinely checked for mycoplasma contamination by PCR Mycoplasma Test Kit I/C (Promokine). Huh7 and SH-SY5Y-traffic light cells were generously provided by Evox. For CD63 overexpression, doxycycline-inducible cell lines, cells were treated with 500 ng/mL doxycycline (Sigma, Catalog no. D9891).

2.2.2 Drug Treatments

Cells were transfected with plasmids for AAV production as described below and 4 h after transfection, media was changed to media containing the chemical inhibitor or DMSO controls. Cells were treated with either 5 nM Bafilomycin (rPeptide, Catalog no. CV-1015-1), 1 μ M Y27632 (Sigma, Catalog no. SCM075) or DMSO control. Media and cell lysates were collected by iodixanol gradient.

2.2.3 AAV Production

HEK293T cells were seeded in 150 cm² polystyrene dishes (Starlab). When cells were at 70-80% confluency, media was changed to OptiMEM (Life Technologies) or DMEM (Life Technologies) supplemented with 2% FBS (Life Technologies) depleted of EVs. Cells were transfected with Helper (Cell Biolabs, Catalog no. 340202) (22.4 μ g), RepCap2/9 (Addgene, Catalog no. 112865) (14.15 μ g) and transgene plasmids (13.37 μ g), totalling 50 μ g DNA/plate mixed with PEI MAX® - Transfection Grade Linear Polyethylenimine Hydrochloride (MW 40,000) (Polysciences, Catalog no. 24765) (1:2.5 ratio). Cells were left to incubate for 8 h or overnight. Then media was changed to either 2%EV-free FBS DMEM or OptiMEM. After 2 days, media was collected. Media was then spun down 400xg for 5 min and supernatant was collected and further spun at 4000xg for 20 min. Media was then concentrated using tangential flow filtration (TFF) using a Pierce™ Crossflow Filtration Cassette with a 100 kDa MWCO

(Thermo Scientific, Catalog no. A57934), followed by 100kDa MWCO spin filters (Merk Millipore). Concentrated media was processed in one of three ways: 1. ultracentrifugation, 2. iodixanol fractionation or 3. size exclusion liquid chromatography. For more details see methods below.

2.2.3.1 *AAV Isolation*

To isolate AAVs from the media, media was processed as described in the iodixanol fractionation method. Fractions 9-11 were pooled into a 100 kDa MWCO filter and washed 4 times with PBS to remove iodixanol. Samples were then concentrated to 200 μ L and stored at 4°C short term or -80°C long term. To collect AAV from the cell lysate fraction, media was decanted from the culture plates for EV and AAV media samples collection. Cells were detached using a cell scraper in 5 mL of fresh OptiMEM per dish. Scraped cells from each condition were pooled into 50 mL falcon tubes. Next, tubes were spun for 10 min at 300 x g to pellet the cells. The supernatant was discarded, and the pellet was resuspended in AAV lysis buffer (0.15 M NaCl, 50 mM Tris-HCl pH8.5). The lysate was subjected to four cycles of freeze-thaw to ensure cell lysis, with thorough vortexing after each thaw. For the freezing step, the lysate was placed on dry ice with ethanol for at least 30 min, while the thaw step was performed at 37°C, ensuring it was kept at this temperature only long enough for complete thawing. After completing the freeze-thaw cycles, the lysate was stored at -80°C until purification. To proceed, the lysate was thawed by placing it at 37°C. Once thawed, benzonase was added to achieve a final concentration of 50 U/mL to remove any DNA which is not protected by a capsid. The mixture was incubated at 37°C for 30–45 min to degrade free nucleic acids. Following this step, the lysate was centrifuged at 3,700 x g for 20 min at room temperature, and the supernatant was filtered through

a 0.45 μ m PVDF filter. At this point, 5 μ L of the lysate was collected for titration, and the total volume of the lysate was recorded. To prepare for ultracentrifugation, an iodixanol gradient was layered into a Beckman Quick-Seal Polyallomer tube using a blunt needle and syringe in the following order: 4 mL of 60% iodixanol, 4 mL of 40% iodixanol, 4.2 mL of 25% iodixanol and 7.8 mL of 15% iodixanol. Iodixanol was diluted in PBS-MK buffer. The filtered lysate was then gently added on top of the gradient, and lysis buffer was added if necessary to complete the volume. The tubes were sealed, and the samples were centrifuged using a Beckman 70Ti rotor at 59,000 rpm (41,700xg) for 1.5 h at 20°C. Using an 18G needle, the interface between the 60% and 40% iodixanol phases was pierced, and approximately 2–3 mL of the 40% phase was collected without disturbing debris at the top of the phase. Amicon Ultra-15 centrifugal filters (Millipore, 10 kDa MWCO) were pre-rinsed by adding 15 mL of PBS and centrifuging at 4,000 g for 5 min to remove residual components from the filter membrane. The iodixanol fraction containing the AAVs was then combined with PBS and loaded onto the pre-rinsed filter. The sample was centrifuged at 4,000xg for 5–15 min until the remaining volume was reduced to approximately 500 μ L or less. To further purify and concentrate the sample, 15 mL of PBS was added to the filter, and the centrifugation step was repeated. This washing process was performed a total of three times to remove residual iodixanol. During the final (third) centrifugation, the process continued until the remaining volume was reduced to between 250 and 500 μ L. This concentrated solution contained the purified AAV. The final volume was used to rinse the filter membrane thoroughly to maximize recovery, after which the solution was collected and aliquoted into 50–100 μ L portions. Aliquots were stored at -80°C for long-term preservation.

2.2.4 EV Isolation

2.2.4.1 Ultracentrifugation

Cells were cultured and transfected as described above. After 48 h, media was collected and spun at 2,000 x g for 10 min to remove cells and cell debris. Larger vesicles were isolated by centrifugation at 20,000 x g for 30 min. Smaller extracellular vesicles were isolated by centrifugation of the 20,000 x g supernatant from the above step for 90 min at 100,000 x g. 20k and 100k pellets were resuspended in 0.5 mL of PBS for further analysis. All ultracentrifugation steps were performed using the Beckman Coulter Type 70Ti rotor at 4 °C.

2.2.4.2 Iodixanol gradient

Using 100 kDa MWCO (Sigma) spin filters, media was concentrated to 500 µL. An iodixanol gradient (OptiPrep, 60% iodixanol; Sigma) was prepared (2 mL 60%, 2 mL 40%, 2 mL 25%, 1 mL 18%, 1 mL 16%, 1 mL 14%, 1 mL 12%, 1 mL 6%) by pipetting described volumes into the ultracentrifuge tube (Beckman Coulter). 500 µL of concentrated media was carefully pipetted to the top of the gradient. Tubes were spun at 28,500 rpm (18, 700 xg) for 16 h at 4 °C. Once centrifugation was complete, 1 mL fractions were collected. 5 µL of each fraction were set aside for titration of viral genomes. Fractions 4-6 were combined to isolate extracellular vesicles, whereas the AAV particles were found in fractions 9-12. To remove the iodixanol, samples are placed into the 100kDa MWCO spin filter columns and washed 4 times with PBS. After washes were complete, sample was concentrated down to 200 µL and removed from the spin filter.

2.2.4.3 Size Exclusion Chromatography

Proceeding from the steps described above, media was concentrated to 1 mL using the 100 kDa MWCO spin filters. Sample was then run on a Sepharose 4 Fast Flow size exclusion chromatography column (GE Healthcare), bed volume 120 mL (HiPrep 16/60 format) connected

to the ÄKTA Pure system (GE Healthcare) and eluted at 0.5 mL/min flow rate using PBS as the eluent. Chromatograms were recorded using absorbance at 280 nm. 0.5 mL fractions were collected, and EV-containing fractions, as determined by the UV spectra, were pooled.

2.2.4.4 High-Resolution Size Exclusion Chromatography

Eluted SEC fractions containing EVs were pooled, mixed and subjected to fractionation through high-resolution size exclusion liquid chromatography (HR-SEL). For this purpose, an XK 16/70 SEC column (GE Healthcare) packed with Sephacryl S-500 Superfine SEC medium (GE Healthcare) was used. Elution was performed in PBS at a flow rate of 0.5 mL/min and monitored through absorbance at 280 nm. Thirty individual HR-SEC fractions of 4.0 mL were collected using a fraction collector (F9-R Ge Healthcare) and stored at 4 °C.

2.2.5 Western Blot

Samples were resuspended in NuPAGE LDS Sample Buffer (4X) (Invitrogen) with freshly added NuPAGE Sample Reducing Agent (10X) (Invitrogen). Samples were subsequently heated for 10 min at 70 °C and separated on a 1.5 mm, 10% NuPAGE Bis Tris (Invitrogen) polyacrylamide gel alongside a molecular weight marker (Precision Plus Protein Dual Colour Standard, Biorad). Blotting was performed on an Immobilon-FL membrane (Merck Millipore). Membranes were blocked in 5% skimmed milk in TBS-Tween for 1 h, and immunoblotting was performed using the antibodies listed in Table 2.1 by incubation overnight in 5% milk in TBS-Tween at 4 °C. After incubation, membranes were washed three times in TBS-Tween for 10 min at RT. Membranes were incubated with secondary HRP-labelled antibodies listed in Table S1 in TBS-Tween for 2 h at RT. After incubation, membranes were washed two times with TBS-

Tween for 10 min. Proteins were detected with Clarity™ Western ECL Substrate (Bio-Rad) and detection was performed with the ChemiDoc Imager (Biorad).

2.2.6 qPCR Titration

5 µL of the sample to be titrated was aliquoted into 0.2 mL PCR strip tubes. First, samples were incubated with DNase (Promega) to remove any DNA not protected by a capsid at 37 °C for 30 min. To stop DNase action, 2 µL of stop solution was added to each sample, and samples were incubated for 10 min at 65 °C. Next, samples were incubated with proteinase K (ProK, Roche) to release the AAV genome from the capsid. 100 µL of ProK Buffer (2X) (0.1M EDTA, 0.5% SDS), 10µL ProK (Roche) and 70 µL RNase/DNase free H₂O, was added to each sample, and samples were incubated for 1 h at 50 °C. 2.67 µL of each sample was transferred to a new tube and mixed with 17.33 µL RNase/DNase free H₂O, resulting in a final 1:300 dilution. Standards were generated in the range of 1x10³-1x10⁹ vg/mL, using the respective transgene plasmid to be titrated. qPCR was performed using primers against the transgene, using a master mix containing 100 nM of forward and reverse primers, 5 µL RNase/DNase free H₂O and 10 µL Fast SYBR green (2X) (Applied Biosystems). 17 µL of master mix was combined with 3 µL of sample or standard. A StepOne real-time PCR instrument was used. Primer sequences are listed in Table 2.

2.2.7 ddPCR

Digital droplet PCR (ddPCR) was used in addition to qPCR to verify the concentrations of samples before in vivo injections and viral particle (vp) detection. The BioRad QX200™ Droplet Digital™ PCR System was used according to the manufacturer's instructions. Samples were processed as described above for qPCR. After proteinase incubation, the sample was

further diluted 1:1000 and 1:10,000, for a final dilution of 1:3000 and 1:10000. Reaction mixes were prepared with 1 µL of diluted sample, 250 nM primers, 9 µL of RNase/DNase free H₂O and 10 µL of 2x QX200 ddPCR EvaGreen Supermix (BioRad, Catalog no. 186-4033). Sample was vortexed and spun down. 20 µL was transferred to the BioRad slide, and droplets were generated. Using a multichannel pipet sample was transferred to a 96-well plate. The sealed plates were placed into a thermal cycler (Bio-Rad C1000 Touch). Following thermal cycling, the plates were transferred to the Bio-Rad QX200 Droplet Reader. Droplets were analysed for fluorescence signals, distinguishing between positive droplets (amplified target sequences) and negative droplets (no amplification). Data acquisition and analysis were performed using Bio-Rad QuantaSoft software, which calculates absolute copy numbers (copies/µL) based on the fraction of positive droplets and the Poisson distribution. Quality control involved ensuring sufficient droplet counts per well (>10,000 droplets) and clear separation between positive and negative droplets. Wells failing to meet these criteria were excluded from further analysis. Optimization of reaction conditions, including primer/probe concentrations and annealing temperatures, was performed as necessary for each target.

2.2.8 AAV9 Capsid Quantification MSD ELISA

An ELISA assay was used to quantify AAV9 capsids. In combination with qPCR or ddPCR results, this was used to determine empty vs full capsid ratios. Performing this assay with and without Triton-X preincubation determines the percentage of viral particles encapsulated by EVs. MSD GOLD 96-Well Streptavidin Plate (MSD, Catalog no. L15SA) was incubated with 0.1 µg/mL CaptureSelect Biotin Anti-AAV9 Conjugate (Thermo Fisher, Catalog no. 7103332100) at 700 rpm for 1 h at 21°C on a thermomixer (Eppendorf). Once incubation was

complete, the plate was washed three times with 0.05% Tween-20 in PBS using a plate washer (Biotek), and the supernatant was discarded.

Samples were diluted in diluent 100 (MSD, Catalog no. R50AA-3), along with an AAV9 empty capsid standard (Progen, Catalog no. 66V090). For EV-AAV9 samples and standards, an aliquot was incubated with 0.5% Triton-X-100 in PBS with agitation at 400 rpm for 30 min at 37 °C.

Both samples were further diluted to a final dilution factor of 1:1000 and 1:10,000 in Diluent 100. 50 µL of each sample, the standard and the standard curve were loaded in triplicate in the coated plate. The plate was incubated at 700 rpm for 1 h at 21 °C on a thermomixer (Eppendorf). Once incubation was complete, the plate was washed as described previously, and the supernatant was discarded. Next, the plate was incubated with 50 µL 0.5ug/mL of ADK9-1R(Progen, Catalog no. 610178) at 700 rpm for 1 h at 21°C on a thermomixer (Eppendorf). The supernatant was discarded, and the plate was washed using the Biotek plate washer.

50 µL of 0.5 ug/mL MSD SULFO-TAG labelled anti-mouse antibody (Goat) (MSD, Catalog no., R32AC-1) was added to the plate. The assay plate was incubated at 700 rpm for 1 h at 21°C on a thermomixer (Eppendorf). The plate was washed using the same settings as above on the plate washer (Biotek). Then 150 µL of MSD Gold Read Buffer B (MSD) was added, and the plate was immediately read on the MSD plate reader (MESO QuickPlex SQ120; MesoScale Discovery).

Data was analysed using the MSD Discovery Workbench Software. The assay was repeated if the standard curve $R^2 < 0.9$, $CV > 20\%$ or if the calculated concentration of the reference with and without triton-X-100 was not within two standard deviations of the internal standard.

2.2.9 Electron Microscopy

EV-AAV and AAV samples were normalized in concentration to 1×10^{12} particles/mL or 1×10^{12} vg/mL, respectively. Particle concentration was determined using NanoFCM particle

measurements and qPCR was used to measure vg/mL. Samples were fixed in 2% PFA + 0.1% glutaraldehyde in 0.1M PIPES buffer, pH 7.2-7.4 at room temperature for 30 min, then stored at 4 °C. Imaging was performed by Charlotte Melia, at the Dunn School Bioimaging Facility.

2.2.10 Magnetic Pulldown Experiments

2.2.10.1 Anti-AAV Pulldown

Dynabeads magnetic beads (Dynabeads™ MyOne™ Streptavidin T1, Catalog no. 65601) were resuspended by vortexing for 1 minute. 25 µL of Dynabeads magnetic beads were transferred to a microcentrifuge tube. Dynabeads magnetic beads were washed 3 times with PBS by resuspending beads in PBS and then placing a tube on the magnet for a 1-minute pulldown. Supernatant was discarded. After the last wash, Dynabeads magnetic beads were resuspended in 100 µL PBS and incubated with 2.5 µL of anti-AAV9 biotinylated Ab for 30 min, rotating at RT. Dynabeads magnetic beads were then placed on the magnet for a 2min pulldown, and supernatant discarded. Dynabeads magnetic beads were washed 4 times with 0.1% BSA in PBS. After the last wash, the supernatant was discarded and the sample added. Dynabeads magnetic beads and sample mixture were resuspended thoroughly by vortexing and incubated overnight rotating at 4°C. The next day supernatant was collected, and beads were resuspended in 100 µL of PBS. 5 µL of each sample was taken for qPCR analysis.

2.2.10.2 Anti-Tetraspanin Pulldown

Exosomes were isolated using the MACS® Exosome Isolation Kit (Miltenyi Biotec) following the manufacturer's protocol. Purified EVs were used as the sample. For magnetic labelling, 50 µL of Exosome Isolation MicroBeads conjugated to CD9, CD63, CD81, or a pan-tetraspanin cocktail was added to 2 mL of pre-cleared sample and incubated for 1 h at room temperature

with gentle vortexing. The magnetically labelled exosomes were applied to a μ Column placed in a μ MACS™ Separator mounted on a MACS® MultiStand. The column was equilibrated with 100 μ L of Equilibration Buffer and washed three times with 100 μ L Isolation Buffer. The labelled sample was loaded, and unbound vesicles were removed with four washes of 200 μ L Isolation Buffer. Elution of intact exosomes was performed by removing the column from the magnetic separator, placing it onto a 1.5 mL tube and flushing 100 μ L of Isolation Buffer through the column using a plunger. All buffers were pre-warmed to room temperature to ensure proper flow through the column and prevent air bubble formation. The following day, the supernatant was collected, and the beads were resuspended in 100 μ L of PBS. A volume of 5 μ L from each sample was taken for qPCR analysis.

2.2.10.3 Anti-Phosphatidylserine Pulldown

Dynabeads magnetic beads (Dynabeads™ MyOne™ Streptavidin T1, Catalog no. 65601) were prepared by resuspending the beads via vortexing for 30 seconds. A 25 μ L aliquot of the beads was transferred to a new tube and washed three times with 1 mL of PBS-based washing buffer by resuspending, then pulldown and discarding the supernatant. For antibody coating, the washed beads were incubated with TIM-4 biotinylated antibody (Adipogen, Catalog no. AG-40B-0180B-C010) in PBS for 1 h at room temperature with gentle rotation. Following incubation, the antibody-coated beads were separated with a magnetic pulldown for 2-3 min and washed 4-5 times in PBS containing 0.1% BSA to remove unbound antibody. Finally, the beads were resuspended with 90 μ L of 1×10^{10} EV particles and 10 μ L of 10X binding enhancer and incubated overnight. For 500x binding enhancer (1 M CaCl_2), 14.7 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was dissolved in 80 mL of TBS-T and adjusted to a final volume of 100 mL with TBS-T. This was then further diluted to have a 10X binding enhancer buffer. The next day, supernatant was

collected, and beads were resuspended in 100 μ L of PBS. 5 μ L of each sample was taken for qPCR analysis.

2.2.11 Transduction Studies Using the Operetta High-Content Imaging System

Transduction efficiency was analysed using the Operetta High-Content Imaging System. Target cells were seeded into 384-well plates at 4000 cells per well and allowed to adhere overnight under standard culture conditions (37°C, 5% CO₂, 90% Humidity). Cells were incubated with 10 μ M Hoechst (Thermo Fisher, Catalog. no. 34580) for 15 min at 37°C. Viral vectors were prepared and added to the wells at the desired multiplicity of infection (MOI) in OptiMEM. Plates were gently agitated to ensure even distribution and incubated for 12–24 hours to allow for viral uptake. Following incubation, the plate was placed into the Operetta and incubated for 75 h, taking images every 4 h. Images were analysed using the Harmony software to quantify transduction efficiency. Data were normalized to control wells and analysed for statistical significance.

2.2.12 Mouse studies

In this study, mice were used to assess transgene expression following AAV and EV-AAV9 delivery. For studies with IgG: on day 0, mice received an intraperitoneal injection of IgG in a maximum volume of 320 μ L. The following day (day 1), the mice were injected intravenously with either AAV, EV-AAV9, AAVR-EVs or a combination of EVs and AAVs at a dose of 1×10^{11} viral genomes per mouse, in a maximum volume of 120 μ L. Concentrations of all treatments were adjusted with PBS so that all treatments received an equal injection volume. After two weeks, the mice were euthanized, and major organs were harvested for downstream

analysis. 2 male and 2 female C57BL/6 mice were used for each condition. Studies without IgG started on day 1 as detailed above, with the day 0 IgG injections omitted. To investigate the effects of repeated dosing with CD63-EV-AAV9 on transgene expression, a study was conducted using four female mice per condition. On day 0, mice were injected intravenously with either AAV9 or CD63EV-AAV9 carrying the Nanoluc-eGFP transgene at a dose of 1×10^{12} vg. Two weeks later, the mice received a second injection with either PBS (control), AAV9 or CD63EV-AAV9 carrying the Nanoluc-mScarlet transgene.

At four weeks post-initial injection, mice were euthanized, and tissues were harvested for analysis. RNA was extracted from the collected tissues, and qPCR was performed to measure transcript expression levels of eGFP, mScarlet and Nanoluc transgenes. Serum samples were collected at two and four weeks following the first injection to assess the presence of neutralizing antibodies. The Meso Scale Discovery (MSD) immunoassay was used to detect anti-AAV9 mouse IgG.

2.2.12.1 Meso Scale Discovery (MSD) Serum NAb Detection

To detect anti-AAV9 mouse IgG in the serum, we used a protocol published by Haar et al¹⁸². The MSD GOLD 96-Well Streptavidin Plate (MSD, Catalog no. L15SA) was incubated with 0.1 µg/mL CaptureSelect Biotin Anti-AAV9 Conjugate (Thermo Fisher, Catalog no. 7103332100) at 700 rpm for 1 h at 21°C on a thermomixer (Eppendorf). Once the incubation was complete, the plate was washed three times with 0.05% Tween-20 in PBS using a plate washer (Biotek), and the supernatant was discarded. The plate was coated with the reference AAV9 empty capsid standard (Progen, Catalog no. 66V090) at a concentration of 5×10^8 viral particles (vp) per well. The assay plate was incubated at 700 rpm for 1 h at 21°C on a thermomixer

(Eppendorf). The plate was washed using the same settings as before on the plate washer (Biotek). Serum samples and standards ADK9-1R (Progen, Catalog no. 610178) were diluted 100-fold in Diluent 100 (MSD, Catalog no. R50AA-3). Samples were loaded in triplicate and incubated at 700 rpm for 1 h at 21°C. 50 µL of 0.5 µg/mL MSD SULFO-TAG labelled anti-mouse antibody (Goat) (MSD, Catalog no. R32AC-1) was added to the plate. The assay plate was incubated at 700 rpm for 1 h at 21°C on a thermomixer (Eppendorf). The plate was washed using the same settings as before on the plate washer (Biotek). Then, 150 µL of MSD Gold Read Buffer B (MSD) was added, and the plate was immediately read on the MSD plate reader (MESO QuickPlex SQ120, MesoScale Discovery). Data were analysed using the MSD Discovery Workbench Software.

2.2.13 RNA extraction

Maxwell® RSC simply RNA Tissue Kit (Promega, Catalog no. AS1340) was used for RNA extraction. Maxwell Resuspension Solution master mix was made by combining homogenization solution (180 µL /sample), thioglycerol (5 µL/sample), and Proteinase K (15 µL/sample) in appropriate volumes based on sample count, stored on ice or at –20°C for long-term use. 0.5 mL elution tubes were labeled, with 50 µL nuclease-free water added to each elution tube. Homogenization tubes (Precellys, Catalog no. P000973-LYSK0-A.0) were preloaded with 200 µL of Thioglycerol/Proteinase K/Homogenization Solution and frozen on dry ice. Frozen tissue (~20 mg) was added on top of the frozen solution, with samples stored at –70°C if needed. Before processing, samples were thawed, homogenized twice using the Precellys tissue homogenizer, incubated at 60°C for 10 min, and returned to ice. DNase I was prepared by adding 275 µL of nuclease-free water and 5 µL of blue dye, mixed by inversion, and

stored at -20°C . Maxwell cartridges were set up by adding 10 μL DNase I to well #4, placing labelled elution tubes with 50 μL nuclease-free water next to the cartridges, and inserting plungers into well #8. Samples were mixed with 200 μL Lysis Buffer, vortexed briefly, spun down (1 min, 10,000xg), and 400 μL of homogenate was transferred to well #1, avoiding bubbles.

2.2.14 Nano-Glo Luciferase Assay for cells and tissues

Approximately 20 mg of tissue (± 1 mg) was isolated, weighed, and placed into tubes containing pre-chilled 10% Triton X-100 solution. Samples were partially thawed on ice and homogenized twice using the Precellys program 5 (5000 rpm, 2×20 s). The homogenized samples were centrifuged at 10,000 x g for 1 min, and the supernatants were transferred to fresh 0.6 mL tubes on ice. For further lysis, samples were sonicated for 5 min in a water sonication bath and returned to ice. Cell debris was removed by centrifugation at 14,000 xg for 5 min, and the resulting lysates were transferred to clean tubes on ice. Protein concentrations were measured using the DC protein assay (see below), and all samples were diluted to the lowest common concentration or 1 mg/mL, whichever was lower. 30 μL of lysate at 1 mg/mL lysate was added to duplicate wells. Using a multichannel pipette 30 μL of Assay mix was added to duplicate wells. Assay mix consisted of Nano-Glo Assay buffer and Nano-Glo Substrate. The plate was placed into the plate reader (CLARIOstar Plus), agitated and read at 460 nm within 30 min. PBS injected animals were used as controls. Nano-Glo® Luciferase Assay System (Promega, Catalog no. N1110) was used.

2.2.15 Detergent Compatible (DC) Assay

The assay was performed using DC protein assay kit I (Biorad, Catalog no. 5000111). For sample preparation, 5 μ L of BSA standards, ranging from 0.25 to 2.0 mg/mL, or 5 μ L of unknown protein samples (diluted as necessary) were added to duplicate or triplicate wells. The appropriate volume of solution A' was prepared to accommodate the total number of samples. During assay setup, 15 μ L of A' solution was added to each standard and sample well, followed by 120 μ L of solution B. The wells were then incubated at room temperature for 20–40 min until a pale blue colour developed. Absorbance was measured at 750 nm.

2.2.16 AAV Staining

Anti-AAV9 biotinylated antibody (Thermo Fisher, Catalog no. 7103332100) was incubated with monovalent Streptavidin-Alexa 647 magnetic beads (Thermo Fisher) for 5 min protected from light on a rotator. 500 μ L of LC-purified EVs were incubated with 0.1% BSA, 0.001% TritonX-100 in PBS, and 8 μ L of anti-AAV9-BIO-Alexa647 antibody for 1h rotating at RT protected from light. Samples were then purified by SEC (see above). EV fractions were pooled, and staining was evaluated using the flow nanoanalyzer from NanoFCM.

2.2.17 In Vitro Transduction and Neutralization Assay

150,000 HEK293T cells/well were seeded into 12-well plates and cultured in DMEM with 10% FBS and 1x antibiotic/antimycotic. The next day, dilutions of human IgG (1mg/mL, 2mg/mL, 4mg/mL or 8mg/mL in PBS) were incubated with free AAVs, WT EVs mixed with free AAVs or AAVR-EVs+AAVs for 30 min at 37°C. Media was removed and replaced with 450 μ L of fresh DMEM. 50 μ L of Human IgG-Sample was then added to the wells. 48 hours later, wells were washed with PBS and then lysed using 400 μ L of Glo Lysis Buffer (Promega)

for 5 min. 100 μ L of lysate (in triplicate) was transferred to a black-walled 96 well plate (Corning). GFP fluorescence was measured using a CLARIOstar Plus Plate reader.

2.2.18 Stable Cell Lines

EV-marker mClover3 fusion cell lines were generated and provided by Claire Staton, a previous student in the lab. WT-VP and pX-VP2 plasmids were generated using a doxycycline-inducible piggyBac vector, which has a puromycin resistance gene to allow for the selection of transfected cells.

2.2.19 AAV production in VP2-pX or VP2-WT

HEK293T cells were seeded in 15 cm polystyrene dishes. When cells were at 70-80% confluency, media was changed to OptiMEM containing 500 ng/mL doxycycline (Life Technologies). Cells were transfected with Helper (20 μ g), RepCap2/9 with WT VP1 and VP3 but no VP2 (8 μ g), WT- or pX-VP2 (11 μ g) and transgene iRFP or GFP (11 μ g) totalling to 50 μ g DNA/plate mixed with PEI. Cells were left to incubate for 8hr or overnight. Media was then changed to OptiMEM containing 500 ng/mL doxycycline. After 2 days, media was collected and processed as described above.

2.2.20 Surface Binding of AAVs

50 μ L of AAV sample was combined with 450 μ L of EVs or the indicated EV:AAV ratios and incubated on a rotator for 30 min-1 h at RT, then isolated. Iodixanol gradient fractionation was then used as described above.

2.2.21 Proteomics

EV and EV-AAV samples were collected from HEK293T cells and isolated by iodixanol gradient. Sample particle concentration was analyzed by NanoFCM, and adjusted to 1×10^{10} particles/ml. These samples were analysed by label free quantification. To each approximately 100 μ l of sample, 100 μ l of Lysis buffer was added (4% SDS, 50 mM HEPES pH 7.6, 1 mM DTT). Protein digestion (trypsin, sequencing grade modified, Pierce) was performed using a modified protocol for SP3 protein clean up¹⁸³ followed by SP3 peptide clean up. Each sample was separated using a Thermo Scientific Dionex nano LC-system in a 3 hr 5-40 % ACN gradient coupled to Thermo Scientific High Field QExactive. All searches were done against the *Homo Sapiens* protein database UniProt 110 using our proteomics workflow (<https://github.com/lehtiolab/ddamsproteomics> vs2.6)¹⁸⁴. Protein false discovery rates were calculated using the picked-FDR method using gene symbols as protein groups and limited to 1% FDR¹⁸⁵. Venn diagrams were generated using JVenn¹⁸⁶. PCA and heatmaps were generated using ClustVis analysis tools¹⁸⁷. Boxplots and groupwise statistical analysis was performed using Perseus¹⁸⁸.

2.2.22 Statistical Analysis

All statistical analyses were performed using GraphPad Prism (version 10.4.2). Normality of data was assessed using the Shapiro–Wilk test, and when necessary, confirmed with additional tests including D’Agostino & Pearson, Anderson–Darling, and Kolmogorov–Smirnov. Homogeneity of variance was evaluated using F-tests and Brown–Forsythe tests. A significance threshold of $p < 0.05$ was used for all analyses.

For datasets meeting the assumptions of normality and equal variance, parametric tests were used. F-tests were used for comparing variances, which were performed alongside every ANOVA, and are reported in the figure legends. Ordinary one-way ANOVA followed by

Tukey's (for comparisons between multiple groups) or Dunnett's multiple comparisons tests (when multiple groups were compared to a control) was applied to compare group means. Two-way ANOVA was used to evaluate the effects of multiple factors (e.g., timepoint $\times$ treatment, vector type $\times$ fraction), with post hoc tests including Tukey's, Šidák's, Dunnett's, or uncorrected Fisher's LSD, depending on the comparison. Two-way repeated measures ANOVA to assess interactions across multiple levels of repeated factors. When variance was unequal, Welch's t-test was used in place of standard unpaired t-tests. In one instance Log transformation was performed prior to ANOVA in cases where variances were unequal between groups.

For non-parametric data or when assumptions of normality were violated, non-parametric tests were used. Kruskal–Wallis tests were used for comparing multiple independent groups, followed by Dunn's multiple comparisons test. Friedman tests were used for repeated-measures or matched designs without normality, followed by Dunn's post hoc test where appropriate. Mann–Whitney tests were used for pairwise comparisons of non-parametric data.

All results are expressed as mean $\pm$ standard deviation (SD) or standard error mean (SEM), and individual data points are shown in plots where feasible. Sample sizes (N) and precise statistical tests are provided in the figure legends.

Power calculation and sample-size estimations were run in G*Power 3.1.9 (F tests $\rightarrow$ ANOVA: fixed effects, omnibus, one-way). Inputs used were $\alpha = 0.05$, target power = 0.80, and two effect-size scenarios—medium (Cohen's $f = 0.25$) and small ($f = 0.10$)—based expected effect size. These returned theoretical requirements to reach 80% power of 45 mice per group (total sample size = 190) for a medium effect and 274 mice per group (total sample size = 1290) for a small effect. Experiments in this thesis used four mice per group due to limitations in EV-AAV manufacturing capacity. Consequently, the experiments are recognized as under-powered.

2.2.23 AAV9 Capsid Library

2.2.23.1 Late Domain Library Cloning

AAV9 Rep/Cap plasmid was cleaved using BsmBI-v2 (NEB) and gel purified. Randomized oligos were ordered as ssDNA oligos from IDT. 1 μ L of 100 μ M ssDNA oligos was combined with 0.25 μ L Klenow-exo, 2 μ L of reverse primer, 1 μ L of 0.1 mM dNTPs and 13.75 μ L of RNase/DNase free H₂O. Oligos were incubated at 37 °C overnight to convert the ssDNA library to dsDNA with the appropriate overhangs. Next, dsDNA oligos were purified using Monarch PCR DNA Clean Up kit (NEB) following the small oligo kit protocol. Vector and purified double-stranded insert were eluted in RNase/DNase free H₂O. Concentration of vector and dsDNA oligos was determined by NanoDrop. Gibson assembly Master Mix (2X) (NEB) was combined with 50 ng of vector and 4.2 ng of insert and incubated for 1 h at 50 °C. Ligated material was then concentrated and purified using Monarch Spin PCR & DNA Cleanup Kit (New England Biolabs, T1130S). Competent bacterial cells were thawed on ice without vortexing. Pre-warmed ampicillin plates and SOC medium (37 °C) were prepared, and electroporation cuvettes were kept on ice. 1 μ L of ligated material was added to 25 μ L of electrocompetent bacteria (New England Biolabs, Catalog no. C3020K) and gently mixed by flicking. The mixture was added to the cuvette, ensuring no bubbles were made. Bacteria were electroporated using the Ec1 setting on the MicroPulser Electroporator (Biorad, Catalog no. 1652100). After electroporation, SOC medium was added within 30 s, and bacteria were incubated in a 2 mL tube at 37 °C with shaking. Around 40 μ L of bacterial solution was plated on ampicillin plates and incubated upside down at 37 °C overnight. Transformation efficiency was estimated by plating a 1000-fold dilution and counting colonies. Bacterial colonies were scraped

from ampicillin plates using 2–5 mL of cold LB, transferred to 50 mL Falcon tubes, and pelleted (4000 x g, 15–20 min, 4°C). Supernatants were discarded, and pellets (~0.45 g) were processed using Qiagen or Macherey-Nagel Maxiprep kits per manufacturer protocols. Eluates from multiple columns were combined if necessary. Samples were sent for Sanger sequencing for confirmation.

2.2.23.2 AAV library Evolution screen

Virus was produced as described in AAV production section above, however the amount of plasmids used for transfection was different. In this case 1 µg of transgene plasmid (RepCap2/9-ITR) and 25 µg of helper plasmid per plate was used. Then AAV was produced as described above. EV-AAVs and AAVs were isolated by iodixanol gradient. DNA was extracted from the EV-AAVs, cloned back into the RepCap2/9-ITR plasmid, and subjected to repeated rounds of selection. Samples from each round were PCR amplified for sequencing, and motif analysis was performed to identify amino acid sequences correlated with improved EV incorporation. From this analysis, the top 100 hits were selected and ordered as an OligoPool. (IDT) for further evaluation. Three biological replicates of EV-AAV production were performed and sent for Amplicon EZ Sequencing. Sanger sequencing was used to validate the presence of specific motifs, such as PT/SAP, within the randomized library.

For each 30 µL sample (prepared 4x for EV samples and 2x for AAV media), a DNase treatment was performed by adding 3 µL DNase buffer and 2 µL DNase. The mixture was incubated at 37°C for 30 min. To stop DNase action, 3µL of stop solution (20mM EGTA (pH 8.0)) was added to each sample, and samples were incubated for 10min at 65 °C. Next, samples were incubated with proteinase K (ProK, Roche) to release AAV genome from capsid. 100 µL of ProK Buffer (2X) (0.1M EDTA, 0.5% SDS), 10 µL ProK (Roche) and 50 µL RNase/DNase H₂O, was added

to each sample, and samples were incubated for 1 h at 50 °C. The Monarch DNA Clean Up kit was used following the ssDNA protocol. Next, 2X KAPA Hifi (Roche, Catalog. no. 0795892700) was used with primers to amplify the inserts. An aliquot of extracted DNA was retained to send for sequencing. This was purified and then ligated back into the RepCap2/9 vector as described in the library cloning section above. This process was repeated for 3 rounds, and extracted DNA was prepared for sequencing. The region of interest was PCR amplified using primers and purified using the Monarch DNA Clean-Up kit. Insert size was confirmed by gel electrophoresis before sending to Beijing Genomics Institute (BGI) for sequencing.

Enrichment scores were calculated by dividing the number of sequences after enrichment by their frequency in the original library, normalized to total read counts. An enrichment score cutoff was applied, and enriched sequences were analysed for motif content. Motif analysis was performed to identify patterns based on the size of insertions and the inclusion of random or rationally designed late domain motifs. Results were visualized with letter sizes indicating amino acid frequencies at specific positions. The top 120 enriched variants were selected for a more refined screen that also included the AAV9 wildtype sequence and a negative control, which encoded several stop codons. These sequences were ordered as an OligoPool (IDT) and cloned into the RepCap2/9-ITR plasmid as described previously. Next, EV-AAVs were produced and isolated in three separate biological replicates. DNA was extracted and prepared for sequencing as previously described. Samples were sent for Amplicon EZ sequencing (Azenta Lifesciences).

Enrichment scores were calculated by dividing the number of sequences after enrichment by their frequency in the original library and normalizing to total read counts. The top five enriched sequences across all replicates were selected, ordered as individual oligos and cloned back into the RepCap2/9-ITR plasmid as described above for individual validation.

2.2.23.3 AAV library Bioinformatics

The bioinformatic analysis for the AVV library screen was conducted by Marina Luchner; here are the detailed methods.

2.2.23.3.1 Deriving read counts from raw fastq files

The AAV9 capsid protein library screen discussed above outputs 300 KB (first run) to 50 GB (second run) of raw fastq data. Fastq data consists of information about the sequencing machine, a unique index, the DNA sequence and a quality score per base. The manufacturer has already trimmed adapter sequences and discarded reads below a quality threshold. We first merged the fastq files corresponding to the same sample. Next, we merged corresponding paired end reads using an in-house python script. To validate our in-house python script for merging paired end reads, we visually inspected the results of the merging process for three different reads, which showed the expected sequence. We utilized the bioinformatic tool Cutadapt (<https://doi.org/10.14806/ej.17.1.200>)¹⁸⁹ to filter the resulting reads containing the expected flanking regions (both left and right of the semi-random and fully random inserts) in an error-tolerant manner. Additionally, we trimmed the flanking regions to extract the variable region of each variant (later referred to as insert).

To validate the effectiveness of the bioinformatic processing with Cutadapt¹⁸⁹, two sanity checks were performed. Firstly, a histogram of the variant length was plotted with the frequencies peaking at the expected length of 24, 27, 36, 39, 60, and 63 nucleotides (nts). The results demonstrate that the bioinformatic processing using Cutadapt¹⁸⁹ yielded the anticipated insert length distribution. As an additional sanity check, we compared five filtered and trimmed sample sequences with the raw sequences. For each of the five example sequences, the inserts

isolated by the bioinformatic processing were flanked by the expected regions and exhibited the expected length.

To calculate the read count per variant, we initially discard variants shorter than 24 nts or longer than 63 nts. Subsequently, we count the occurrences of each sequence in the data, thus deriving the read count per variant.

2.2.23.3.2 Deriving enrichment scores from read counts

To compute the enrichment score, we first normalized the read count per variant by the total number of reads per sample. Then, we divide the normalized read count of the cell lysate, EV fraction, and AAV fraction by the normalized read count of the original library. Sequences with the top 10 enrichment scores were extracted.

3 Chapter 3. Results 1- Characterization of EV-AAVs

3.1 Abstract

AAVs are widely used as vectors for gene therapy, but their clinical application is hindered by limitations such as immunogenicity, low transduction efficiency, and the need for large doses. EV-AAVs present a promising alternative, published to exhibit enhanced transduction and protection from neutralizing antibodies. However, challenges remain, particularly in terms of increasing EV-AAV yields and understanding the mechanisms of AAV incorporation into EVs. This study compares high-resolution size exclusion liquid chromatography (HR-SELIC) and iodixanol density gradient techniques for isolating EV-AAVs and characterizing their biogenesis and composition. Results indicate that larger vesicles are more efficient at incorporating AAVs. Additionally, EV-AAVs are enriched in phosphatidylserine but exhibit low levels of tetraspanins, which are known exosome markers. Knockdown of ESCRT components and the use of chemical inhibitors suggest that ectosomes play a key role in EV-AAV biogenesis. Importantly, EV-AAV transduction was found to be dependent on the AAV receptor (AAVR). These findings provide valuable insights into the optimization of EV-AAV production and highlight potential engineering strategies to improve their clinical viability.

3.2 Introduction

Adeno-associated viruses (AAVs) are the leading viral vector for gene therapy, with several FDA-approved therapies^{70,190}. AAVs are small, non-enveloped and part of the genus *Dependoparvovirus* in the family *Parvoviridae*. They have a 4.7 kb single-stranded DNA genome flanked by 145bp inverted terminal repeats (ITRs). The genome has 4 main open

reading frames (ORFs) encompassing Rep, Cap, AAP and MAAP. The 60-subunit capsid consists of VP1, 2, and 3, in a ratio of 1:1:10²⁶. The variable region of VP3 results in the different tropism of AAV serotypes, which are targeted to specific cell types through their interaction with specific proteoglycan receptors on the cell membrane of recipient cells. In this way the targeted delivery of the therapy is limited to the tropism of the virus. There are 13 naturally occurring primate serotypes and many recombinant AAVs that have been developed, where the Rep and Cap genes have been removed allowing the virus to effectively deliver the gene therapy but preventing AAV replication in the host cells. Although AAVs are not pathogenic and have lower immunogenicity in comparison to other viral vectors, they can elicit an immune response. For example, activation of cytotoxic T lymphocytes (CTLs) against AAV capsid peptides presented on hepatocyte MHC class I molecules can lead to targeted destruction of transduced liver cells, reducing transgene levels in the tissue and decreasing clinical efficacy^{102,191}. Prior exposure to AAVs results in the production of neutralizing antibodies (NAbs), which prevents efficient gene delivery with AAVs, thus precluding repeated treatment¹⁹². Furthermore, current AAV therapies require large doses, which increases cost and the risk of adverse effects, and in severe cases even death^{5,193,194}.

Extracellular vesicles (EVs) are a heterogeneous population of nanosized, membrane-bound particles secreted by eukaryotic and prokaryotic cells, which can be subdivided into groups based on their biogenesis and cell of origin, as discussed in more detail in Chapter 1. EVs play important roles in intercellular communication and various physiological processes. The three main subtypes are exosomes and ectosomes. Exosomes are small EVs, 30-150nm in size that are formed through the inward budding of the endosomal membrane, which forms multivesicular bodies (MVBs). These MVBs then fuse with the plasma membrane releasing the intraluminal

vesicles which are now exosomes. These processes can be ESCRT (Endosomal Sorting Complexes Required for Transport)-dependent or independent^{195–197}. Ectosomes have a larger size range of 50 nm-1 μ m in diameter and are formed by the outward budding of the plasma membrane. Apoptotic bodies are a subtype of ectosomes which are released by cells undergoing cell death and are typically larger vesicles 50 nm-2 μ m in size^{197,198}.

EVs are being investigated as an effective delivery vehicle for multiple therapeutic cargos^{140,141}. Interestingly, several non-enveloped viruses have been shown to take advantage of the EV sorting machinery to become quasi-enveloped particles¹⁷³. In line with these observations, literature has shown that a proportion of the AAVs naturally incorporates into EVs, forming EV-AAVs¹⁷⁷. EV-AAVs have been shown to have better transduction in the heart, liver, retina, nervous system, and inner ear, in comparison to AAVs^{178–181,199}. EV-AAVs protect AAVs from NABs which are a major barrier to effective gene therapy. By preventing immune detection and neutralization, EV-AAVs enable safe and repeated administration of AAV-based therapies, significantly enhancing their clinical utility and therapeutic potential^{178,200}. It also has the added benefit of the ability to co-package additional molecules (e.g., targeting and immunomodulatory molecules), however EV-AAVs efficacy as a candidate for clinical therapeutics is limited by low levels of endogenous incorporation of AAVs into EVs and lack of a scalable isolation method. Conventional ultracentrifugation is insufficient to separate free AAVs from EV-AAVs. Therefore, this study evaluates alternative isolation methods, including high-resolution size exclusion liquid chromatography (HR-SELIC) and iodixanol density gradients, to more effectively separate and characterize EV-AAVs. This chapter aims to characterize EV-AAVs to better understand their biogenesis, composition and mechanisms of cell entry.

3.3 Results

3.3.1 Iodixanol density gradient selected for EV-AAV isolation

Many EV isolation methods rely on size or density differentiation; however, the overlap in size between the smallest EVs (30–100 nm) and AAVs (25 nm) makes it particularly challenging to fully remove free AAVs from EV samples^{201,202}. It has been shown in our lab that HR-SELIC can be used to separate EVs into several different-sized populations that have distinct proteomic profiles^{202,203}. To investigate if the same protocol can be used to separate EV-AAVs from free AAVs, HEK293T producer cells were triple transfected with RepCap2/9 (Addgene, Cat no.112865), Helper (Cell Biolabs, Cat no.340202) and iRFP transgene(Addgene, Cat no.64887), or an empty plasmid at the same concentration to serve as a control. After 2 days media and cell lysate were collected. Media from both conditions was concentrated by TFF and spin filter, and free AAVs were spiked into the EV only sample. Samples were first run on a column packed with Sepharose Fast Flow 4 resin to remove the majority of small proteins, and EV fractions were collected and pooled based on UV absorption peaks based on EV collection protocols, previously developed in the Wood Lab. Next, sample was run on a Sephacryl S-500 HR columns overnight and fractions were collected and analysed for EV and AAV concentration as determined by NanoFCM and qPCR of the transgene, respectively (Fig 3.1A). It was observed that eluted volumes 44.5-88.5mL show an enrichment of EV-AAV content (Fig 3.1). These results were confirmed by western blot looking for EV markers (TSG101, AnnexinA1, Alix and CD81), and AAV capsid proteins (VP1-3) in the 44.5-88.5mL fractions. Particle numbers measured by NanoFCM and viral genomes measured by qPCR also corresponded to these fractions (Fig 3.1C). Although both separation steps rely on size exclusion, the use of two columns with different matrix compositions and pore sizes increases resolution and allowed for collection of an enriched EV-AAV9 population. HR-SELIC is an attractive purification method,

as it is more scalable to larger volumes in comparison to the 12-step iodixanol gradient discussed below, thus simplifying downstream applications. Variability was observed in the location of the EV-AAV particle peak but not the viral genome peak (Fig 3.1B).

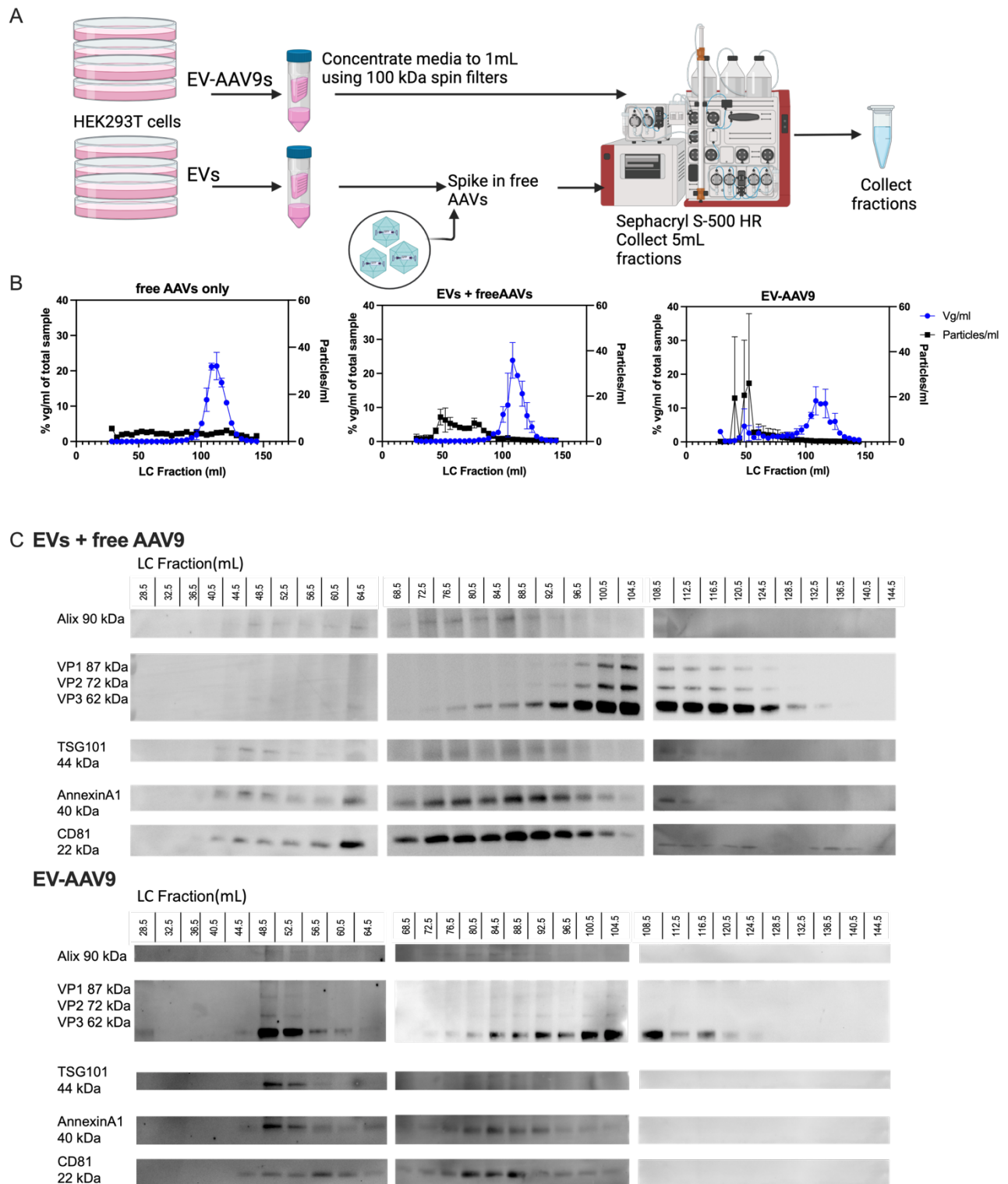


Figure 3.1 High-resolution size exclusion chromatography can be used to collect EV-AAV enriched populations.

Media from HEK293T cells was collected and concentrated using TFF and spin filters down to 2 mL. Free AAVs collected from the cell lysate were spiked into the empty EV sample. Sample

was then loaded into the HR-SELFC columns. For each sample of free AAVs, Empty EVs and mixed with free AAVs 30 fractions of 4 mL were collected after HR-SELFC (A). Particle concentrations (particles/mL) were determined by NanoFCM, and viral genome concentration (vg/mL) was determined by qPCR of iRFP transgene. Viral genome concentration (vg/mL) is plotted in black on the left y-axis, and particle concentration (particles/mL) is plotted in blue on the right y-axis, N=3 (B). Western blot of EV markers and AAV capsid proteins of Empty EVs mixed with free AAVs, and EV-AAVs, showing EV markers correlating with the particle counts from the NanoFCM and viral genome counts from the qPCR analysis (A-C). AAV=Adeno-associated virus, EVs=Extracellular vesicles, HR-SELFC=High Resolution-Size Exclusion Liquid Chromatography.

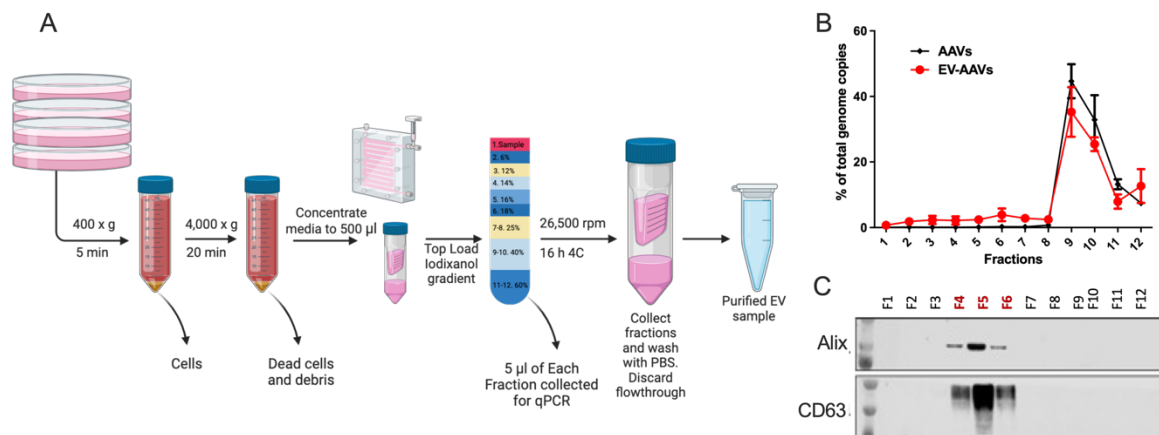


Figure 3.2 Iodixanol density gradient effectively separates free AAVs from EV-AAVs.

Extracellular vesicles were produced in HEK293T cells and purified using TFF, 100kDa spin filtration, iodixanol gradient fractionation, followed by 100kDa spin filtration to remove iodixanol and concentrate sample (A). Fractions 4-6 contain EVs as confirmed by Western Blot of the EV-AAV sample (C) detecting EV markers Alix and CD63, and free AAVs are found in fractions 9-12 as determined by qPCR for detection of viral genomes of purified AAVs or concentrated media containing EV-AAVs (B). AAV=Adeno-associated Virus, EV= extracellular vesicles.

Alternatively, a 12 step iodixanol density gradient was also evaluated as a method to separate EVs from AAVs based on their buoyant densities (Fig 3.2A). In this approach, fractions 4-6 contain EVs, confirmed by the presence of ALIX and CD63 in western blot analysis, while fractions 9-12 hold free AAVs, as indicated by qPCR results (Fig 3.2B). Iodixanol gradient was selected as the method used for the remainder of this thesis, due to its reliability and ability to process 6 samples simultaneously rather than just 1 at a time as with HR-SELFC.

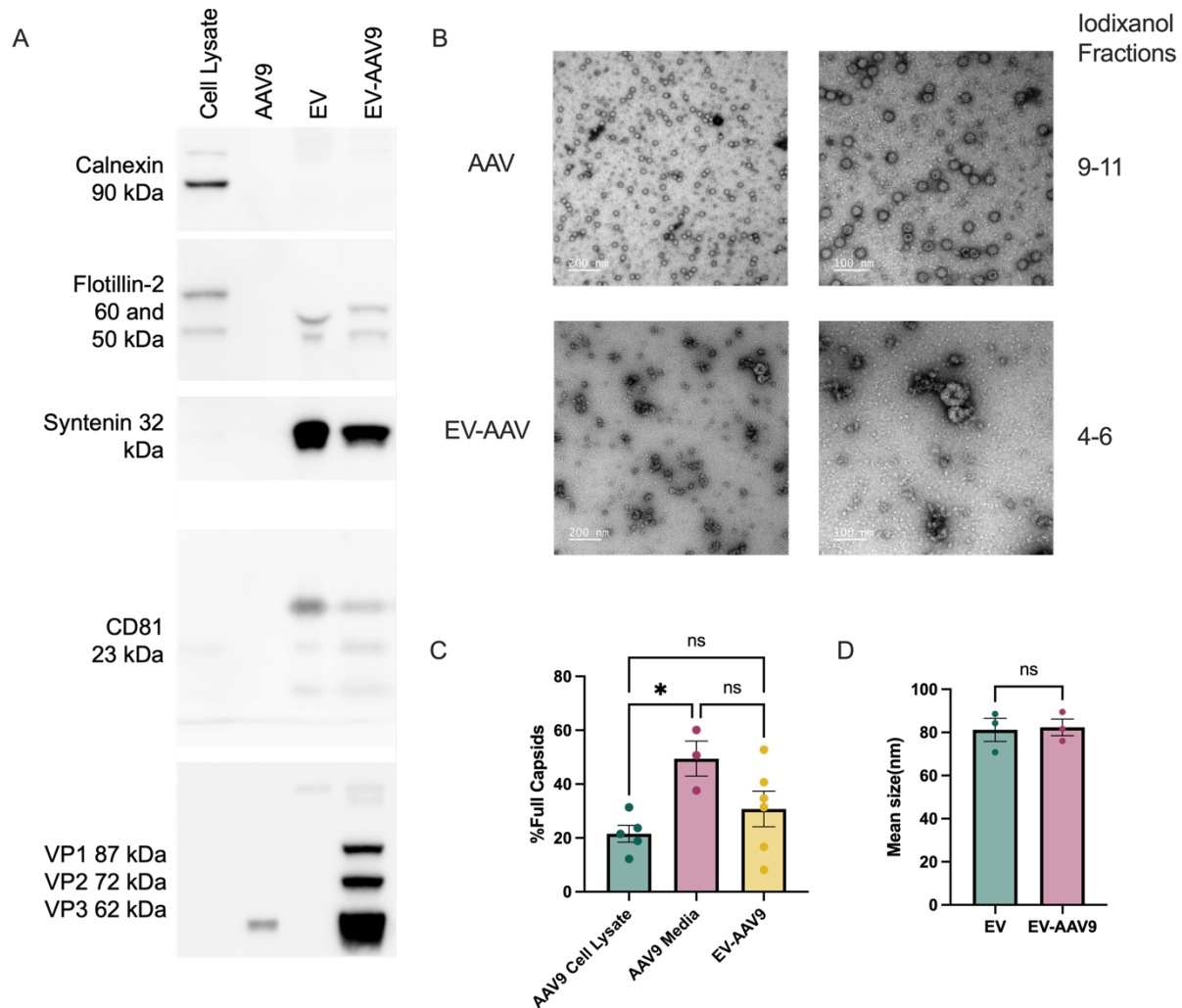


Figure 3.3 EV-AAV9s collected by iodixanol gradient are positive for EV markers and negative for cytoplasm marker Calnexin and have similar full capsid ratios to AAV9 collected from the cell lysate.

EV-AAV9s are negative for calnexin, and positive for EV markers Flotillin-2, Syntenin and CD81 as confirmed by Western Blot (A). Electron Microscopy images of AAVs from fraction 9 and 10 showing no EVs and images of EVs collected from iodixanol fraction 4-6 (B). The percentage of full vs empty capsids was calculated using ddPCR of viral genomes and MSD AAV9 virus particle detection (C). No significant difference between EVs and EV-AAVs in mean size as evaluated by NanoFCM (D). All values presented as Mean $\pm$ SEM. Statistical Analysis: One-way ANOVA ($F(2, 11) = 4.589, p = 0.0355$); Tukey's multiple comparisons test identified a difference between AAV9 Cell Lysate and AAV9 Media ($p = 0.0288$), with no significant differences observed between AAV9 Cell Lysate and EV-AAV9 ($p = 0.4758$), or between AAV9 Media and EV-AAV9 ($p = 0.1367$) (C). Unpaired t -test found no size difference between EV and EV-AAV9 ($t(4) = 0.1789, p = 0.8667$); variances were similar ($F(2, 2) = 1.897, p = 0.6903$) (D).

* $P < 0.05$.

EV-AAV9s collected via iodixanol gradient (from pooled fractions 4-6) were positive for EV markers Flotillin-2, Syntenin, and CD81 and negative for the cytoplasmic marker Calnexin, confirming their EV identity (Fig 3.3A). Electron microscopy further demonstrated that AAVs were isolated without EVs in fractions 9 and 10, while EVs were present in fractions 4-6 (Fig 3.3B). The analysis of full versus empty capsids revealed no significant difference between EV-AAV9s and AAV9s purified from cell lysates, indicating comparable quality in terms of viral packaging (Fig 3.3C). Additionally, there was no significant difference in the mean size of EVs collected from AAV-producing HEK293T cells and transfected controls as determined by NanoFCM, suggesting similar particle dimensions across both groups (Fig 3.3D).

3.3.2 Characterization of EV-AAVs

The level of AAV secretion into the media varies significantly depending on the serotype, with certain serotypes like AAV9 being more efficiently secreted, while others, such as AAV2, tend to be retained in the cell lysate. Building on this observation, we investigated whether the incorporation of AAVs into extracellular vesicles also exhibited serotype specificity, aiming to understand whether specific serotypes preferentially associate with vesicles to influence their packaging and delivery⁴¹. AAV2, 8 and 9 were produced in HEK293T and isolated using a 12 step iodixanol gradient. No significant differences in viral genomes accumulating in each fraction for the different serotypes were observed (Fig 3.4A). To evaluate the number of protected genomes, anti-AAV pulldown of EV-AAV samples, collected from fraction 4-6 of the iodixanol gradient, was performed where free AAVs or AAVs bound to the surface of the beads would be pulled down to the bead fraction, whereas AAVs inside EVs would be protected from

pulldown and thus remain in the supernatant; no significant differences between serotypes were observed (Fig 3.4B).

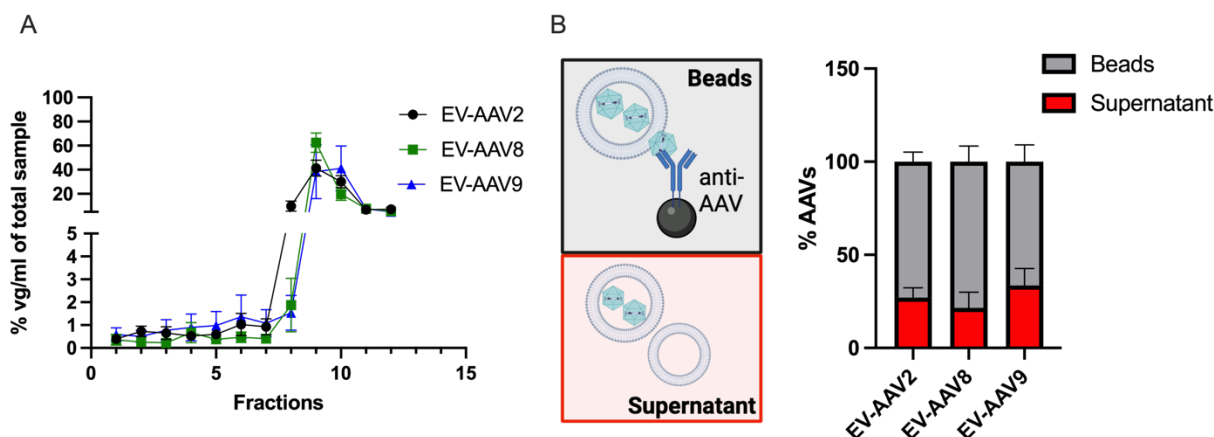


Figure 3.4 The incorporation of AAVs into vesicles is not serotype dependent.

Extracellular vesicles were produced in HEK293T cells and purified using TFF, spin filtration and iodixanol gradient fractionation. qPCR for detection of viral genomes in each fraction shows no significant difference across serotypes 2, 8 and 9 (A). Anti-AAV pulldown revealed there was no significant difference between the number of protected genomes for the AAV serotypes tested $N=3$ (B). All values presented as Mean $\pm$ SEM, Statistical Analysis: Groups failed normality (Shapiro–Wilk $p < 0.01$), so a Friedman test was used and found no significant difference ($p = 0.1738$) (A). Normality was confirmed ($p > 0.9$), and two-way ANOVA showed significantly higher viral genomes in bead-bound fractions ($F(1, 12) = 51.17, p < 0.0001$), with no effect of serotype ($F(2, 12) = 3.04, p > 0.9999$) or interaction ($F(2, 12) = 1.21, p = 0.3324$). Tukey’s test found no differences between groups (all adjusted $p > 0.5$) (C). Schematic made using BioRender.

To determine what size of EVs incorporate AAVs, vesicles were isolated using ultracentrifugation, where the 20k pellet contains larger vesicles (ectosomes and larger exosomes) and the 100k pellet contains smaller vesicles (Fig 3.5A)²⁰⁴. qPCR analysis of the iodixanol fractions demonstrated that in fractions 4-6, percentages of total genomes from 20k-EV-AAV9 were 20–43 times higher than those from the AAV9-only control, and 4.7–6.2 times higher than those from 100k-EV-AAV9 (Fig. 3.5B). Particles isolated from fractions 4-6 of the iodixanol gradient were evaluated using anti-AAV9 pulldown alongside AAV9 only control, demonstrating significantly more viral genomes encapsulated within EVs and shielded from

pulldown in the 20k-EVAA9s (Fig. 3.5C), indicating that larger vesicles incorporate AAVs.

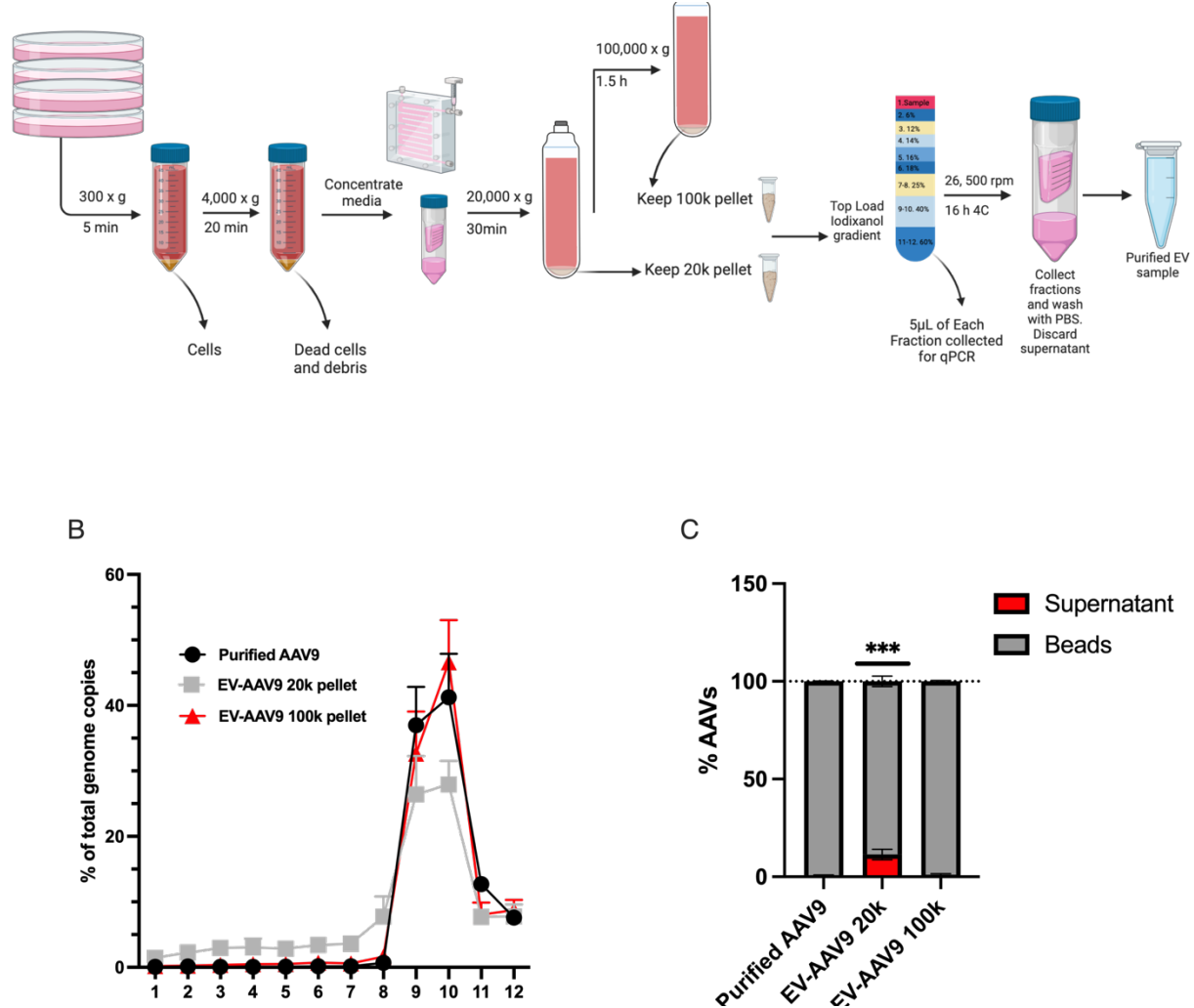


Figure 3.5 Larger vesicles incorporate AAVs.

Extracellular vesicles were produced in HEK293T cells and purified using TFF, spin filtration and ultracentrifugation, first at 20, 000 x g for 30 minutes. The pellet was collected, and the supernatant was then further spun at 100, 000 x g for 1.5 h, this pellet was also collected and the supernatant discarded. Pellets were then resuspended in 500 μ L of PBS and run on a 12 step iodixanol gradient (A). qPCR of iodixanol fractions shows increased viral genome accumulation in EV containing fractions 4-6 after 20k UC, whereas 100k UC results in little to no association of AAVs with EVs (B). Anti-AAV9 pulldown reveals a significantly increased number of viral genomes encapsulated in EVs and protected from pulldown in the 20k pellet (C). All values presented as Mean $\pm$ SEM, Statistical Analysis: Groups failed normality (Shapiro–Wilk $p < 0.0005$); Friedman test showed a significant difference ($p = 0.0388$). Dunn’s test revealed a difference between EV-AAV9 20k pellet and Purified AAV9 ($p = 0.0429$); all other comparisons were not significant ($p > 0.19$)(B). Two-way ANOVA showed significant effects of fraction ($F(1, 14) = 6341, p < 0.0001$) and interaction with AAV condition ($F(2, 14) = 35.98, p < 0.0001$), but no effect of AAV condition alone ($p > 0.9999$). Tukey’s test revealed differences

between Purified AAV9 and EV-AAV9 20k, and between EV-AAV9 20k and 100k in both fractions ($p < 0.001$). No differences were observed between Purified AAV9 and EV-AAV9 100k ($p > 0.98$)(C).

To further characterize the particles, EVs (obtained from cells transfected with empty plasmid) or EV-AAVs, were both purified by iodixanol fractionation, and sent for proteomic analysis. The first set of samples analysed revealed clear separation in the principal component analysis (PCA) between EVs and EV-AAVs (Fig 3.6.1A-C). For the second round of proteomics four experimental replicates of empty EVs, EV-AAV2s, EV-AAV8s, and EV-AAV9s were submitted, however results from the first round were not reproducible, as shown in Fig 3.6E, where the Venn diagram of proteins identified as unique to EV-AAVs had no overlap (Fig 3.6D-G).

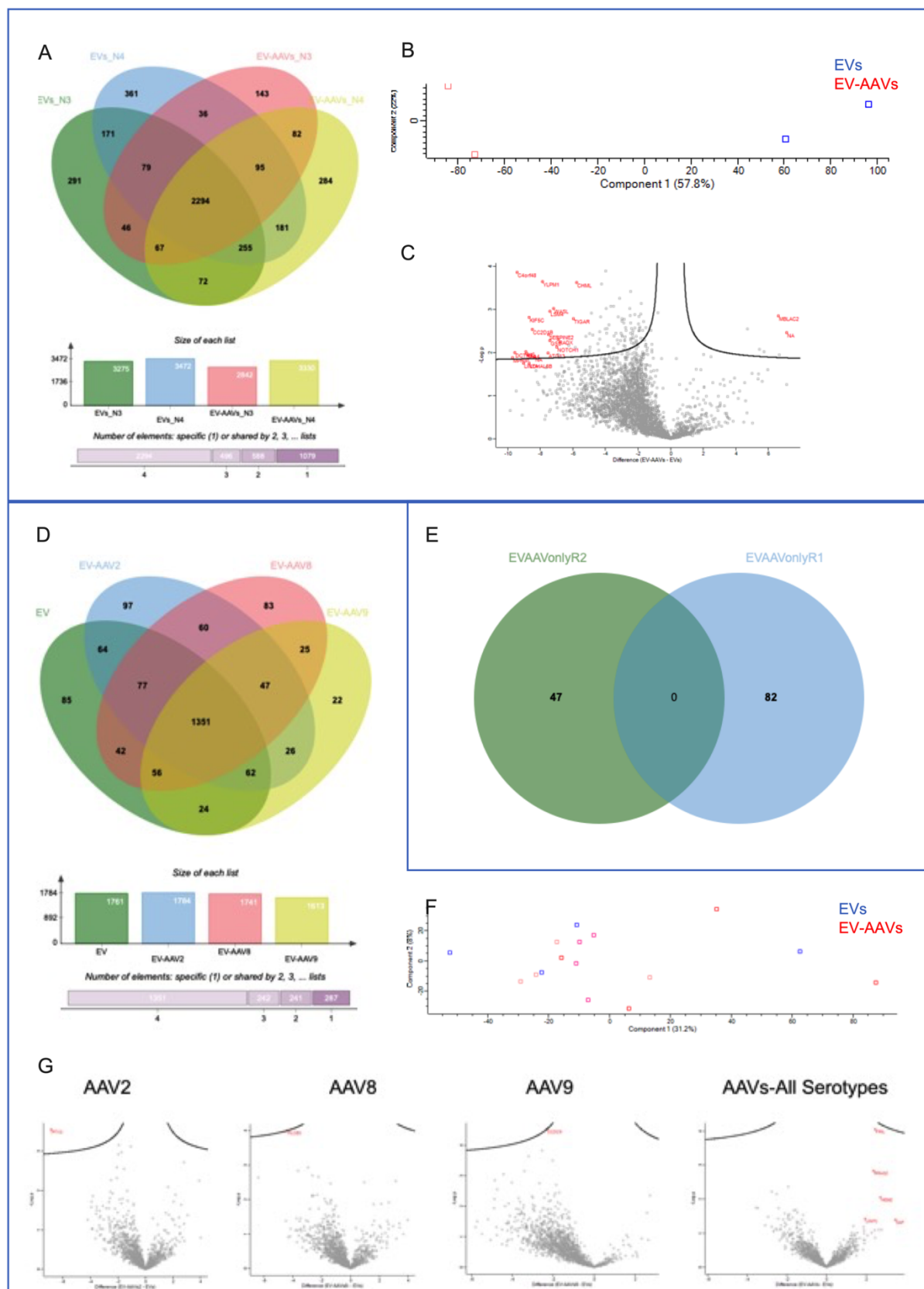


Figure 3.6 Proteomic comparison of EVs and EV-AAVs was not reproducible.

Venn diagram of proteomic hits across the two Ns of EVs and EV-AAVs (A). PCA analysis shows a clear separation between EVs (blue) and EV-AAV9s (red) samples N=2(B). Volcano plot of enriched proteins (C). Venn diagram of proteomic hits across the EVs, EV-AAV2, EV-AAV8, EV-AAV9 samples N=4 (D). Venn diagram comparing the EV-AAV unique genes found between rounds 1 and round 2 of proteomics, demonstrating zero overlap (E). PCA analysis shows no clear separation between EVs (blue) and EV-AAVs (red) samples (F). Volcano plots of enriched proteins with no significant hits (G). Proteomic samples were normalized to particle concentration and sent for label-free proteomics at the Karolinska institute. Venn diagrams were made using online jvenn tool. PCA and volcano plot analysis was performed using Perseus.

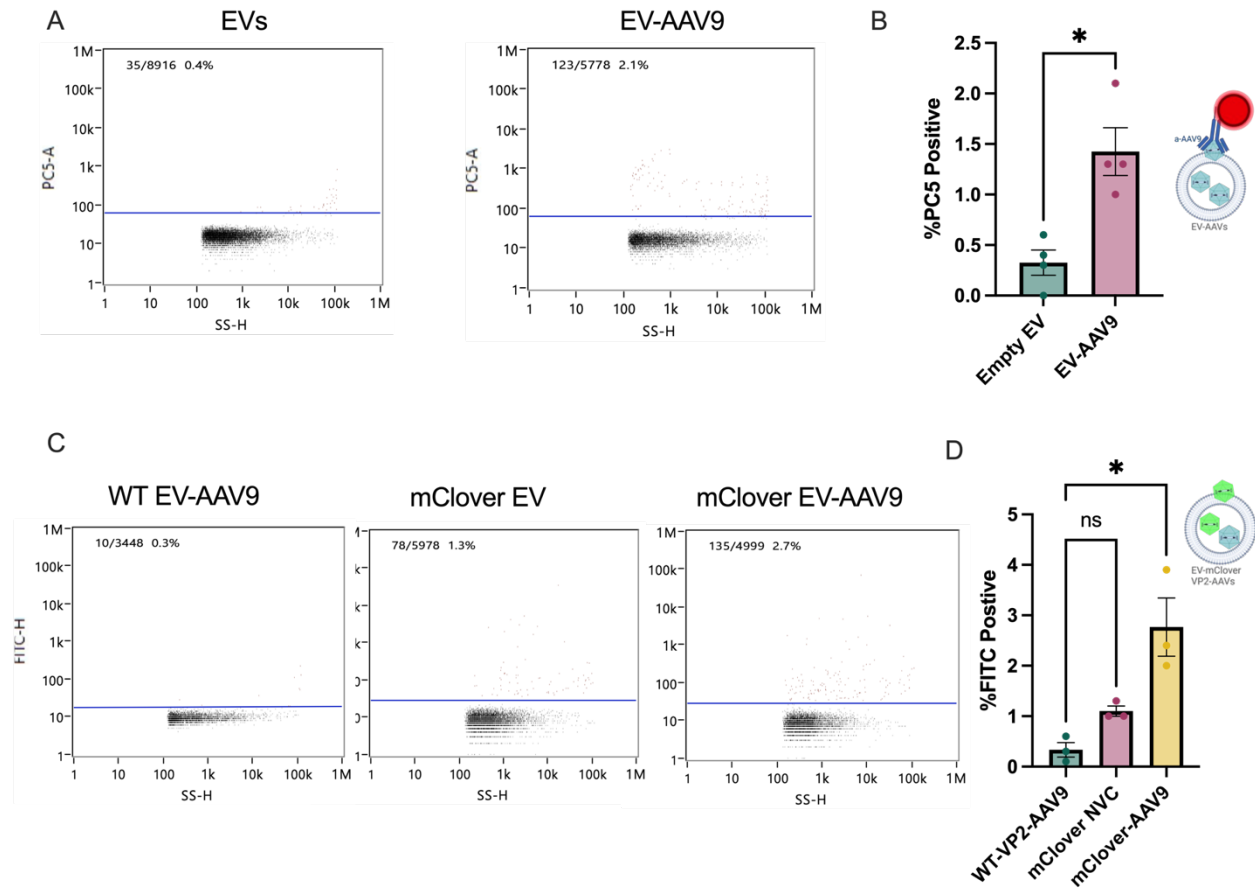


Figure 3.7 Low endogenous levels of EV-AAV9s.

Representative gating of EV or EV-AAV9 stained with anti-AAV9-Alexa 647 (PBS, 0.1% BSA, 0.001% Triton-X-100) (A). Only 1.2% of EVs are associated with AAVs (B). Representative gating of EVs collected from cells producing WT-VP2 AAVs, cells transfected VP2-mClover only (NVC: No virus control), or cells producing VP2-mClover AAVs (C). On average, 1.6% of EVs are positive for AAVs (after subtracting NVC background) (D). All values are presented as Mean $\pm$ SEM. NVC: No Virus Control. Statistical Analysis: F-test for equality of variances: $F(3, 3) = 3.560$, $p = 0.3248$. Unpaired two-tailed t -test: $t(6) = 4.121$, $p = 0.0062$ (B). Ordinary one-way ANOVA detected a significant difference among groups ($F(2, 6) = 12.70$, $p = 0.0070$). Variances were similar based on Brown-Forsythe ($p = 0.3758$). Tukey's test showed significant differences between WT-VP2-AAV9 and mClover-AAV9 ($p = 0.0063$), and between mClover NVC and mClover-AAV9 ($p = 0.0344$) (D).

Anti-AAV antibody staining, performed after permeabilizing the EVs, followed by NanoFCM analysis, revealed that once the background of EVs only staining was subtracted from the total signal only 1.2% of EVs contain AAVs(Fig 3.7A-B). To confirm the percentage of EVs that associate with AAVs, the VP2 capsid protein of AAV was modified to contain mClover3²⁰⁵. To produce EV-AAVs cells were transfected with Helper, Rep/Cap with unmodified VP1 and VP3 and with either VP2-mClover3 or VP2-wildtype plasmid. As a control, cells were also transfected with VP2-mClover3 plasmid only, to provide the background for any unassembled VP2-mClover3 that may get packaged into detected vesicles, this value was subtracted from the EV-mClover3-AAV value. EVs and EV-AAVs were purified and measured using the NanoFCM. Based on detection of EV-mClover3-AAVs, approximately 1.6% of EVs collected are EV-AAVs (Fig3.6D). The low yields of EV-AAVs observed with these two independent strategies highlight the need for engineering to improve AAV incorporation into vesicles and to provide a purer EV-AAV9 sample for more nuanced analysis.

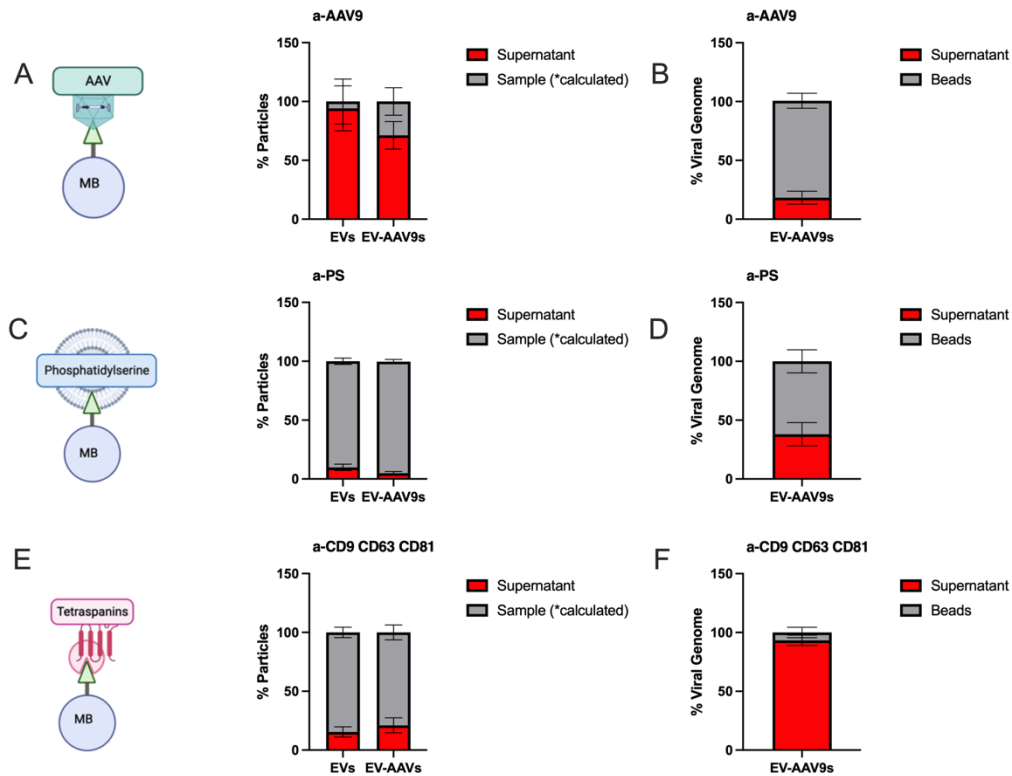


Figure 3.8 EV-AAV9s are enriched in phosphatidylserine, but not tetraspanins.

Magnetic pulldowns were performed with either anti-AAV9 antibody (AB), TIM-4 (CD), or a-CD9 CD63 CD81 (EF); for each case, number of particles was determined using NanoFCM, and the percentage viral genomes was calculated using qPCR, of the transgene. All values shown as Mean±SEM, N=3.

Affinity purification of EVs can enrich for a specific population and help to identify surface characteristics. Magnetic beads were conjugated with either anti-AAV9, TIM-4 which binds phosphatidylserine, or commercially available anti-CD9/CD63/CD9 magnetic beads were prepared (Fig 3.8A, C, E). Magnetic beads were then incubated with EVs or EV-AAV9 samples overnight, and the next day beads and supernatant were analysed by qPCR to detect viral genomes, and by NanoFCM to determine particle concentration. Anti-AAV9 showed that most EV particles remained in the supernatant, whereas 28.7% of the EV-AAV9 fraction remained bound to the beads, and 82.25% of the viral genomes were pulled down by the beads (Fig 3.8A-B). This suggests that many of the wildtype EV-AAV9s might have AAVs bound to the surface as suggested by our electron micrographs (Fig 3.3B) and by others¹⁷⁷. TIM-4 binds

phosphatidylserine in a calcium dependent manner. 90% of EVs were pulled down whereas 95% of EV-AAV9 particles were pulled down, as were 62% of viral genomes, indicating that EV-AAV9s are phosphatidylserine rich (Fig 3.8C-D). Lastly pan-tetraspanin beads were used, which pulled down close to 20% of EV and EV-AAV9 particles, and less than 10% of viral genomes (Fig 3.8E-F). Together these results suggest that EVs that are associated with AAVs are phosphatidylserine enriched and tetraspanin low, and many have virus bound to the surface.

3.3.3 Biogenesis of EV-AAV9s

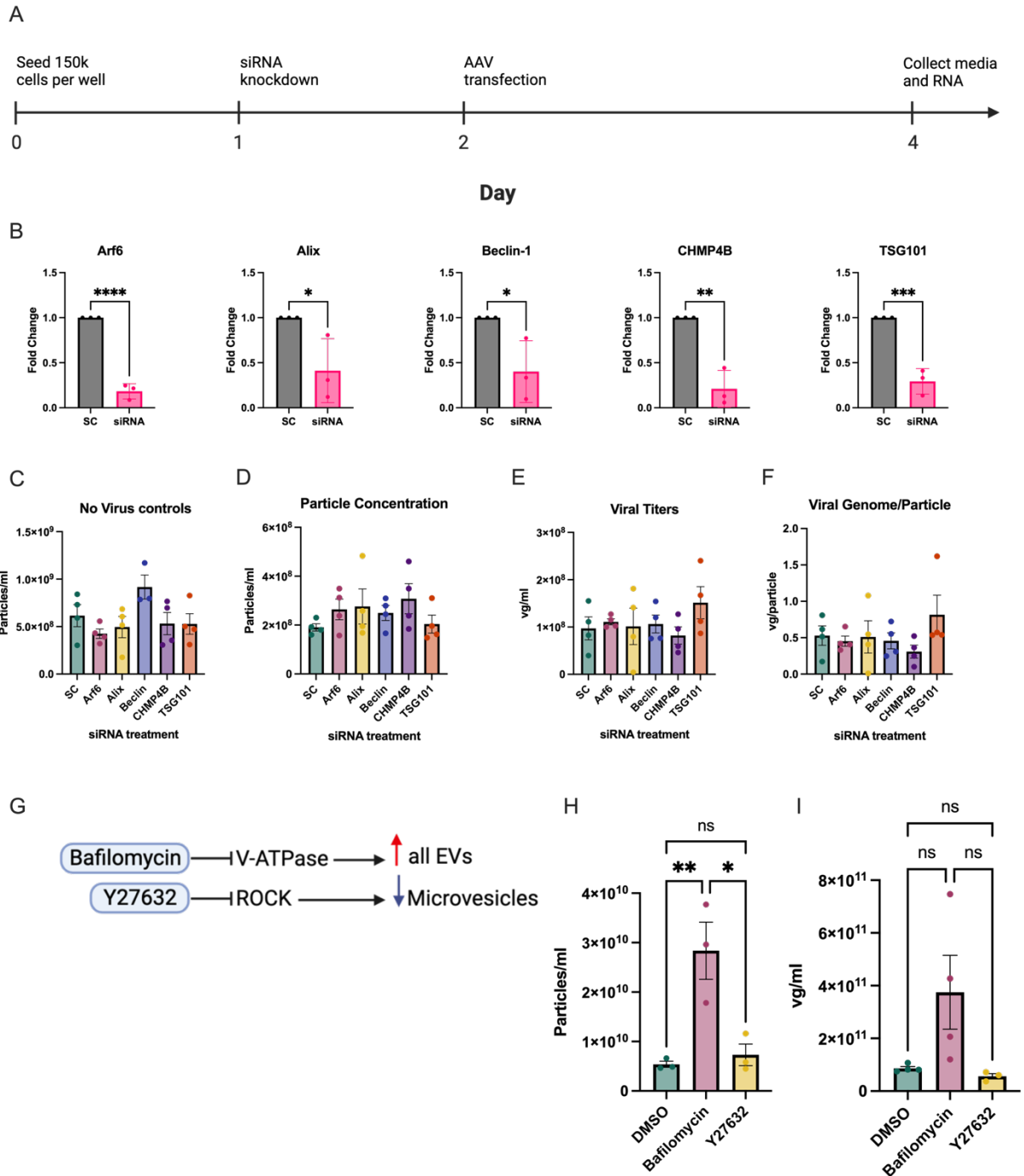


Figure 3.9 Chemical inhibition of ectosome release tended to decrease EV-AAV9 yield, while chemical activation of extracellular vesicle release using Bafilomycin tended to increase EV-AAV9 yield.

HEK293T cells were seeded at 150,000 cells per well on Day 0, followed by iRNA knockdown of Arf6, Alix, Beclin-1, CHMP4B or TSG101 using siRNA pool from Dharmacon on Day 1. On Day 2, cells were transfected with either AAV plasmids or control plasmid, and media and RNA were collected on Day 4 for downstream analysis (A). Knockdown of target genes was confirmed by

qPCR of target gene (B). Particle concentration in the media of HEK293T cells treated with siRNA and transfected with either empty vector plasmids: no virus controls (NVC) (C) or with plasmids to produce AAVs (D) was evaluated using the NanoFCM. Viral titers in the media were evaluated by qPCR (E). Ratio of viral genome per particle was calculated using values from D and E. Schematic of drugs used in the study (G). Normalized viral genomes of EVs were collected using liquid chromatography from drug treated cells (H). All values presented as Mean $\pm$ SEM. Statistical analysis: Unpaired two-tailed *t*-tests were used to compare siRNA-treated vs. scrambled control (SC) conditions for Alix ($t(4) = 2.871$, $p = 0.0454$), Beclin1 ($t(4) = 3.025$, $p = 0.0390$), CHMP4B ($t(4) = 6.667$, $p = 0.0026$), and TSG101 ($t(4) = 8.665$, $p = 0.0010$). F-tests indicated unequal variances for all ($p < 0.0001$; $F(2, 2) = \infty$). Normality was assessed using the Shapiro–Wilk test; Beclin failed ($p = 0.0392$), so Kruskal–Wallis was used ($H = 6.975$, $p = 0.2225$)(C). For siRNA particle and genome analysis (D–E), groups passed Shapiro–Wilk ($p > 0.19$), and variances were homogeneous (Brown–Forsythe $p = 0.4287$). One-way ANOVA showed no differences ($F(5, 18) = 0.8961$, $p = 0.5046$)(D). For viral titers (E), all groups passed normality ($p > 0.29$) and variance tests (Brown–Forsythe $p = 0.1205$), and ANOVA showed no differences ($F(5, 18) = 0.8270$, $p = 0.5469$). For viral genome/particle (F), one group failed Shapiro–Wilk ($p = 0.0030$), so a Kruskal–Wallis test was used ($H = 4.870$, $p = 0.4320$). Drug effects on particle concentration (G) met normality ($p > 0.05$) and were analyzed by one-way ANOVA ($F(2, 6) = 12.65$, $p = 0.0070$, $R^2 = 0.8083$); Tukey’s test identified significant pairwise differences (adjusted *p*-values: Bafilomycin vs. DMSO = 0.0094; Y27632 vs. Bafilomycin = 0.0141; Y27632 vs. DMSO = 0.9259). For Drug effects on viral genomes (H), Shapiro–Wilk ($p > 0.07$), Brown–Forsythe ($p = 0.5283$) test confirmed assumptions, and one-way ANOVA showed no differences ($F(3, 8) = 1.582$, $p = 0.2682$).

Understanding the biogenesis pathway of EV-AAVs would be valuable for guiding future engineering strategies. To explore this, two approaches were employed: transient knockdown of ESCRT machinery pathways using siRNA and the application of chemical inhibitors. Due to material limitations, a smaller-scale setup was required for the siRNA experiments, resulting in the use of less sophisticated isolation techniques that did not separate EV-AAVs from free AAVs. HEK293T cells were seeded into a 12-well plate, allowed to attach overnight, and the following day were treated with siRNA pools targeting Arf6, Alix, Beclin-1, CHMP4B, TSG101 or a scrambled control. The next day, cells were transfected with AAV9 production plasmids or empty vector controls (Fig 3.9A). Two days later, media was collected and analysed by qPCR and NanoFCM, and RNA was extracted from cell lysates to confirm efficient gene knockdown (Fig 3.9B). While qPCR analysis verified effective knockdown of Arf6, Alix, Beclin-1,

CHMP4B, and TSG101, no significant impact on secreted AAV9 genomes was observed in the media (Fig 3.9A-F).

To further investigate EV biogenesis, chemical inhibitors were utilized to overcome the reagent limitations of the siRNA approach. Bafilomycin inhibits vacuolar-type ATPase (V-ATPase), and as a result total EVs secretion is increased²⁰⁶. Y27632 decreases ectosome secretion by inhibiting Rho-associated protein kinase (ROCK)¹⁸⁷. No longer limited by the amount of reagent, a larger scale production was made, and EVs were isolated by iodixanol gradient purification, enabling us to look at the EV population specifically rather than only the total titer in the media. Chemical inhibition of ectosome production using Y27632 led to a trend toward a decrease in EV-AAV yields (Fig 3.9G-H).

3.3.4 EV-AAV9 transduction in Huh7 cells is dependent on AAVR expression.

AAV9 transduction is dependent on its interaction with the receptor AAVR, which plays roles in both cell entry and post-entry processes. To investigate the role of AAVR in EV-AAV9 transduction, Huh7 wild-type (WT) and Huh7 AAVR knockout cells (provided by Evox Therapeutics) were used (Fig 3.10A). AAVR expression was restored in the knockout cells through transfection with an AAVR plasmid before transduction with either AAV9 or EV-AAV9. AAV9 and EV-AAV9 carried an mNeonGreen transgene. Operetta live cell imaging was used to detect cells expressing the delivered transgene, and percentage of cells transduced was calculated. It was observed that AAVR knockout significantly decreased the transduction efficiency of both AAV9 and EV-AAV9, which was restored by overexpression of AAVR (Fig 3.10B). This suggests that AAVR does play an important role in EV-AAV9 transduction.

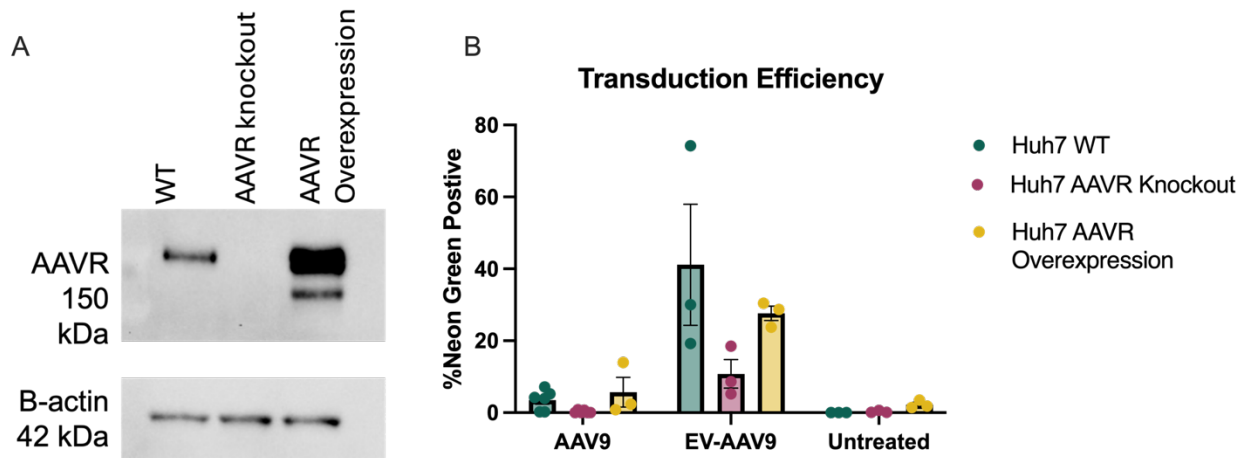


Figure 3.10 EV-AAV9 transduction in Huh7 cells is dependent on AAVR expression. Huh7 WT and Huh7 AAVR knockout cells were provided by Evox Therapeutics. AAVR expression was restored by transfection with an AAVR plasmid, before transduction with AAV9, or EV-AAV9. Expression of AAVR was confirmed for each biological replicate by western blot (A). Transduction efficiency was evaluated by calculating percentage of cells expressing the mNeonGreen transgene using the Operetta High Content Imaging System (B). All values presented as Mean $\pm$ SEM, N=3.

3.4 Discussions and conclusion

EV-AAVs have been shown to have multiple advantages over AAVs *in vivo* and *in vitro*, including improved transduction and protection from NABs^{177,178,180,181,200,207–211}. To improve EV-AAVs viability in the clinic, reliable purification and improvements in yield are needed. It is also poorly understood how AAVs are loaded into EV-AAVs, making designing engineering strategies challenging.

Here, two different isolation methods have been proposed which were both effective at separating EV-AAVs from AAVs and characterizing EV-AAV particles. One disadvantage of HR-SELFC is that large volume dilution of the sample may rid the EVs of their protein corona which may play an important role in EV particle distribution, transduction, and interaction with the immune system. Iodixanol density gradient, on the other hand, has been shown to preserve the protein corona^{212–214}. Iodixanol density gradient was selected as the technique for the

remaining studies in this thesis. EVs collected using iodixanol gradient were positive for EV markers, and negative for endoplasmic reticulum protein calnexin. The average population size of EVs and EV-AAV9s was not different. It was also found that EV-AAV9s have a similar percentage of full capsids as the AAV9s collected from the same producer cells. This is important for gene therapy efficacy as empty capsids can increase risk of adverse immune response, without adding increased delivery of the transgene.

There was no difference found among AAV2, AAV8 and AAV9 in the percentage of viral genomes in EV containing fractions 4-6 or percentage of protected genomes. The secretion levels of AAV serotypes into the media vary, with AAV8 exhibiting the highest secretion, followed by AAV9, and AAV2 showing the least⁴¹. Given that there is no difference in the percentage of AAVs associated with EVs with the different serotypes, it suggests that the mechanism by which AAVs are loaded into EVs is different. The membrane-associated accessory protein (MAAP), encoded by the AAV cap gene, was shown by Elmore et al., 2021 to reduce AAV secretion into the media when mutated, without impacting the overall viral yield. They also found evidence to suggest that MAAP facilitates association of AAVs with EVs²¹⁵. We did not investigate MAAP in this project, however MAAP is conserved across the serotypes, so engineering MAAP may play a role in future strategies to increase EV-AAV yields. Given that there were no differences in EV association between serotypes, AAV9 was selected for further characterization for its broad tropism and proven transduction in heart and central nervous system.

Larger vesicles from the 20k pellet had increased EV-AAV9 yield. Samples were prepared and sent for proteomics to compare the profiles of EVs collected from empty plasmid-transfected cells and EV-AAV9s from HEK293T producer cells under serum-free conditions. The samples were normalized based on particle concentration using NanoFCM and then sent for proteomic

analysis. The goal was to identify proteins in EV-AAVs that could help pinpoint the subpopulation of EVs most effective at associating with EV-AAVs. However, after performing two rounds of proteomics, we found there was no overlap in the data. To investigate potential reasons for the inconsistent results, I examined what percentage of the total EV population collected from HEK293T virus-producing cells was associated with AAVs. Using two different staining techniques, I found that only 1.2-1.6% of the total EV population were EV-AAVs, which might explain the difficulty in detecting differences in the proteomic data. Further separation or enrichment of EV-AAVs from the broader EV population could provide better insights into their composition and/or biogenesis.

To investigate the biogenesis pathway of EV-AAVs for future engineering strategies, HEK293T cells were treated with siRNA targeting components of the ESCRT machinery, as well as with chemical inhibitors of V-ATPase and ROCK (bafilomycin A1 and Y27632), which affect total EV secretion and ectosome secretion, respectively. Although knockdown of Arf6, Alix, Beclin-1, CHMP4B, and TSG101 showed no significant effect on secreted AAV9 genomes, chemical inhibition of ectosome production using Y27632 led to a trend toward a decrease in EV-AAV yield, suggesting ectosomes (and not exosomes) may play a role in the biogenesis process. It may be that the lack of differences seen in the EV-AAV9 yields, despite the confirmed knockdown of targets, is because this was done at a smaller scale and as such the conditioned media was examined for EV-AAV9 yields without any advanced purification steps, and nuanced differences were masked by the heterogeneity of the sample. Future experiments could employ shRNA lentiviral knockdown of these components to generate sufficient yield for isolation using an iodixanol gradient.

Further investigation into the composition of EV-AAV9s through magnetic separation of particles demonstrated that EV-AAV9s are enriched in phosphatidylserine but low in tetraspanins CD63, CD81 and CD9 (Fig 3.9A-F). Interestingly, another nonenveloped virus, poliovirus was shown to be loaded into phosphatidylserine rich vesicles, and their transduction was more efficient than the free particles, but still depended on the virus-specific receptor¹⁷⁶. Phosphatidylserine is in the plasma membrane and typically found on the inner leaflet of the membrane, however during cellular stress it can be translocated to the outer leaflet²¹⁶. One of the mechanisms that can lead to phosphatidylserine translocation is transfection²¹⁷. Anti-AAV pulldown results suggest that many of the EV-AAVs have virus bound to the surface, which based on literature are still protected from neutralizing antibodies, perhaps by a protein corona, or by the way the AAVs are bound there¹⁸¹. If binding AAVs at the surface of EVs is enough to protect them from neutralizing antibodies, this presents a potential engineering strategy that overcomes the unknowns of lumenally loaded EV-AAVs, such as their unclear biogenesis pathway. This idea is explored further in the next chapter.

The AAVR plays a critical role in the transduction process of AAVs, influencing both the entry of the virus into host cells and its subsequent intracellular trafficking^{34,218–220}. AAVR knockout in Huh7 cells resulted in significantly decreased transduction in both AAV9 and EV-AAV9, which could then be rescued by overexpressing AAVR in knockout cells. This suggests that AAVR is required for efficient EV-AAV9 transduction, and future studies are needed to determine whether this is due to its interaction with AAVs bound to the surface of EVs or to the intracellular trafficking of AAVs post cell entry^{102,220}.

In conclusion, this study highlights the complexity and potential of EV-AAVs as a therapeutic platform, particularly in gene therapy. While EV-AAVs offer several advantages

over AAVs, such as enhanced transduction efficiency and protection from neutralizing antibodies, significant challenges remain in optimizing their yield and understanding their biogenesis. The lack of overlap in proteomic data and the low percentage of EV-AAVs in the total EV population underscore the need for further refinement in isolation and purification methods. Larger size, increased phosphatidylserine expression and sensitivity to ectosome biogenesis inhibition suggest that ectosomes and not exosomes are better at incorporating AAVs. Additionally, the involvement of AAVR in EV-AAV transduction suggests that optimizing interactions between EV-AAVs and host cell receptors could enhance therapeutic efficacy. Future research should focus on unravelling the mechanisms underlying AAV loading into EVs, improving EV-AAV yields and exploring engineering strategies that leverage these insights to overcome current limitations, ultimately improving the clinical applicability of EV-AAV-based therapies. Several such engineering strategies will be explored in the next chapter.

4 Chapter 4. Results-2 Engineering Strategies to improve EV-AAV yields

4.1 Abstract

This chapter focused on exploring engineering strategies to improve EV-AAV9 yields to enhance their therapeutic potential. A range of approaches was investigated, including surface-loading AAVs onto EVs, engineering producer cells to increase EV secretion and AAV9 incorporation, and modifying AAV capsids to enhance their loading. Surface-loading strategies utilizing AAVR showed partial protection against NAbs *in vitro* but were less effective *in vivo*. Engineering producer cells by overexpressing of CD63 significantly improved EV-AAV yields and the amount of lumenally loaded AAV9s. Engineered CD63-EV-AAV9s performed similarly or better to EV-AAV9 *in vitro* but exhibited decreased transduction efficiency *in vivo*.

Incorporation of the full-length pX domain from hepatitis A virus into AAV9 capsids significantly enhanced EV loading and transduction *in vitro* in SH-SY5Y-TL cells; however, pX-EV-AAV9s did not outperform the wildtype *in vivo*. A high-throughput directed evolution screen was conducted to optimize AAV capsid variants for enhanced EV incorporation, using libraries with randomized late domain motifs and linker lengths. After multiple rounds of selection and sequencing, top variants exhibited increased EV loading and protection of viral genomes, with specific motifs like LSGGLDILPKLC and LSDRKIERQGNVF showing significant enrichment and superior performance in subsequent validation studies. This chapter highlights the potential of EV-AAV-based approaches to shield AAVs from NAbs and enable repeated dosing in gene therapy. Future strategies combining capsid and producer cell engineering, as well as machine learning-guided optimization, can be explored to further refine EV-AAV platforms for enhanced

therapeutic delivery. These findings provide a comprehensive framework for advancing EV-AAV technology and overcoming current limitations in gene therapy applications.

4.2 Introduction

Optimizing EV-AAV production remains a key challenge, as efficient loading of AAVs into EVs is necessary to maximize therapeutic yields. This chapter explores various engineering strategies aimed at increasing EV-AAV yields, which can be broadly categorized into surface loading techniques, modifications targeting the producer cell and direct alterations of the AAV capsid itself.

In Chapter 3 anti-AAV pulldown using passively loaded EV-AAV samples demonstrated that most viral genomes are outside the EV, yet studies show that the AAV is protected from NAbs^{11,178}. This raised an interesting opportunity. Binding AAVs at the surface allows future therapies to take advantage of established isolation processes for AAV production and combine the AAV product with the engineered EVs at a known ratio. Surface loading also allows for more flexibility in choosing the cell of origin for the EVs. AAVs bind to specific receptors on the cell surface to facilitate entry. For AAV9, 2 such receptors are laminin receptor (LamR) and AAV receptor (AAVR). AAV9 and AAV8 bind to the 67kDa LamR for efficient entry into cells. LamR acts as a co-receptor binding AAV9 at the surface of the cell and enhancing interactions with its primary receptor⁴⁶. The AAVR, or KIAA0319L protein, is essential in facilitating AAV entry, trafficking and infectivity, acting as a primary receptor for multiple AAV serotypes. Research has shown that AAVR aids in AAV endocytosis and intracellular transport, highlighting its potential as a target for enhancing AAV-based gene therapies^{220–222}. Additionally, AAVR may act as a co-receptor, interacting with other cellular factors to support AAV movement through the host cell's pathways, suggesting a complex role beyond initial viral

binding²²³. Both receptors were selected as potential candidates to efficiently bind AAV9 at the surface of EVs.

NAbs pose a significant challenge for AAV gene therapy. These antibodies develop either from prior exposure to natural AAV serotypes or from earlier AAV-based treatments. All four IgG subclasses (IgG1, IgG2, IgG3, IgG4) can exhibit neutralizing activity²²⁴. In this study, intravenous immunoglobulin (IgG) derived from pooled human sera was used to replicate the effect of NAbs. Evaluating the effectiveness of surface-loading strategies in overcoming NAbs is important for future clinical efficacy. rAAV engineering studies have used directed evolution and rational design to identify capsid variants with enhanced resistance to NAbs, including the development of chimeric capsids and peptide-modified surfaces that evade antibody recognition^{80,225,226}. Additionally, pre-treatment strategies such as plasmapheresis, IVIG column depletion, or transient immunosuppression have been explored to reduce circulating antibodies and enable treatment of patients which have NAbs^{227–229}.

We engineered EVs by modifying the cells that produced them. One approach is to focus on proteins that are known to be loaded into EVs. Tetraspanins are a family of proteins which have 4 alpha-helical transmembrane domains with 2 extracellular loops²³⁰. Tetraspanins have been shown to be involved in cell signalling and to act as scaffolds to anchor multiple proteins to a single location in the plasma membrane. Tetraspanins CD63, CD81, CD9, TSPAN2 and TSAPN14 are known to be enriched in EVs and thus were selected for evaluation. Single-pass proteins enriched in EVs that were evaluated included PTTG1IP (Pituitary Tumor-Transforming Gene 1 Protein-Interacting Protein), PTGFRN (prostaglandin F2 receptor inhibitor), LAMP2B (Lysosomal Associated Membrane Protein 2B) and N-TransferrinR1 (N-terminal portion of Transferrin Receptor 1). PTTG1IP has been recently shown to be efficiently sorted into

exosomes, and modification of this protein resulted in efficient cargo loading into EVs¹⁶⁸.

Cytosolic proteins evaluated were HSPA8 (Heat Shock Protein Family A (Hsp70) Member 8), N-syntenin (N-terminal portion of Syntenin-1) and BASP1 (brain abundant membrane attached signal protein 1). These cytosolic proteins are all lumenally loaded and do not have transmembrane domains. Both PTGFRN and BASP1 have been shown to efficiently load into vesicles and have been modified to surface-display and lumenally load cargo into EVs, respectively¹⁷².

Of particular relevance to this project, HAV virus is a non-enveloped virus that can be loaded into EVs due to pX, an 8 kDa protein in its capsid, which has been shown to interact with ALIX to move the virus into EVs¹⁷⁴. Therefore, overexpression of EV markers could lead to increased EV-AAV yields. Late assembly domains (L-domains) are crucial peptide motifs found in the structural proteins of enveloped viruses, facilitating their budding and egress from host cells. These domains serve as signals that recruit host cellular machinery, particularly the ESCRT components, which are essential for membrane fission events during viral release^{160,231}. The identification and characterization of L-domains have significantly advanced our understanding of viral assembly and the mechanisms by which viruses exploit host cellular processes. There are several types of late assembly domains, each characterized by specific amino acid motifs that interact with different components of the ESCRT pathway.

Table 4.1 Amino Acid Motifs Associated with Enveloped Virus Egress, Late Domain Activity, or Protein Targeting to Extracellular Vesicles.

Amino Acid Motif	Virus Type	Example Viruses or Proteins	Known Interacting Partners
PT/SAP	Retroviruses, Paramyxoviruses	HIV-1, MLV (Murine Leukemia Virus), Human Parainfluenza Virus (HPIV), Hepatitis C Virus	TSG101 (Tumor Susceptibility Gene 101) ^{160,231–233}

YPXnL	Retroviruses, Picornaviruses	HIV-1, SIV (Simian Immunodeficiency Virus), Hepatitis A Virus (HAV)	ALIX (PDCD6IP), ESCRT-III components ^{174,231,234,235}
PPXY	Arenaviruses, Coronaviruses	Lassa Virus, Sars-CoV-2	NEDD4 family ubiquitin ligases (e.g., ITCH, WWP1) ^{236,237}
PxxP	Hepatitis E Virus	Hepatitis E Virus	TSG101 ^{160,231–233}
LXXL	Lentivirus	Feline immunodeficiency Virus (FIV)	ALIX ²³⁸
PPLPV	Retroviruses	Mouse mammary tumor virus (MMTV)	Possibly interacts with ESCRT components ²³⁹
FPIV	Paramyxoviruses	Newcastle disease virus (NDV)	Charged multivesicular body protein 4 (CHMP4) ^{240,241}
YXXL	Deltaretrovirus	Bovine leukemia virus (BLV)	AP-2 complex, Clathrin- mediated endocytosis components ²⁴²
KFERQ	Various Proteins	HIF1A	LAMP2A and HSC70 ²⁴³

This table summarizes amino acid motifs with functional roles in enveloped virus egress, late domain interactions, or the targeting of proteins into EVs. The first column lists the amino acid sequences, the second column provides examples of viruses or proteins where these sequences are found, and the third column identifies potential interacting partners that facilitate cargo loading into EVs. Abbreviations for amino acids: P = Proline, T = Threonine, S = Serine, A = Alanine, Y = Tyrosine, X = Any amino acid, L = Leucine, V = Valine, F = Phenylalanine, K = Lysine, E = Glutamate, R = Arginine, Q = Glutamine.

In addition to L domains there are motifs that are known to be sorted into extracellular vesicles. One such motif is the KFERQ motif, which has been shown to interact with LAMP2A, a protein integral to the lysosomal membrane and involved in the transport of proteins into EVs²⁴³. The incorporation of such sorting motifs into AAV capsids presents a promising strategy to leverage existing cellular pathways to increase EV-AAV yields. The position and length of the insertion of these targeting motifs can significantly influence their functionality. For instance, the spatial arrangement of the KFERQ motif within the capsid structure may affect its accessibility

to LAMP2B and other interacting partners, thereby impacting the efficiency of EV loading⁸¹. Similarly, variations in the length of linkers connecting the targeting motifs to the capsid proteins can alter the conformational dynamics of the capsid, which may either enhance or hinder the interaction with the cellular machinery responsible for vesicle formation and trafficking. Given the multitude of potential outcomes arising from different combinations of motifs, linker lengths and compositions, a high-throughput approach to evaluate these variables is needed. One potential method is to generate a comprehensive library of AAV capsids, each displaying a unique combination of targeting motifs and linkers. This library can then be subjected to an evolution screen, allowing for the identification of capsids that exhibit superior loading capabilities into EVs. Such an approach has been successfully employed in AAV engineering to enhance targeting to specific tissues, demonstrating its versatility and effectiveness in optimizing vector performance²⁴⁴. By employing massively parallel sequencing and other high-throughput methodologies, researchers can map functional residues within the capsid proteins, providing insights into how specific modifications influence the overall performance of AAV vectors²⁴⁵. This iterative process of screening and selection can lead to the development of AAV variants that are finely tuned for enhanced delivery of therapeutic payloads via EVs. In conclusion, the integration of sorting motifs like KFERQ or L domains into AAV capsids represents a novel strategy to exploit cellular pathways for improved EV-AAV yields. The careful consideration of motif positioning and linker length, along with the application of high-throughput screening methods can significantly enhance the loading of AAV into EVs.

In summary, the strategies explored in this chapter outline avenues for enhancing EV-AAV9 yields through surface loading, producer cell engineering and targeted modifications of AAV9 capsids. By binding AAVs to EV surfaces, it is possible to leverage existing isolation

protocols and maintain EV cell origin flexibility. Engineering producer cells to overexpress proteins known to enhance EV loading, such as tetraspanins and specific single-pass proteins, can further optimize EV-AAV9 production. Additionally, the incorporation of late assembly domains and EV-sorting motifs like KFERQ into AAV capsids represents a possible approach to harness cellular pathways for more efficient AAV packaging. The high-throughput evolution screening of capsid variants presents a robust framework for identifying configurations that maximize EV loading without compromising AAV functionality. Together, these approaches comprise a comprehensive engineering toolkit aimed at improving EV-AAV9 efficacy and overcoming immunological barriers, ultimately contributing to the advancement of AAV-based gene therapies.

4.3 Results

4.3.1 2A. Surface Loading Strategy

While loading AAVs into the EV lumen would presumably best protect them from NABs, the yields are still limited by the cells' poorly understood endogenous AAV loading pathways. An alternative strategy to overcome this limitation is to bind AAVs to the surface of EVs, leveraging the proximity of AAVs to the EV surface and its protein corona, which may protect against NABs^{246,247}. During the characterization of EV-AAV9 particles, an anti-AAV9 pulldown of purified EV-AAV9 particles demonstrated that the majority of viral genomes are located on the surface of the particle and are available for pulldown, further supporting the feasibility of surface loading strategies. AAVR and LamR are two receptors that have been shown to bind AAVs at the cell surface. AAVR and LamR were overexpressed in HEK293T cells, and EVs were collected from fractions 4-6 of the iodixanol gradient. They were then incubated with

isolated AAV9 which was prepared separately and separated using iodixanol fractionation. AAVR was significantly more effective than LamR in binding AAVs, as evidenced by qPCR titration of iodixanol fractions (Fig 3.4.1A-B). To reduce the number of purification steps needed to eliminate free AAVs, various ratios of EV to AAV were tested, and mixing AAV9s with EVs at a 1:1 ratio minimized the presence of free AAV9s in the solution, while ensuring maximal loading of AAV9s onto EVs (Fig 3.4.1C). To determine whether binding AAVs to the surface of EVs provides protection against NAbs, an *in vitro* neutralization assay was performed. AAV9, AAV9 plus either AAVR- or LamR- engineered EVs and AAV9 plus wildtype EVs were preincubated with either 0, 2, 4, or 8 mg/mL of IgG and then added to HEK293T cells. Levels of fluorescent transgene were evaluated using a SpectraMax plate reader. Fluorescent signal persisted in AAVR-EVs plus AAV9 but not in AAV9 only or wildtype EV plus AAV9 treatments demonstrating that AAV9s bound to the surface of EVs are partially protected from NAbs (Fig 3.4.1D).

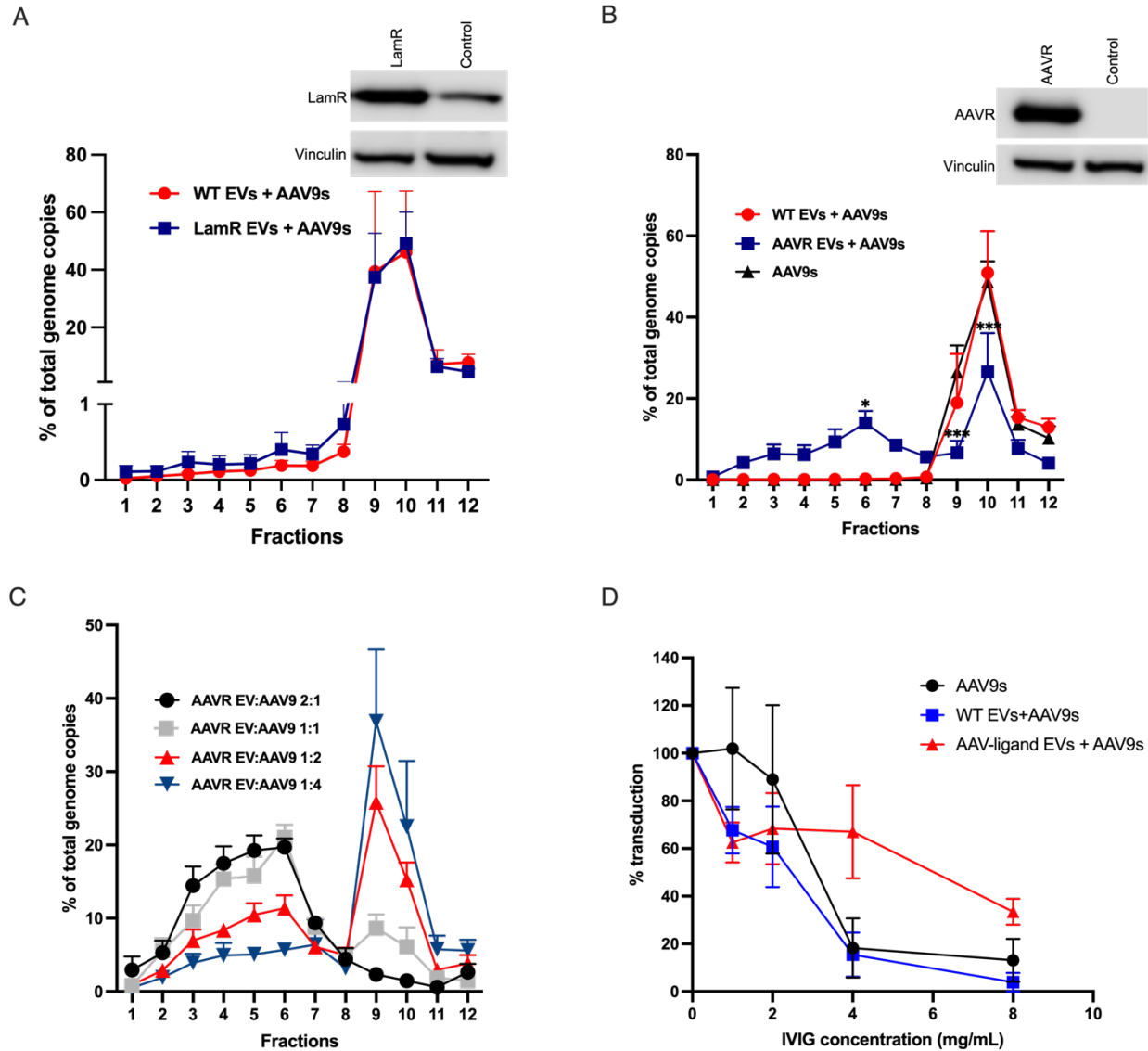


Figure 4.1 AAVR is more effective than LamR at binding AAVs to the surface of EVs and confers some protections from NAb in vitro.

EVs were produced in cells overexpressing either Laminin Receptor (LamR) (A) or AAV Receptor (AAVR) (B). EVs were isolated by iodixanol fractionation, incubated with AAV9s for 1 hour, and subsequently purified using iodixanol gradient fractionation. Western blot confirmed the overexpression of LamR and AAVR (A-B). EV-AAVRs were incubated with AAV9 at AAVR-EV:AAV9 ratios of 2:1, 1:1, 1:2, and 1:4 and then purified using iodixanol gradient. Viral genomes were measured in each fraction by qPCR (C). Cells were incubated with neutralizing antibodies (NAb) at varied concentrations prior to analyzing transduction *in vitro*, using GFP transgene expression (D). Mean+SEM, N=3. Statistical Analysis: Multiple unpaired *t*-tests comparing WT EVs + AAV9s to LamR EVs + AAV9s across 12 conditions revealed no significant differences after false discovery rate correction ($Q = 1\%$) ($q > 0.67$) (A). Two-way ANOVA of AAV9 vs. AAVR EVs + AAV9s showed a significant interaction ($F(22, 72) = 3.25, p < 0.0001$) and row effect ($F(11, 72) = 31.42, p < 0.0001$), but no column effect ($F(2, 72) = 4.1, p > 0.9999$); Fisher's LSD post hoc tests identified significant differences in Rows 6 ($p = 0.0088$), 9 ($p = 0.0003$), and

10 ($p < 0.0001$) (B). Two-way ANOVA of AAVR EV:AAV9 ratios revealed a significant interaction ($F(33, 96) = 7.1, p < 0.0001$) and row effect ($F(11, 96) = 18.2, p < 0.0001$), but no column effect ($F(3, 96) = 0.01, p = 0.9964$); Tukey's post hoc test found significant pairwise differences in Rows 3–6, including AAVR EV:AAV9 1:4 vs. 1:1 ($p = 0.0201$), 1:2 vs. 1:1 ($p = 0.0036$), 1:2 vs. 1:4 ($p = 0.0217$), and 1:4 vs. 1:1 (Row 5, $p < 0.0001$; Row 6, $p = 0.0009$) (C). One-way ANOVA on area under the curve values showed no significant differences among groups ($F(2, 6) = 2.06, p = 0.2084$) (D) Row in these analyses represent the fractions, and different serotypes were separated into columns.

To evaluate whether surface-loaded AAVs are protected from neutralizing antibodies *in vivo*, we first determined the optimal concentration of NABs. Briefly, mice were injected on Day 0 intraperitoneally with 0, 2, 4, 6 or 8 mg IgG, and the next day, mice were injected intravenously with AAV9 (at 1×10^{11} viral genome per mouse) carrying a Nanoluciferase transgene (Fig 4.2A). Two weeks later, organs were collected and analysed for the presence of the delivered transgene, Nanoluciferase, at the transcript level using qPCR (Fig 4.2B) and at the protein level using the Nano-Glo Luciferase Assay (Fig 4.2C). Transcript analysis showed highly variable expression (Fig. 4.2B). Notably, the liver consistently exhibited the highest Nanoluciferase transcript levels across IgG doses, and 2 mg was selected as the minimal dose that neutralized AAV9 (Fig. 4.2B). No significant expression was detected in lung, kidney, spleen, cerebellum, brainstem or cortex (Fig 4.2B). Protein-level luciferase activity (Fig. 4.2C) demonstrated the liver and heart showing higher Nanoluciferase activity in comparison to spleen, and significant suppression of luciferase signal in IgG treatment groups compared to PBS. No significant expression was detected in the lung, kidney, spleen, cerebellum, brainstem, or cortex (Fig. 4.2B–C).

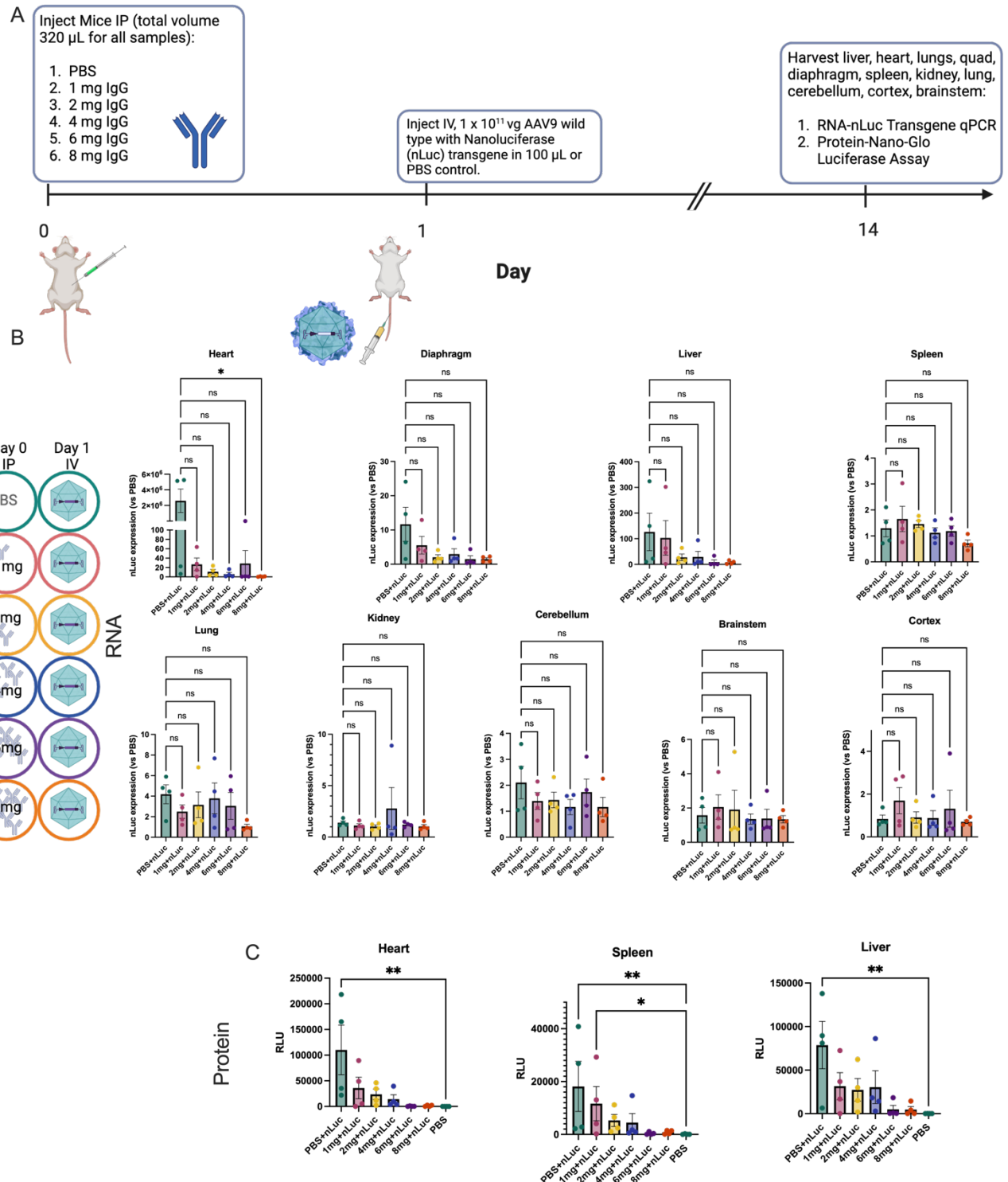


Figure 4.2 2mg IgG is sufficient to significantly neutralize a 1×10^{11} viral genome dose of AAV9 in vivo.

Mice were injected IP with PBS, 1 mg, 2 mg, 4 mg, 6 mg or 8 mg IgG. The next day, the mice received an injection of 1×10^{11} vg AAV9 carrying the Nanoluciferase transgene (nLuc). Organs were harvested on day 14 and analysed by qPCR and Nano-Glo Luciferase Assay (A). RNA was extracted and further analysed using qPCR to show significantly decreased transgene expression

in the presence of IgG (B). The Nano-Glo Luciferase Assay determined fluorescence per mg of tissue lysate (C). Mean $\pm$ SEM, N=4. Abbreviations: IgG=immunoglobulin, NanoLuc=Nanoluciferase, IP=intraperitoneal, IV=intravenous, vg=viral genome. Statistical Analysis: Normality was assessed using the Shapiro-Wilk test, which indicated non-normal distribution. Kruskal-Wallis test followed by Dunn's multiple comparisons was used, significance shown as *** p <0.001, ** p <0.01, * p <0.05 (B-C).

Next, a comparison was made between surface-loaded and lumenally-loaded EV delivery systems. Mice were injected on Day 0 intraperitoneally with 2mg IgG, and the next day, mice were injected intravenously with AAV, EV-AAV, AAVR-EVs or EVs plus AAVs, at a dose of 1×10^{11} viral genomes per mouse. Two weeks later, organs were harvested and analysed by qPCR and Nano-Glo Luciferase Assay to look for transgene expression at RNA and protein levels, respectively (Fig 4.3A). RNA expression levels are shown in comparison to the PBS only controls, and was evaluated in heart, diaphragm, pancreas and liver. In the heart, Nanoluciferase transcript expression was highly variable with no significant differences between groups (Fig 4.3B). In the Diaphragm Nanoluciferase expression of EV+AAV9 and EV-AAV9s injected animals were significantly above PBS controls, whereas AAV9 and AAVR-EV +AAV9 injected animals did not have significant expression (Fig 4.3B). In the pancreas EV-AAV9 injected animals had a 2.3 fold significantly higher Nanoluciferase expression in comparison to AAV9 injected animals, however treatment with EV-AAV9 with 2mg IgG had significantly decreased expression (Fig 4.3B). Nanoluciferase transcript levels in the liver were significantly higher in the EV +AAV9 injected animals (Fig 4.3B). The protein analysis (Fig 4.3C) showed a trend towards increased expression in EV-AAV9 injected animals in comparison to AAV9 only animals in the diaphragm, quadriceps, pancreas and liver. In the quadriceps and liver there is a trend towards similar mean fluorescence values between EV-AAV9 and EV-AAV9+2mg IgG conditions suggesting potential protection from NAb. No transduction was detected at the protein activity level with surface-loaded AAVR-EVs. The data also reveal that AAVs surface-

loaded onto AAVR-EVs were less effective in transducing tissues and protecting AAVs from NAbs. Overall, passively loaded EV-AAV9s demonstrated superior performance in both protection from NAbs and tissue transduction efficiency in comparison to surface-loading strategies. Empty EVs combined with free AAVs showed a trend toward better transduction than AAV alone in the heart and liver at the protein level (Fig4.3C). The low transduction efficiency of AAVR-EV-AAV9s *in vivo* led to the abandonment of surface-loading strategies, shifting the focus instead to engineering approaches aimed at increasing EV-AAV9 yields.

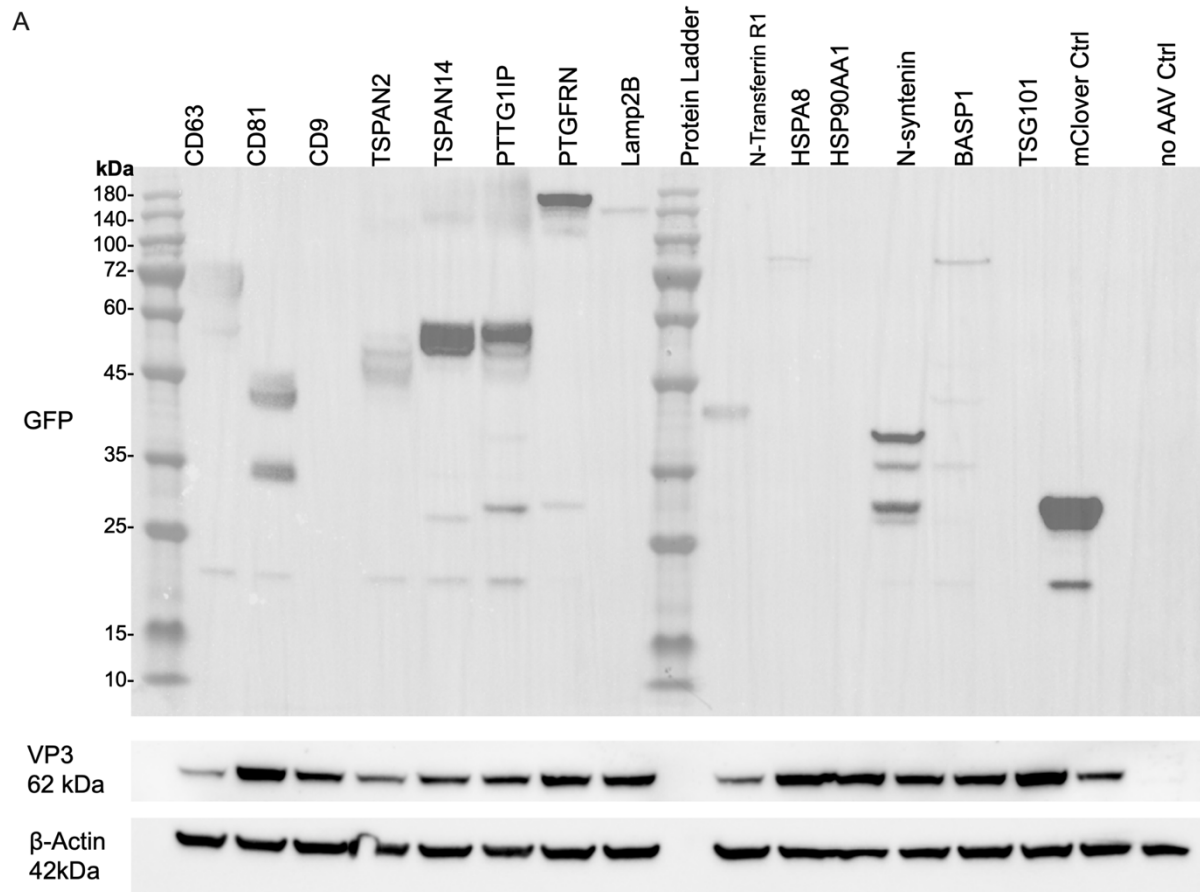
Figure 4.3 Luminally loaded EV-AAV9s show improved transduction and protection from NAbs in vivo.

On day 0 mice were IP injected with either PBS, or 2mg IgG, in a total volume of 320ul. Next day animal were IV injected with 1×10^{11} vg of either AAV9, AAV9 bound to the surface of AAVR-EVs, empty EVs + AAV9s or luminally loaded EV-AAV9s carrying Nanoluciferase (nLuc) transgene. Tissues were collected 2 weeks later (A). RNA expression of Nanoluciferase in different mouse tissues normalized to PBS only controls (B). Nanoluciferase protein activity evaluated by Nano-Glo Luciferase Assay (Promega) (C). All data shown as Mean $\pm$ SEM N=4. Statistical Analysis: Shapiro-Wilk test indicated data was not normally distributed. Brown-Forsythe test and Bartlett's test demonstrated unequal variance. Kruskal-Wallis test followed by Dunn's multiple comparisons was used, significance shown as *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

4.3.2 2B. Engineering the Producer Cells

It is known that proteins can be sorted into EVs based on interaction with EV-associated proteins. To investigate whether AAVs incorporate into EVs through interaction with common EV-resident proteins, EV-AAV9s were collected from HEK293T cells stably overexpressing common EV markers fused to mClover3. These EV proteins can be subdivided into three groups: tetraspanins, single pass transmembrane proteins and cytosolic proteins. A stable cell line expressing mClover3 only, was used as a control. EV-AAV9s were generated from HEK293T stable cell lines expressing mClover3 fusion proteins and isolated using SELC (see Methods section 2.2.4.3) across three independent experiments. Western blot using anti-GFP antibody was used to confirm the overexpression of the mClover fusion proteins (Fig 4.4) Expression levels of the fusion proteins varied across constructs in the cell lysate, potentially influencing the observed differences in EV-AAV9 yield if expression and yield are correlated. Notably, expression in the cell lysate did not consistently reflect incorporation into EVs. For instance, although TSPAN2 showed lower expression than TSPAN14 in the cell lysate (Fig. 4.4), both had comparable EV association, with 93.4% and 91.2% of EVs positive for TSPAN2 and TSPAN14, respectively (Fig. 4.5B). PTGFRN-mClover fusion overexpression resulted in a 15-20 nm increase in EV size, and although it was efficiently sorted into EVs with 91.1% EVs being GFP positive, it did not increase EV-AAV9 yield (Fig 4.5A-B, E). Overexpression of CD63- and PTTG1IP-

mClover3 fusions resulted in a 55- and 32-fold increase of EV secretion in comparison to mClover3 only controls (Fig 4.5C). Overexpression of CD63-, TSPAN2- and PTTG1IP-mClover3 fusions led to significantly higher EV-AAV9 yields (Fig 4.5E) and a higher number of protected AAV genomes, identifying new targets for engineering the EVs to increase association with AAV9s (Fig 4.5F). Overexpression of CD63-mClover fusion resulted in an approximately 10-fold increase in EV-AAV9 yield and a 5-fold increase in protected genomes (Fig 4.5EF). Based on this data, CD63 was selected as a potential candidate to improve EV-AAV9 yields



B

	Molecular Weight (kDa)	mClover3 fusion (kDa)
CD63	26.00	52.80
CD81	26.00	52.80
CD9	24.00	50.80
TSPAN2	24.00	50.80
TSPAN14	30.00	56.80
PTTG1IP	22.00	48.80
PTGFRN	99.00	125.80
Lamp2B	45.00	71.80
N-TransferrinR1	11.00	37.80
HSPA8	70.00	96.80
HSP90AA1	90.00	116.80
N-syntenin	6.65	33.45
BASP1	23.00	49.80
TSG101	44.00	70.80
mClover Ctrl	26.80	
no AAV Ctrl		

Figure 4.4 Western blot confirming overexpression of mClover3 fusions.

Anti-GFP, anti-VP1-3, and anti-β-actin Western Blot of cell lysates collected from stable cell lines transfected to produce AAVs. Protein concentrations were detected by BCA, and 40 µg of lysate was loaded (A). The expected molecular weight of mClover3 fusions (B).

Constitutive overexpression can result in genomic instability or epigenetic modifications that downregulate transgene expression over time. To mitigate these effects, inducible expression systems are often employed, allowing for controlled protein production and minimizing cellular stress²⁴⁸. A doxycycline-inducible HEK293T stable cell line was generated, and overexpression of CD63 was confirmed by western blot (Fig 4.6A). This cell line uses the Tet-On 3G doxycycline-inducible system, in which gene expression, in this case CD63 expression, is activated in the presence of doxycycline via a reverse tetracycline-controlled transactivator (rtTA). In this system when doxycycline is added it binds to rtTA and causes a conformational change, and rtTA can now bind the tetracycline response element in the promoter and activates gene expression. This allows for tight temporal control of CD63 overexpression, preventing constitutive expression during cell expansion and enabling induction only during EV-AAV production²⁴⁹. The results demonstrate that HEK293T cells overexpressing CD63 produced 6-10 times higher yield of EV-AAV9 particles with a significantly increased proportion of luminally loaded AAV9 (Fig 4.6B). EV-AAV9s isolated from this CD63-inducible overexpression cell line will be referred to as CD63EV-AAV9s for the remainder of the text. The engineered cell line did not alter the percentage of empty to full capsids, which was approximately 30% for both, EV-AAV9 and CD63EV-AAV9 (Fig 4.6C), and resulted in ~40% protected viral particles compared to ~10% for EV-AAV9 (Fig 4.6D). Magnetic pull-down experiments confirmed that 70% of the viral genomes were protected in CD63EV-AAV9 particles compared to ~10% seen in EV-AAV9, as verified by qPCR of the transgene (Fig 4.6E).

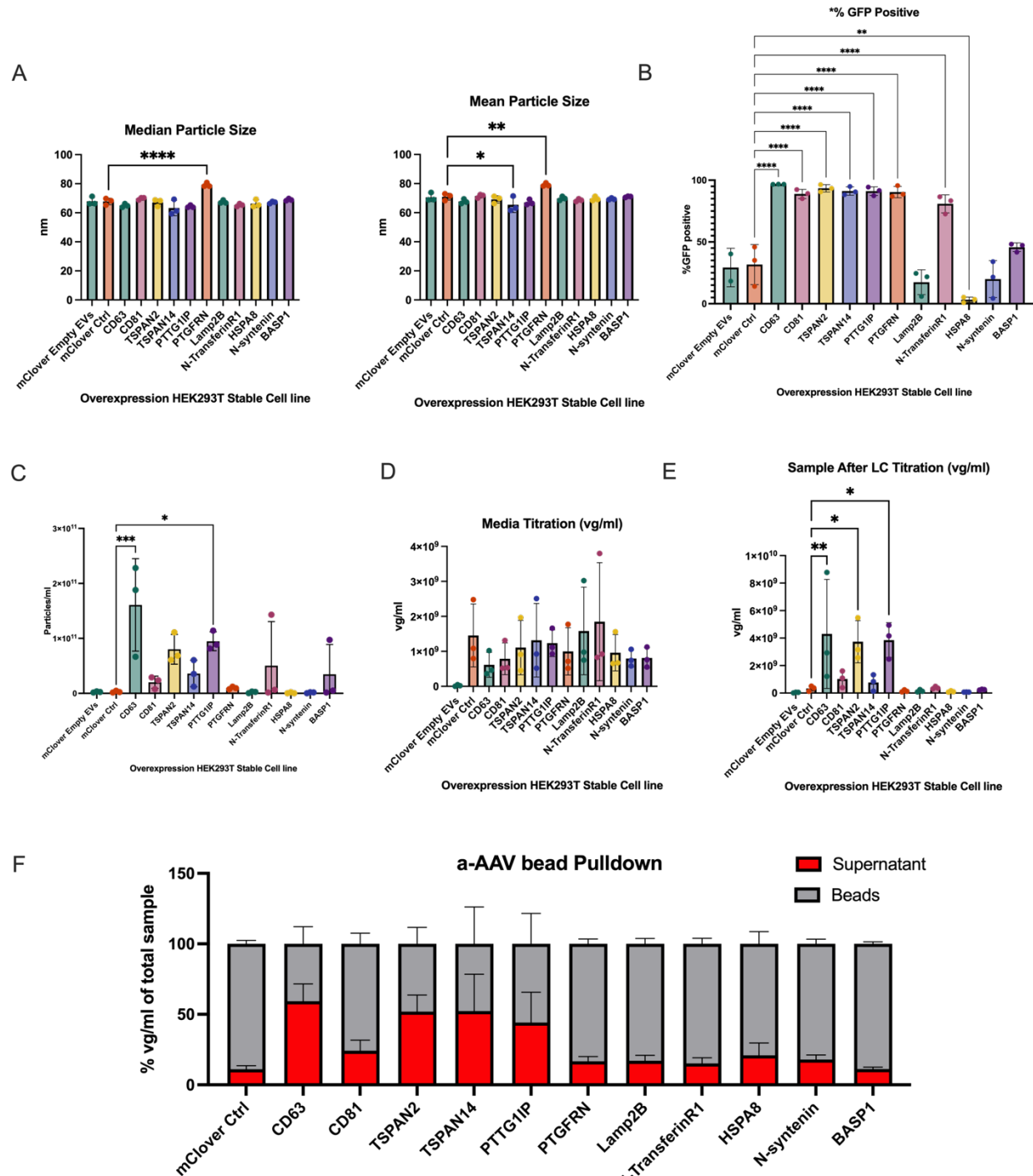


Figure 4.5 Overexpression of CD63, TSPAN2 and PTTG1IP leads to higher EV-AAV9 yields.

Particle size (nm) as measured by the NanoFCM (A). Percentage of particles positive for EV-marker-mClover based on green fluorescence measured by NanoFCM (B). EV particle concentration (particles/ml) of purified EV samples from the EV-marker-mClover overexpression cell lines (C). qPCR titration of viral genomes in the media (D). qPCR titration of EVs purified by size exclusion liquid chromatography (E). Anti-AAV9 pulldown was performed to determine percentages of protected genomes (EV-AAVs) (F). Mean+SEM, N=3 Statistical Analysis:

Ordinary one-way ANOVA was used to compare 13 experimental conditions. Homogeneity of variance was confirmed using Brown-Forsythe test ($p > 0.05$ for all), validating the use of parametric tests. Dunnett's multiple comparisons test was performed post hoc, comparing all groups to the mClover3 control. Significant differences were observed in median diameter ($F(12, 26) = 9.419$, $p < 0.0001$) and mean diameter ($F(12, 26) = 6.512$, $p < 0.0001$) (A), particle concentration ($F(12, 26) = 4.975$, $p = 0.0003$) (B), and % GFP-positive cells ($F(12, 25) = 54.95$, $p < 0.0001$) (C). No significant differences were found in media titration ($F(12, 26) = 1.035$, $p = 0.4484$) (D), while qPCR-based genome quantification showed a significant effect ($F(12, 26) = 5.014$, $p = 0.0003$) (E). Unequal variance and no significant difference in pulldowns (F).*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

To confirm the in vitro efficacy of CD63EV-AAV9s, SH-SY5Y-TL cells—human neuroblastoma cells engineered to express a red fluorescent protein in the absence of Cre recombinase—were plated in a 384-well plate and treated the following day with PBS, AAV9, or CD63EV-AAV9s. These AAVs were prepared using a stable HEK293 cell line producing EVs harbouring AAV9-CMV-Cre, which expresses Cre recombinase under the control of the cytomegalovirus (CMV) promoter. Upon delivery of Cre to the target cells, the red fluorescent protein is excised and replaced with GFP via Cre-mediated recombination, allowing quantification of transduction efficiency using the Operetta CLS High Content Analysis system. CD63EV-AAV9 treatment resulted in improved transduction in SH-SY5Y-TL cells compared to standard EV-AAV9s, although the difference was not statistically significant. Transduction was assessed by measuring changes in GFP expression and quantified using the area under the curve to account for both transduction efficiency and kinetics (Fig 4.7). To compare transduction in vivo EV-AAV9s and CD63EV-AAV9s carrying Nanoluciferase transgene were intravenously injected at 1×10^{11} vg/mouse. Tissues were collected two weeks later and Nanoluciferase protein activity were evaluated using Nano-Glo Luciferase assay. Mice dosed with CD63EV-AAV9s showed an average 4.7, 1.2- and 0.95-times lower transduction in the heart, liver and diaphragm respectively, compared to those treated with standard EV-AAV9s, but these differences were not statistically significant (Fig. 4.8B). Despite the comparable or lower transduction efficiency,

CD63EV-AAV9s were still advantageous for in vivo use due to their ~10-fold increased yield compared to EV-AAV9s and ~100-fold higher total EV production (Fig. 4.6B–C). This increased yield enabled 10-fold higher dosing compared to standard EV-AAV9s, allowing administration of a clinically relevant dose of 1×10^{12} viral genomes per mouse.

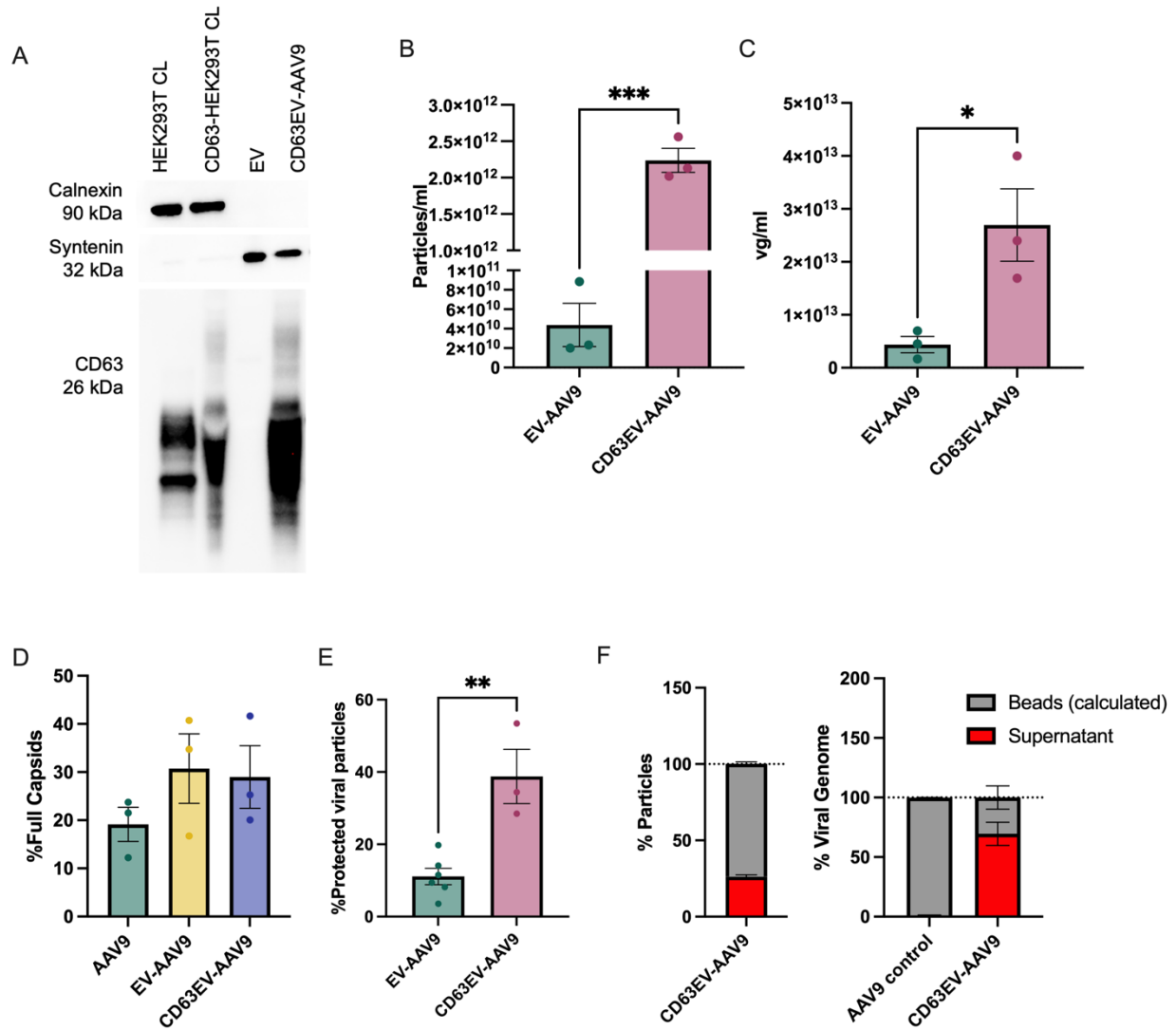
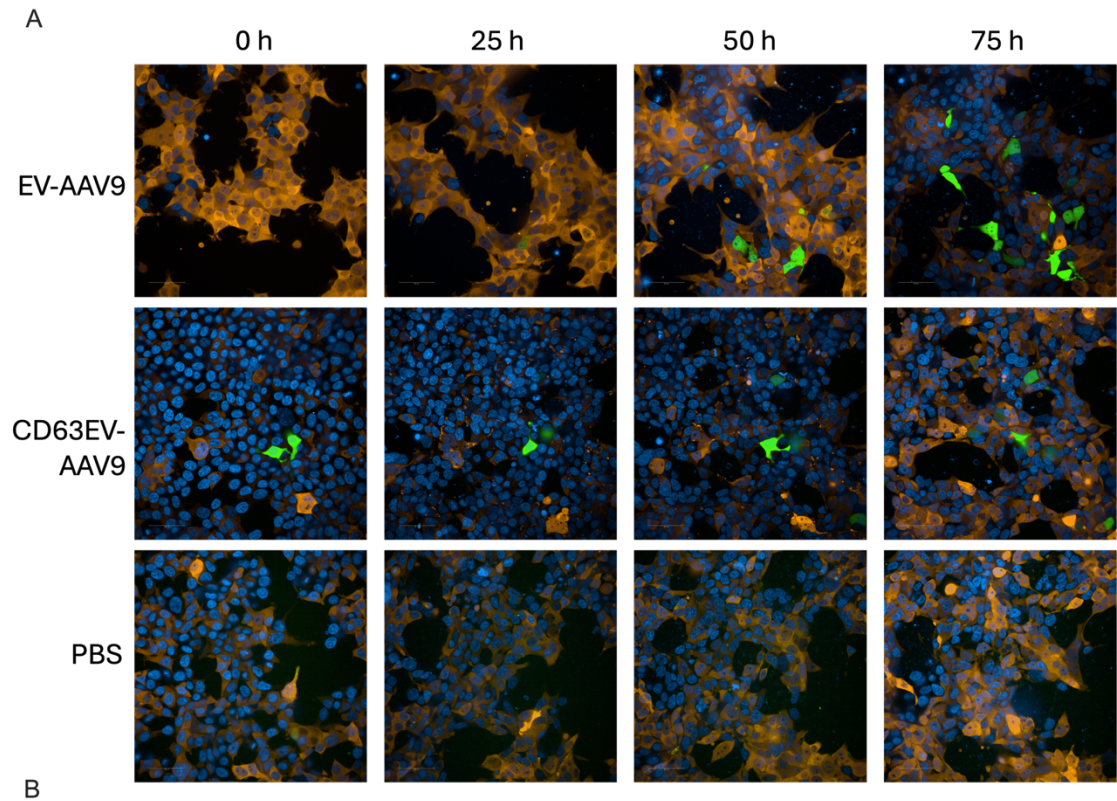


Figure 4.6 CD63 overexpression increased EV-AAV9 yield and luminal AAV9 loading.

Western blot was performed on 20 μ g of cell lysates or 1×10^{10} particles (as measured by NanoFCM) collected from WT HEK293T cells or doxycycline-inducible CD63 overexpression cells (A). EVs were isolated using an iodixanol gradient, concentration was measured using NanoFCM (B), and qPCR of transgene was used to determine vg/mL (C). Empty vs Full capsid ratios were analysed by MSD immunoassay (D). Virus particle concentration before and after treatment with Triton-X-100 (E). Anti-AAV9 magnetic pulldown to assess protected viral

genomes as detected by qPCR of the transgene(F). Mean $\pm$ SEM, N=3, unless otherwise specified. Statistical Analysis: Variance was significantly different between groups as assessed by an F-test ($F(2,2) = 54.65$, $p = 0.0359$), justifying the use of Welch's t-test ($p = 0.0050$, two-tailed) (B). Variance was not significantly different between groups: F-test ($F(2,2) = 19.87$, $p = 0.0959$), supporting the use of a standard unpaired t-test, $p = 0.0321$ ($t = 3.226$, $df = 4$) (C). One-way ANOVA found no significance ($F(4,10) = 1.409$, $p = 0.2997$) (D). F-test was not significant ($p = 0.1075$), Welsh's t-test ($p = 0.0023$, two-tailed). Sample sizes were N=6 for EV-AAV9 and N=3 for CD63EV-AAV9 (E). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. CL=Cell Lysate.



Transduction Efficiency SH-SY5Y-TL cells

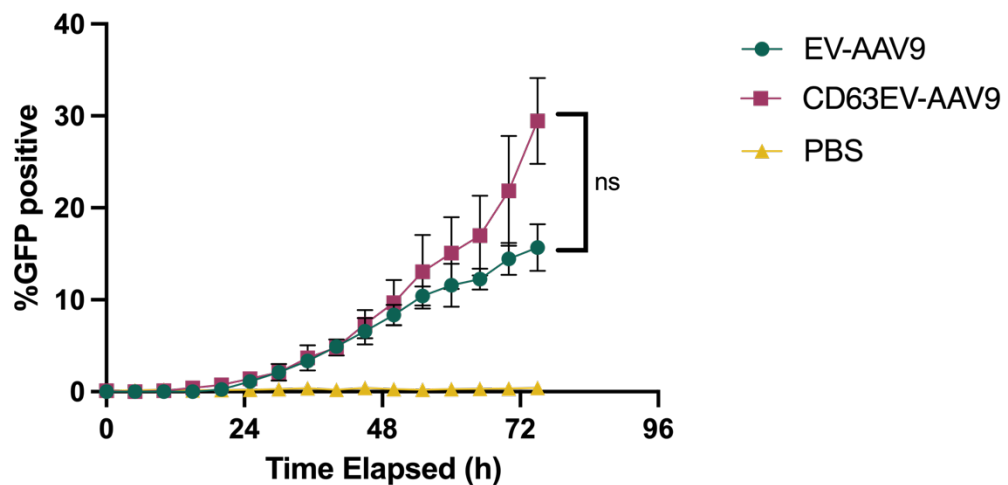


Figure 4.7 CD63EV-AAV9s show improved in vitro transduction in a neuronal cell line.

SH-SY5Y traffic light cells were seeded in a 384-well plate, and the next day the cells were treated with EV-AAV9, CD63EV-AAV9, or PBS, then placed into the Operetta for three days. Images (A) were analysed using Operetta software to determine the percentage of GFP-positive cells for each time point. The data shown is from three biological replicates (B). Mean $\pm$ SEM, N = 3. Statistical analysis: The area under the curve was calculated in GraphPad Prism for all conditions, and significance was evaluated using an unpaired t-test.

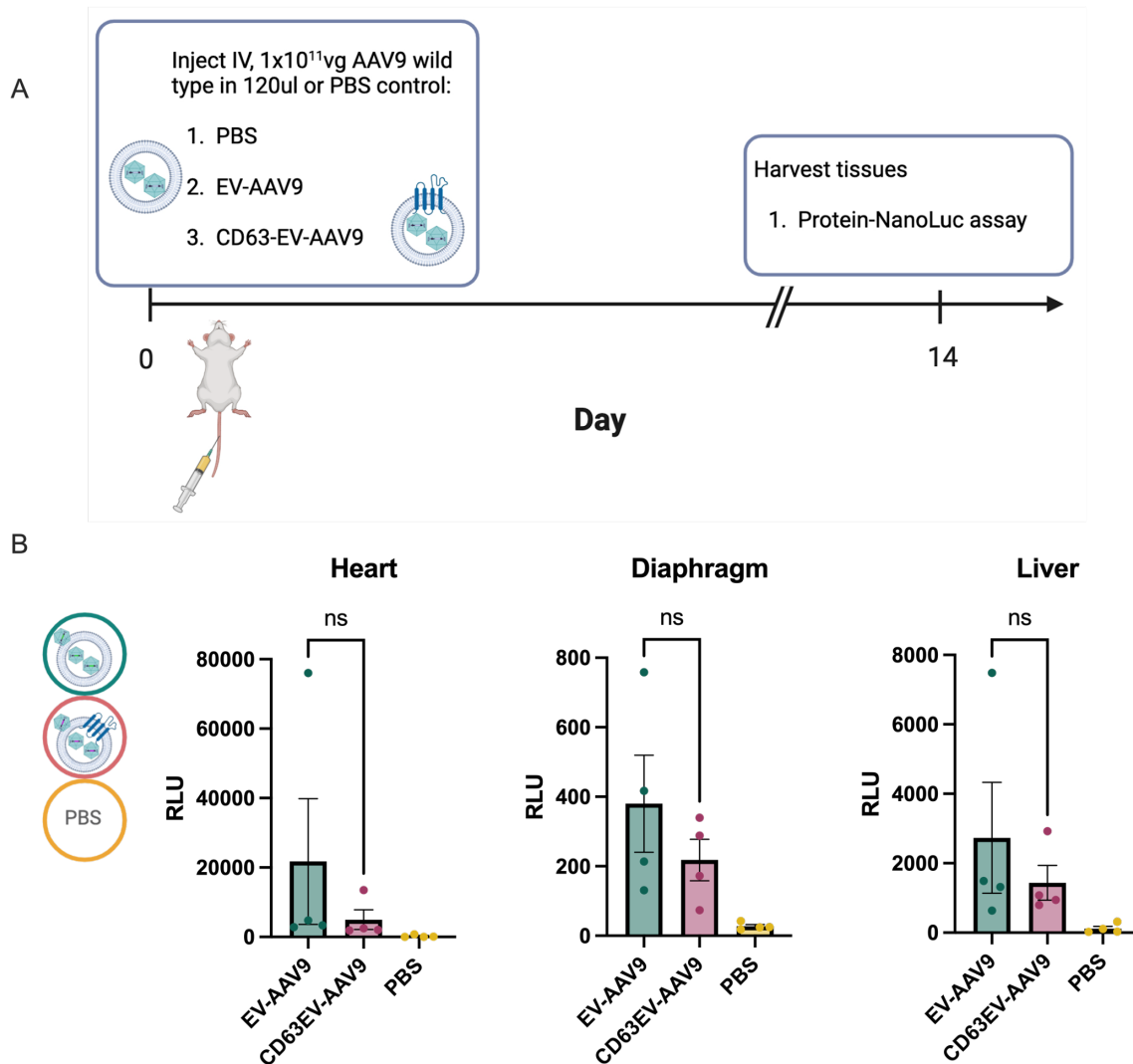


Figure 4.8 No significant difference between EV-AAV9 and CD63EV-AAV9 transduction in vivo.

Schematic of study method, 2 male and 2 female mice were used for each condition. Mice were intravenously injected with either 1×10^{11} vg EV-AAV9, 1×10^{11} vg CD63EV-AAV9 or PBS. Tissues were collected 14 days post injection (A). Nanoluciferase protein expression was evaluated using Nano-Glo Luciferase Assay (Promega), where fluorescence detected is proportional to protein expression, and fluorescence was normalized per mg of tissue (B). Data is shown as Mean $\pm$ SEM, N=4. Statistical Analysis: Due to unequal variances nonparametric t-test, Mann-Whitney test was used to compare EV-AAV9 and CD63EV-AAV9 injected animals, no significance was found (B).

One of the theoretical advantages of using EV-AAVs instead of AAVs is the potential for repeated dosing because the EV membrane may shield the AAVs from NAb, reducing immunogenicity and allowing for multiple administrations without eliciting a strong immune response. Having increased the dose to 1×10^{12} viral genomes per mouse, a repeated dosing study

was designed to investigate this. On day 1, mice were injected with AVV9 or CD63EV-AAV9s carrying a Nanoluciferase-eGFP transgene; on day 14, mice were injected with PBS, AAV9 or CD63EV-AAV9 carrying a Nanoluciferase-mScarlet transgene. Mouse serum was collected at 2 weeks and 4 weeks. In this system, qPCR can be used to determine expression from the first and second dose, which can be distinguished by examining the expression of eGFP and mScarlet, respectively, whereas total expression can be observed by looking at Nanoluciferase expression (Fig 4.9A). This study aimed to assess the increase in transduction achieved through repeated dosing of AAV and EV-AAVs and to evaluate the levels of NAb generated in response to the first and second doses. To evaluate tissue-specific transgene expression following different delivery strategies, NanoLuc, eGFP, and mScarlet expression in heart and liver tissue was quantified by RT-qPCR and normalized to PBS controls. Kruskal–Wallis tests revealed significant differences in median expression across treatment groups for heart NanoLuc, heart eGFP, liver NanoLuc, and liver eGFP, whereas no significant differences were observed for mScarlet in either tissue. In the heart, NanoLuc expression was highest in the AAV-AAV group, which was the only condition to show a significant expression above uninjected control. Heart eGFP expression was highest in the AAV-EV group and was the only group significantly different from uninjected controls, with levels 325-fold higher than those in the EV-AAV9 single-dose group. In the liver, NanoLuc expression was highest in the AAV single-dose group, which was significantly higher than both uninjected controls and the EV-AAV9 group (31-fold higher). Similarly, liver eGFP expression was highest in the AAV single-dose group, and this was the only group significantly different from uninjected controls (Fig. 4.9B). Across both organs, all AAV groups showed higher expression of transgenes in comparison to EV-AAV9 groups, although these changes were not all significant. mScarlet expression was low and not

statistically different among groups, suggesting poor delivery of the second dose regardless of treatment type (Fig 4.9B). Serum taken from the mice was analysed for anti-AAV9 mouse IgG concentrations to evaluate levels of NAb present after the second dose, and at the end of the study. NAb levels were significantly higher at 4 weeks compared to 2 weeks in the AAV single-dose, AAV-AAV, and AAV-EV, but not the EV single dose or EV-EV treatment groups (Fig 4.9C). Improved yields of CD63EV-AAV9s and decreased anti-AAV9 mouse IgG after repeated treatments is an advantage for a potential gene therapy, however, the transduction *in vivo* remains a challenge.

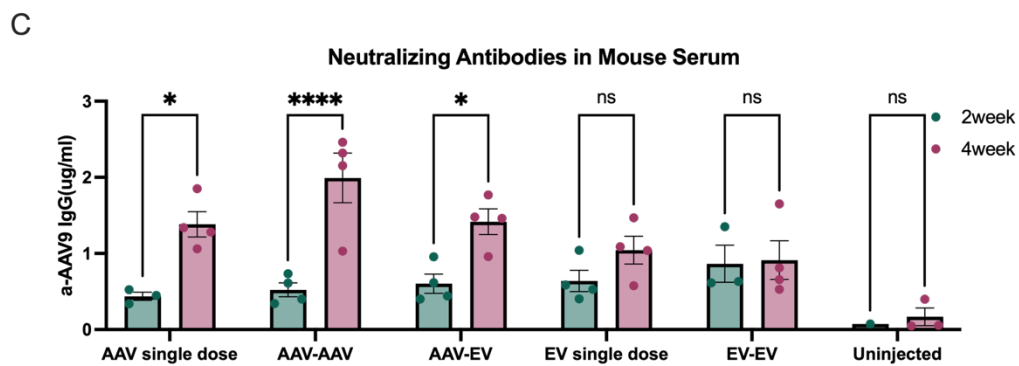
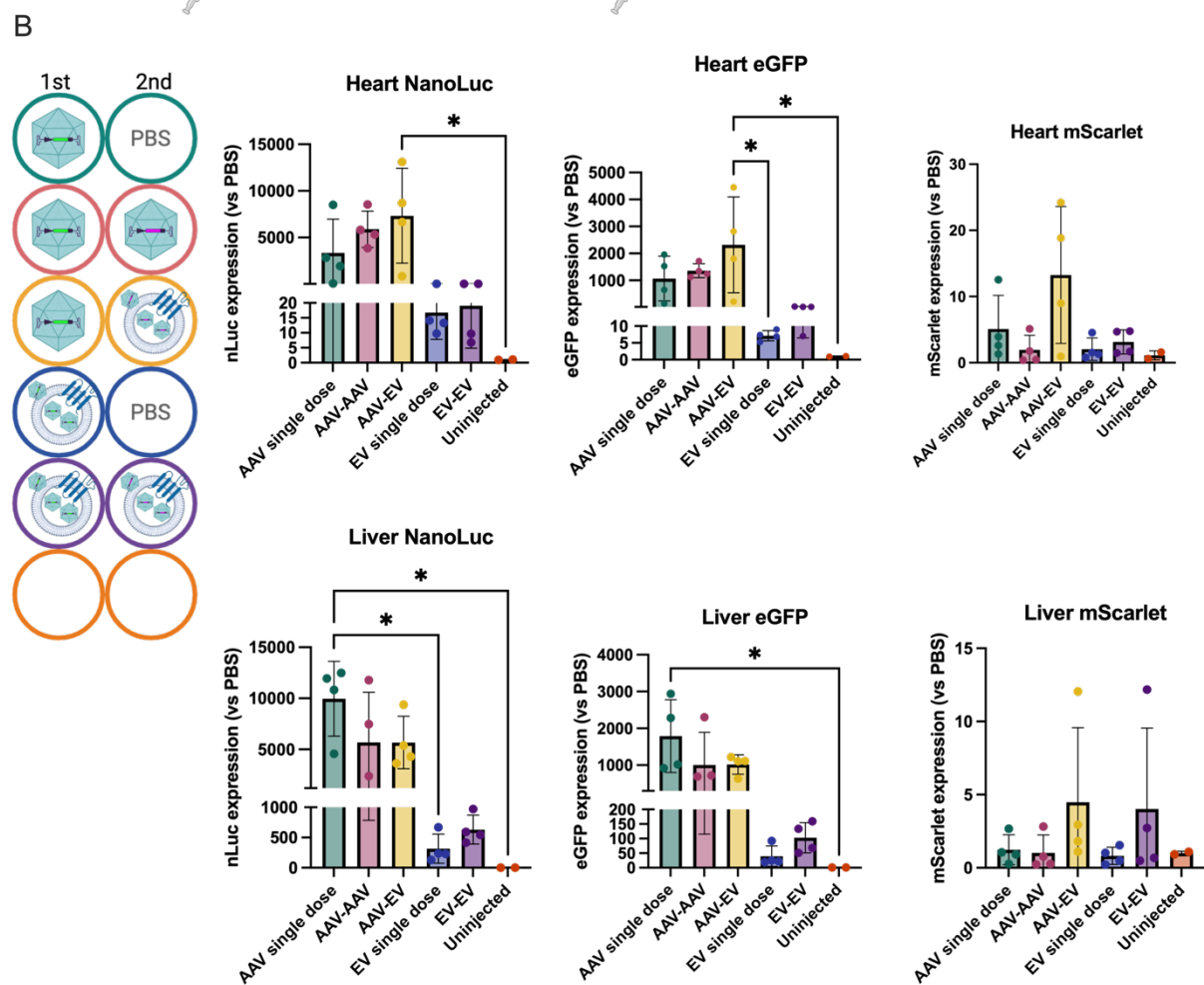
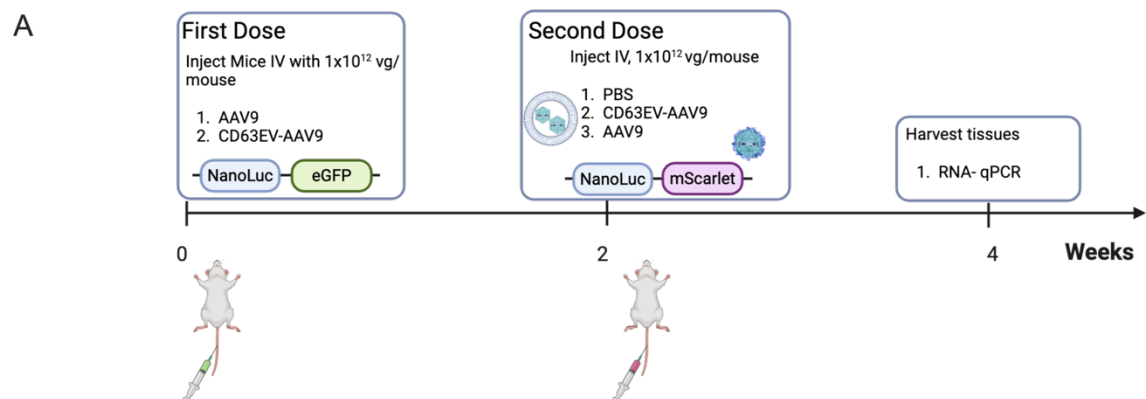


Figure 4.9 Repeated dosing with CD63EV-AAV9, did not increase transduction in the heart, whereas combined treatment with AAV9 then CD63EV-AAV9s showed improved transgene expression.

Schematic of the study method: four female mice were used for each condition. On Day 0 mice were injected with AAV9 or CD63EV-AAV9 carrying the Nanoluc-eGFP transgene. Then, at two weeks, the mice were injected with either PBS or AAV9 or CD63EV-AAV9 carrying the Nanoluc-mScarlet transgene. Tissues were collected four weeks after the first injection (A). RNA was extracted, and qPCR was used to determine transcript expression of eGFP, mScarlet, and Nanoluc transgenes (B). Serum samples were collected at 2 and 4 weeks; neutralizing antibodies were detected using the MSD immunoassay to detect anti-AAV9 mouse IgG (C). Data is shown as Mean $\pm$ SD, N=4. Statistical Analysis: Kruskai-Wallis test Heart: NanoLuc($H = 17.41$, $p = 0.0038$), eGFP ($H = 17.61$, $p = 0.0035$), mScarlet($H = 6.249$, $p = 0.2827$). Liver NanoLuc($H = 17.98$, $p = 0.0030$), eGFP($H = 17.61$, $p = 0.0035$), mScarlet($H = 6.267$, $p = 0.2811$) Dunn's multiple comparisons test was used as a post-hoc test (B). A two-way ANOVA revealed significant effects of both timepoint (2-week vs 4-week) and treatment group on neutralizing antibody (NAb) responses (interaction: $p = 0.0106$; timepoint: $p < 0.0001$; treatment: $p = 0.0045$), with Šidák's post hoc test showing that NAb levels were significantly higher at 4 weeks in AAV single-dose, AAV-AAV, and AAV-EV groups compared to their 2-week counterparts(C). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

4.3.3 2C. Engineering the Virus

Engineering AAVs is challenging due to their compact 4.7 kb genome with overlapping reading frames, where large modifications often lead to low titers or failed capsid assembly²⁵⁰. However, directly modifying AAVs could enhance their incorporation into EVs. Interestingly, other non-enveloped viruses exploit EVs to evade immune detection. For example, the pX domain is an 8 kDa carboxy-terminal extension on VP1 that is unique to hepatitis A virus (HAV) among picornaviruses, and this domain allows this virus to hijack exosomes²¹⁰. Therefore, we hypothesized that adding a pX domain to the N-terminus of VP2 could take advantage of this pathway. Western blot analysis confirmed the successful modification of VP2 with the pX motif (Fig 4.10A). qPCR titration of the transgene in iodixanol fractions revealed ~2-fold higher percentage of total secreted viral genomes in EV fractions 4-6 for pXAAV9 compared to AAV9 controls (Fig 4.10B). Furthermore, anti-AAV9 pulldown assays demonstrated that EV-pXAAV9 particles were significantly enriched in the supernatant

compared to EV-AAV9 (mean = 59.00 vs. 27.60; $p = 0.000839$, $q = 0.000847$) suggesting an increase in protected genomes in EV-pXAAV9 relative to EV-AAV9 (Fig 4.10C). The analysis of empty versus full capsid ratios using an MSD immunoassay indicated no difference between EV-AAV9 and EV-pXAAV9 ratios and no difference in percentage protected capsids (Fig 4.10D-E). It has been shown that a truncated version of pX which contains the Alix interacting domains is able to efficiently load GFP into EVs²¹⁰. AAV9 with either the full length or truncated version of pX were generated, and efficiency of loading into EVs was evaluated by qPCR of the transgene in the iodixanol fractions. The full length, but not the truncated version, showed significantly higher AAV9 loading into EVs compared to AAV9 wildtype, evidenced by higher % of total secreted viral genomes in fractions 4-6 (Fig 4.10F).

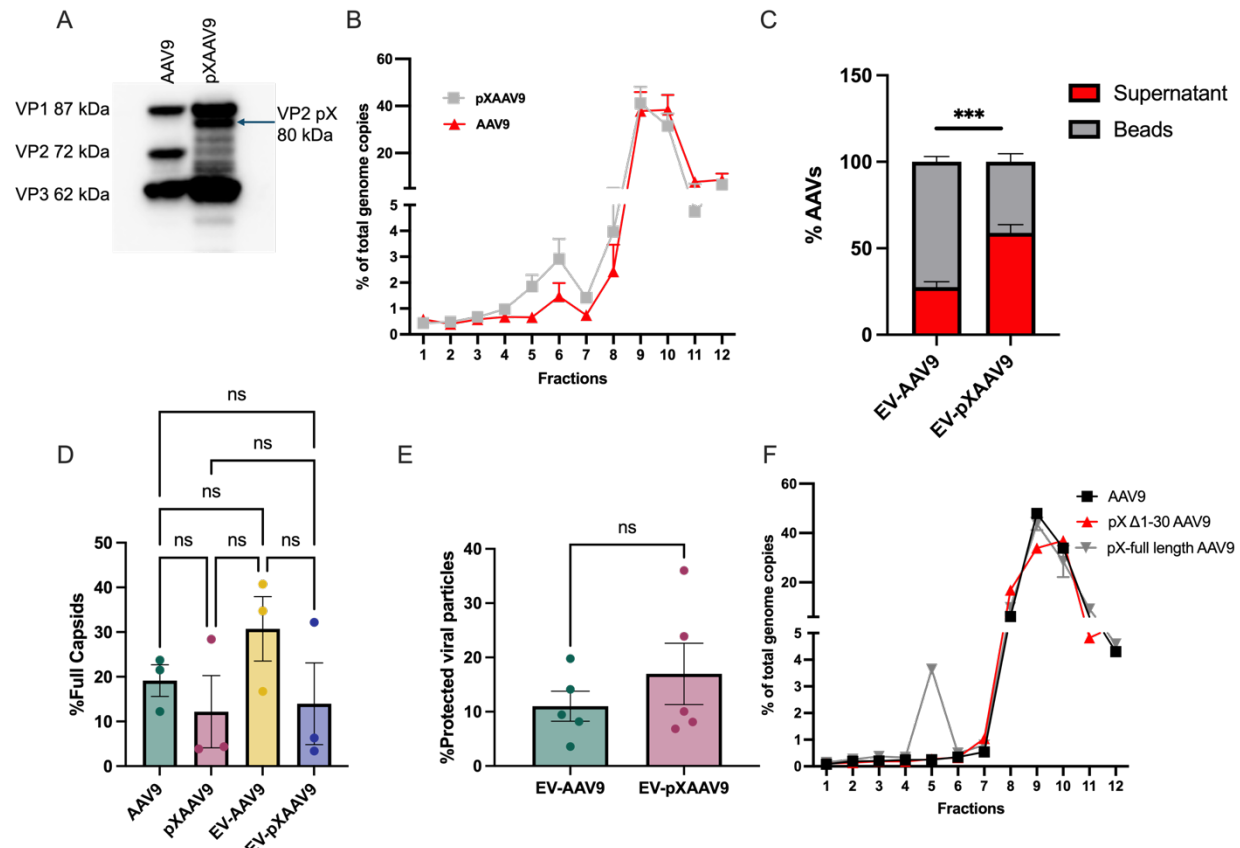


Figure 4.10 AAV9 capsids engineered with pX increase the loading of AAV9 into EVs.

Confirmation of VP2 modification with the pX motif via Western blot (A). qPCR titration of the transgene in iodixanol fractions of AAV9 and pX-AAV9 (N=3) (B). Anti-AAV9 pulldown to determine the percentage of viral genomes which are on the surface of EVs (supernatant) or protected inside the EV(beads) (N=5) (C). Empty vs full capsid ratios as analysed by MSD assay (N=3) (D). Analysis of virus particle concentration before and after treatment with Triton-X-100 demonstrates percentages of protected virus particles of EV-AAV9 and EV-pXAAV9 (N=5) (E). qPCR titration of the transgene in iodixanol fractions of AAV9 and pXAAV9-full length and truncated version; pX Δ 1-30 (N=3) (F). All values were presented as Mean+SEM. Statistical Analysis: Two-ANOVA analysis found no significance, N=5(B). Unpaired t-tests with Welch's correction were used to compare particle levels between EV-AAV9 and E-pXAAV9 in supernatant and bead-bound fractions. A false discovery rate (FDR) correction was applied using the two-stage step-up method of Benjamini, Krieger, and Yekutieli ($Q = 1\%$). E-pXAAV9 particles were significantly enriched in the supernatant compared to EV-AAV9 (mean = 59.00 vs. 27.60; $p = 0.000839$, $q = 0.000847$), whereas EV-AAV9 particles were significantly more abundant in the bead-associated fraction than E-pXAAV9 (mean = 72.40 vs. 41.00; $p = 0.000839$, $q = 0.000847$), N=5 (C). A one-way ANOVA revealed no significant differences in the percentage of full capsids across the five groups ($F(4,10) = 1.409$, $p = 0.2997$), with equal variances confirmed by Brown-Forsythe (D). A Mann-Whitney test revealed no significant difference in the percentage of protected genomes following Triton X treatment between EV-AAV9 and EV-pXAAV9 groups ($p = 0.6905$) (E). Two-way repeated measures ANOVA showed significant interaction between vector type and fraction ($F(22, 66) = 5.718$, $p < 0.0001$), indicating that vector distribution across fractions was dependent on the AAV construct used. Dunnett's post hoc test showed that pX full-length AAV9 differed significantly from AAV9 in fraction 5, while pX Δ 1-30 AAV9 showed no significant difference, N=3 (F).

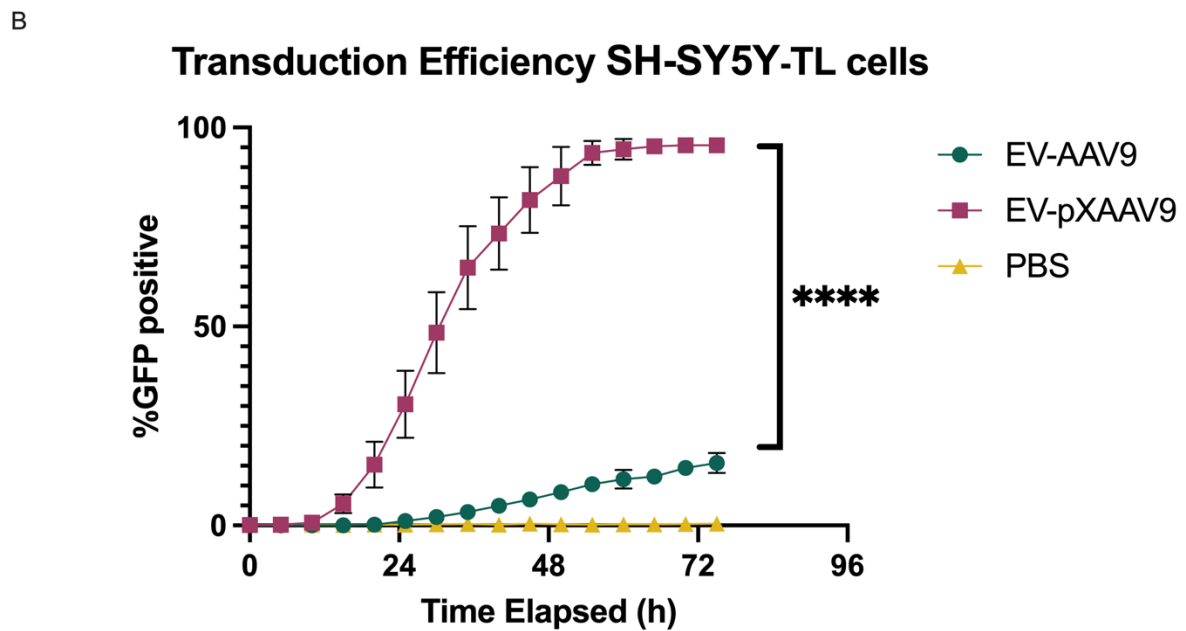
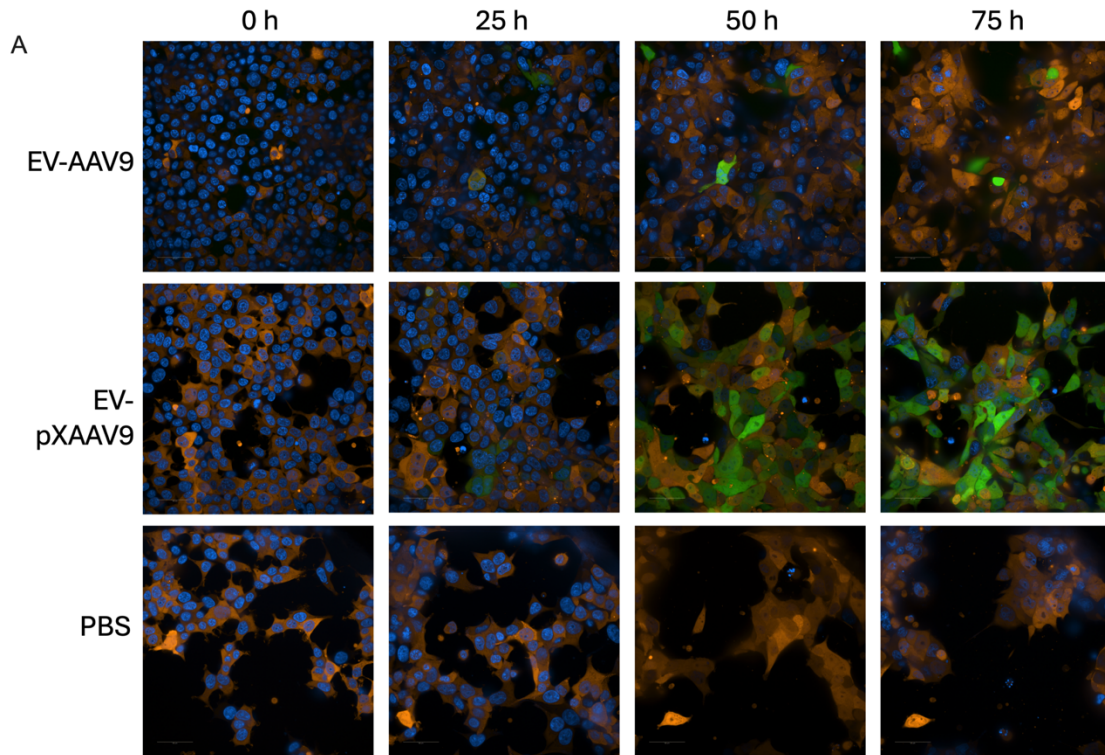


Figure 4.11 EV-pXAAV9s show improved *in vitro* transduction in a neuronal cell line. SH-SY5Y traffic light cells were seeded in a 384 well plate, and the next day, cells were treated with EV-AAV9, pX-EV-AAV9 or PBS and placed into Operetta for 3 days. Images (A) were analysed using Operetta software to determine %GFP positive cells. The data shown are from three biological replicates (B). Data are presented as mean $\pm$ SEM, N = 3. Statistical analysis: GraphPad

Prism was used to calculate the area under the curves for each condition, and significance between conditions was evaluated using an unpaired t-test. **** $p < 0.0001$.

To evaluate the *in vitro* efficacy of EV-pXAAV9s, SH-SY5Y-TL cells were plated in a 384-well plate and treated the following day with PBS, EV-AAV9 or EV-pXAAV9s carrying the *cre* transgene, at a multiplicity of infection (MOI) of 1×10^5 . Concentrations were verified by qPCR and ddPCR. Delivery of the *cre* transgene to target cells resulted in GFP expression, which was subsequently detected using the Operetta CLS High-Content Analysis System. EV-pXAAV9s performed significantly better than wildtype EV-AAV9 *in vitro*, with 13-21% of cells being GFP positive in the EV-AAV9 condition and 94-97% in the EV-pXAAV9 condition (Fig 4.11). Encouraged by the *in vitro* results, EV-pXAAV9 and EV-AAV9, with AAV9 controls carrying the Nanoluciferase transgene were injected intravenously into mice. Mice were sacrificed 2 weeks later and transduction was evaluated using Nano-Glo Luciferase Assay. EV-pXAAV9 performed similarly to wildtype EV-AAV9, with both conditions performing significantly worse than AAV9 (Fig 4.12). This difference was approximately 100-fold in the liver, and approximately 10-fold in the other tissues evaluated (heart, quadriceps and diaphragm) (Fig 4.12B).

Schematic of study method: 4 female mice were used for each condition, and mice were injected with either PBS, AAV9, pXAAV9, EV-AAV9 or EV-pXAAV9 at 1×10^{11} vg per mouse (A). Tissues were collected 14 days post-injection (A). Nanoluciferase protein expression was evaluated using Nano-Glo Luciferase Assay (B). Data shown as Mean $\pm$ SD. Statistical Analysis: Data were log-transformed and analyzed using ordinary one-way ANOVA, which revealed a significant difference among treatment groups in Liver ($F(4,12) = 66.54$, $p < 0.0001$), Heart($F(4,12) = 13.86$, $p = 0.0002$), Quadriceps($F(4,12) = 31.76$, $p < 0.0001$), and Diaphragm($F(4,12) = 28.50$, $p < 0.0001$)(B). Tukey's multiple comparisons test was used, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

An alternative to inserting exogenous motifs into the AAV9 capsid, is to examine the AAV9 capsid itself, for motifs that could affect viral egress. Given that enveloped viruses utilize L domains to exploit the ESCRT machinery for incorporation into extracellular vesicles¹⁶⁰, we examined the AAV9 capsid for similar motifs. We identified five L domains-four YXXL motifs and one LXXL motif (Fig 4.13A). Mutation of the LXXL motif to AXXA resulted in no detectable virus made (no viral genomes were detected in cell lysate and media), indicating that the motif may be important for capsid structure (Fig 4.13B). Interestingly, mutation of YXXL motifs to AXXL resulted in significantly increased AAV9 associated per EV (Fig 4.13C), however there was no significant increase in overall yield (Fig 4.13B).

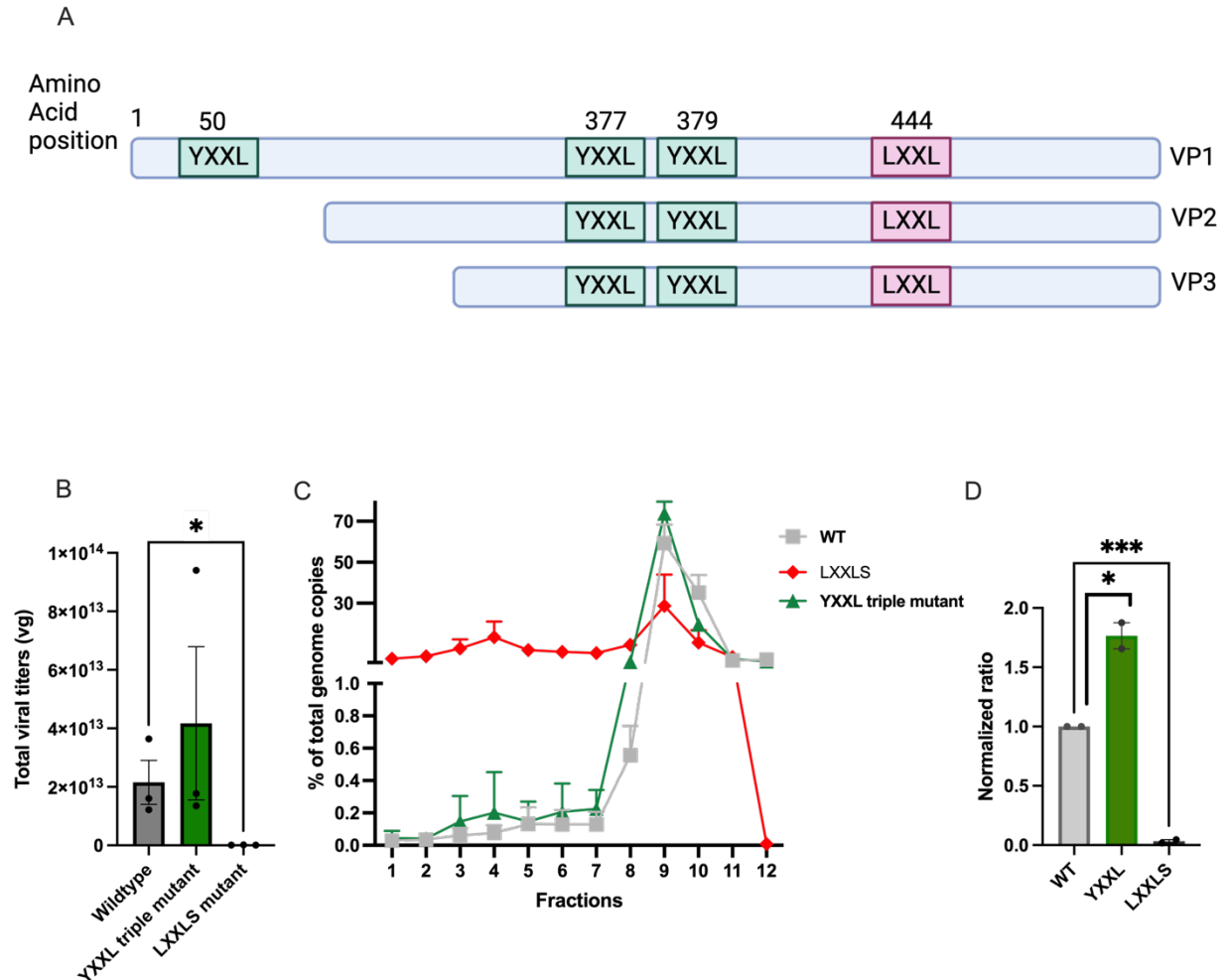


Figure 4.13 Mutation of the YXXL motif results in increased EV-AAV9 yields, while mutation of LXXLS leads to low AAV9 titers.

Schematic of LXXLS motif and YXXL motifs labelled, especially highlighting the motifs in the capsid which were mutated for this study(A). Total viral yields of wildtype controls, and LXXLS and YXXL mutant AAV productions (B). qPCR of transgene in iodixanol fractions of wildtype, YXXL mutant and YXXL mutant (C). Mean $\pm$ SEM, N=3. Statistical Analysis: 2-way ANOVA, Bonferroni posthoc test, no significance. Two-fold increase of AAV loading into extracellular vesicles was calculated using qPCR of transgene to determine viral genomes/ml and NanoFCM analysis for particle concentration(N=4) (D). ***P<0.001, **P<0.01, *P<0.05.

Engineering AAVs with L domains could leverage their ability to serve as cellular adaptors for enveloped viruses to engage with ESCRT machinery and potentially enhance AAV incorporation into EVs. Which L domain, linker length, and determining its position on the AAV capsid may be important. The AAV capsid surface has two protruding loops. One of these loops, VR-IV on the AAV9 capsid, was selected as the site for insertion. To identify the most effective

L domain and determine optimal positioning of the domain, a randomized library was designed, where the late domain motifs (along with one random sequence of the same length) are surrounded by linkers of either 2, 4, or 8 residues. To avoid introducing new stop codons, oligos were designed with NNK codons where N is any nucleotide, and K is any nucleotide but adenosine. The rationale for various linker lengths is to vary the level of protrusion of the loop and thus potentially the accessibility of the motif for binding, knowing that inserting too long of a linker can result in lower viral titers. These oligos were then cloned into the VR-IV region of the AAV9 capsid protein (residue 453 using VP1 numbering). For this library screen, the rep and the cap gene containing the insertion were placed between the ITRs. This way, the viral genome encoding the modified capsids that are better associated with EVs should be more prevalent in the purified EV-AAV sample. Sanger sequencing of cloned vectors confirmed the randomized flanking regions and the non-random late domain motif in the centre (Fig 4.14). The resulting library was used to produce EV-AAV9s, which were then isolated for sequencing as well as re-cloning back into the RepCap2/9-ITR plasmid for the next round of selection, further enriching for viral genomes containing the sequences of AAV capsids that were successfully associated with EVs. In this directed evolution approach, capsids that associate more efficiently with EVs should be enriched in the isolated EV-AAV9 genome pool and carried forward to the next round of selection. Each subsequent round is expected to further enhance the selection of capsids with improved EV association. After three rounds of selection, samples from each round and the original library were sent for NGS sequencing (Beijing Genomics Institute). The resulting data was used to perform motif analysis, and the top variants were selected to conduct an additional oligo screen, which included a wildtype sequence and a negative control sequence. The oligo screen was repeated sequentially three times, and the resulting EV-AAV9 samples were isolated

Figure 4.14 Generation of an AAV9 library to identify variants with improved extracellular vesicle incorporation.

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This figure illustrates the experimental workflow for creating a library of AAV9 variants and selecting for those with enhanced EV incorporation. First, a library was designed with randomized

linkers of 2, 4, or 8 codons with either non-random coding region encoding late domain motifs or random region in the centre. These sequences were cloned into the VR-IV loop of the AAV9 capsid protein (A). The subsequent steps involved co-transfection of HEK293T cells with the AAV RepCap2/9-ITR plasmid, followed by EV-AAV harvest and purification. DNA was extracted from EV-AAVs and cloned back into the RepCap2/9-ITR plasmid for repeated rounds of selection. Samples from each round were then prepared for sequencing by PCR amplification of the insert region. Resulting data was used to perform motif analysis to identify specific amino acid sequences or motifs that correlated with improved EV incorporation. The top 100 hits were selected and ordered as an OligoPool (IDT) for further evaluation. Three biological replicates of EV-AAV harvest were performed and sent for Amplicon EZ Sequencing (Azenta). These results were used to select top 5 candidates to validate for improved incorporation into EVs. Sanger sequencing results of the PT/SAP motif with 8 randomized flanking sequences, confirming successful cloning (B).

Low-depth AmpliconSeq sequencing analysis of three rounds of directed evolution revealed a bias towards smaller capsid inserts in the original library (Fig 4.15A). This bias was further amplified in the NGS data (Fig 4.15B). Enrichment scores, calculated based on the frequency of selected sequences after enrichment relative to the original library, demonstrated a clear preference for certain insert sizes and motifs. Analysis of rationally designed and randomized regions identified specific amino acid sequences that were enriched in the selected capsids. These findings suggest the successful enrichment of capsid variants with desired properties through directed evolution. To ensure that longer sequences which were potentially good hits were not overlooked in the screening, 120 variants were selected based on the motif analysis to maintain an equal number of sequences of each length, including the top 10 overall enriched sequences (Fig 4.15). Negative and positive controls were included as described above. These sequences were synthesized as oligonucleotides (Oligo Pool, IDT) and cloned into the RepCap2/9-ITR plasmid. EV-AAV9s were produced through transfection of HEK 293T cells as described previously. EV-AAV9 fractions were collected in three independent replicates. The original library and isolated EV-AAV9s were prepared for sequencing (AmpliconSeq) by extracting DNA and subsequently using PCR to amplify the region of the insert (Fig 4.15). This screen identified several enriched capsid variants, which were approximately 3-fold more

enriched than the wildtype control sequence. The top five variants, including LSGGLDILPKLC and LDRKIERQGNV, exhibited significant enrichment compared to the negative control and wildtype sequence (Fig 4.16).

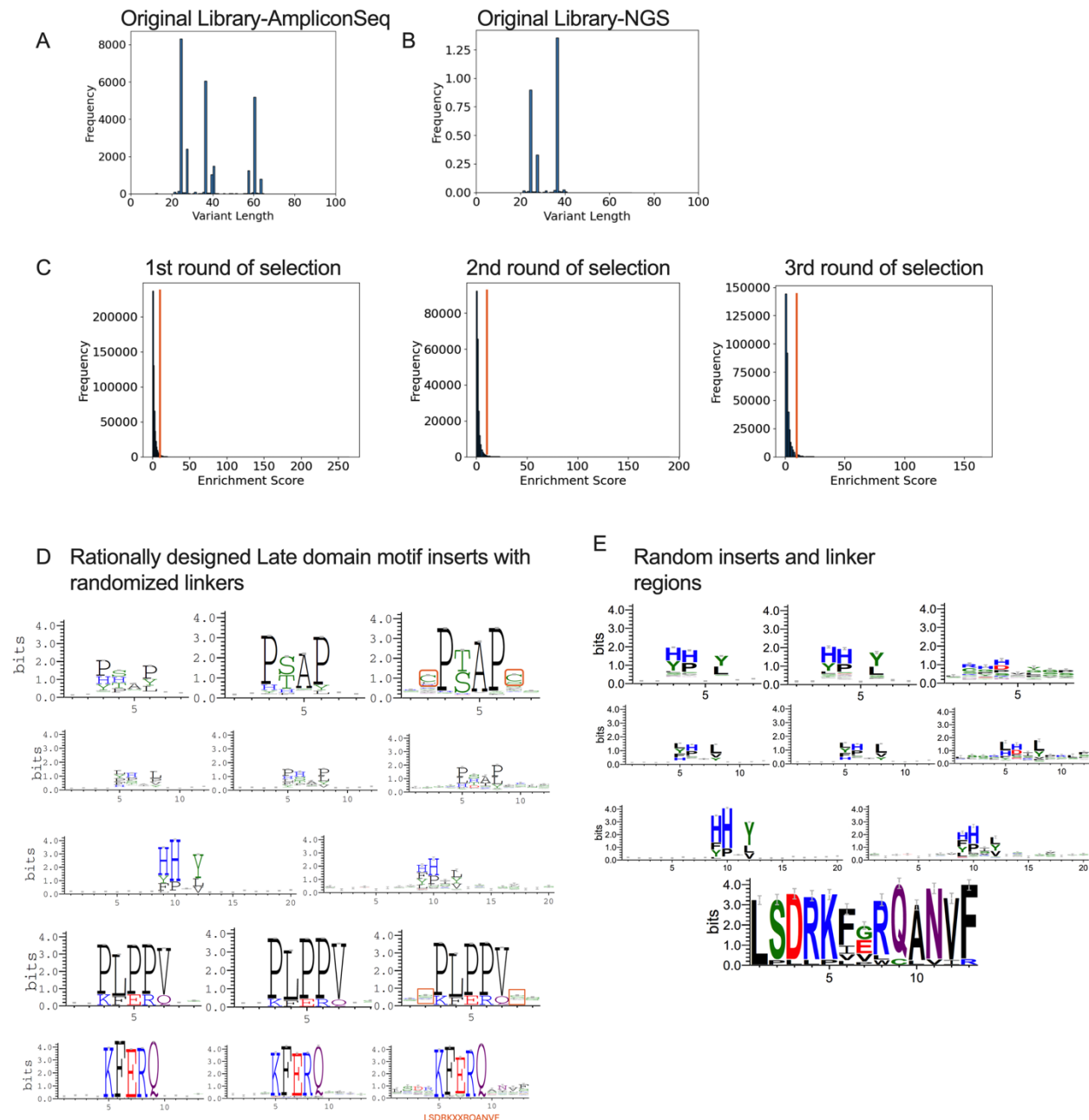


Figure 4.15 Analysis of NGS sequencing results of 3 rounds of directed evolution of capsids that incorporate into EV-AAV9s.

Sequencing of the original input library demonstrated some bias toward smaller inserts based on the frequency of reads per variant (A). After completing several rounds of evolution, samples were sent for NGS with a higher read depth, which showed a much higher read size bias, with very few

[illegible]

Figure 4.16 The refined second round of the capsid variant screen identifies several enriched variants, with the top five selected for further evaluation.

Top variants were selected from the evolution capsid library screen and evaluated in a refined second round of selection, which included negative sequences with stop codons, while wildtype sequences served as controls. EV-AAV9s were produced with the pool of capsids in three biological replicates; viral genomes were extracted from iodixanol-purified EV-AAV9s and the insert region was PCR amplified and sent for sequencing (Amplicon EZSeq (Azenta)). Enrichment

scores were calculated and plotted for each evaluated variant (A). The top five variants with the highest enrichment scores were selected for further examination, their sequences are listed along with the wildtype control, where polar amino acids (G,S,T,Y,C,Q,N) are green, basic amino-acids (K,R,H) are blue, acidic (D,E) are red and hydrophobic (A,V,L,I,P,W,F,M) amino acids are black. (B). All values are presented as Mean+SEM, N=3. P = Proline, T = Threonine, S = Serine, A = Alanine, Y = Tyrosine, X = Any amino acid, L = Leucine, V = Valine, F = Phenylalanine, K = Lysine, E = Glutamate, R = Arginine, Q = Glutamine.

The top five most enriched sequences from the refined screen were selected for further evaluation. These were ordered as individual oligos and cloned into RepCap2/9-ITR, with the wildtype AAV sequence serving as a control. EV-AAV9s were produced in HEK293T cells and isolated by iodixanol gradient (Fig 4.17A). Up to 3-fold higher percentage of the total AAV9 genomes were found in the media of the five engineered variants in comparison to the wildtype control (Fig 4.17B). Nanoflow cytometry analysis did not reveal significant differences in EV particle size or concentration (Fig 4.17C-D). qPCR titration of iodixanol-isolated EV-AAV9s showed a 2-fold significant increase in viral genome copies with the LSDRKIEROGNVF capsid variant having the highest increase (Fig 4.17E). Measurement of the amount of intact AAV9 capsids available for binding before and after Triton-X-100 permeabilization demonstrated an increased number of encapsulated virus particles in engineered variants compared to the wildtype, with SVPPHHAYVPTG having ~50% compared to ~10% found in the wildtype condition (Fig 4.17F). These findings suggest that the directed evolution process successfully generated capsid sequences which have an increased EV association.

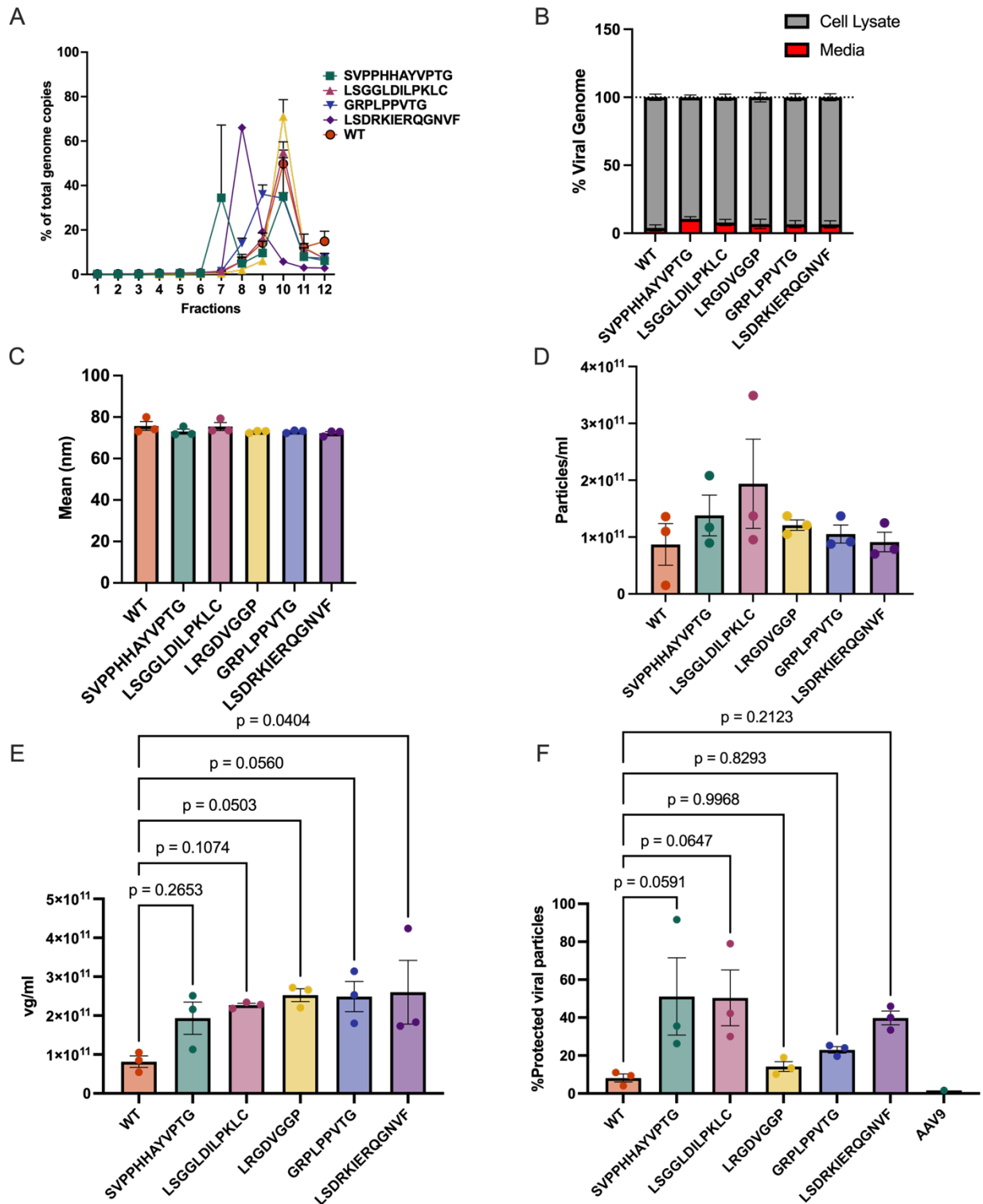


Figure 4.17 Directed evolution capsid library screen identifies capsids with increased EV-AAV9 yield.

The top 5 variants with the highest enrichment scores from the variant screen were selected and produced in HEK293T cells. Titration by qPCR of viral genomes in iodixanol fractions (A), media and cell lysate (B). NanoFCM analysis was used to assess mean EV particle size (C) and

concentration (D). qPCR titration of iodixanol-isolated EV-AAV9s samples (E). Analysis of virus particle concentration before and after treatment with Triton-X-100 (F). All values are presented as mean+SEM, N=3. Statistical analysis was performed using GraphPad Prism, with multiple one-way ANOVA comparisons of engineered capsids to WT-EV-AAV9 control. P = Proline, T = Threonine, S = Serine, A = Alanine, Y = Tyrosine, X = Any amino acid, L = Leucine, V = Valine, F = Phenylalanine, K = Lysine, E = Glutamate, R = Arginine, Q = Glutamine.

4.4 Discussion and Conclusion

Several engineering approaches were employed to improve EV-AAV9 yields, including surface loading, EV engineering, and AAV engineering. Characterization of wildtype EV-AAV9 demonstrated that many of the EV-associated viral genomes were bound to the surface of EVs, rather than in the lumen. To facilitate the surface loading of AAVs onto EVs, two receptors were investigated: AAVR and LamR. AAVR proved significantly more effective than LamR for AAV binding to the EV surface (Fig 4.1A-B). Furthermore, AAVs bound through AAVR offered partial protection against NAbs *in vitro* (Fig 4.1D).

To translate these findings *in vivo*, we first optimized a neutralization assay to determine the effective concentration of intravenous immunoglobulin (IgG) needed to neutralize free AAVs. We identified that a dose of 2 mg IgG effectively neutralized AAVs (Fig 4.2B-C). However, when surface-loaded AAVR-EVs were administered to mice pre-treated with IgG, they did not confer significant protection from NAbs nor enhance tissue transduction compared to free AAVs (Fig 4.3B-C). Poor transduction of AAVR-EVs even in the absence of NAbs suggests that binding the AAVs at the surface interferes with *in vivo* transduction perhaps through blocking AAV interaction with cells' native AAVR and inhibiting AAV intracellular trafficking^{203,204}. This discrepancy between *in vitro* and *in vivo* results highlights the complexity of the *in vivo* environment, where factors such as immune clearance mechanisms and biodistribution may diminish the protective effects observed *in vitro*.

EV-AAV9s showed a tendency of improved transduction in multiple tissues, including the heart, diaphragm, pancreas, and liver, even in the presence of NAbs, when compared to AAV9s (Fig 4.3B-C). These findings align with previous studies suggesting that encapsulation within EVs can shield AAV9s from NAbs and enhance delivery^{179,188,189,192}. The enhanced protection is likely due to the physical barrier provided by the EV membrane, which more effectively shields the AAV capsid from immune recognition compared to surface attachment. Once the AAV is delivered to cells it is also free to interact with AAVR for intracellular trafficking. Interestingly, co-administration of empty EVs with free AAVs also resulted in improved transduction in the heart and liver compared to AAV alone (Fig 4.3C). This suggests that EVs may have intrinsic properties that modulate the immune response or enhance cellular uptake of AAVs, a phenomenon that warrants further investigation but is outside the scope of this study.

Given the limited efficacy of surface loading strategies *in vivo*, remaining work shifted toward improving luminal loading approaches. It has been shown that overexpression of CD9 led to increased EV-AAV yield²⁵¹. We investigated whether overexpression of other common EV-associated proteins could enhance the incorporation of AAVs into EVs and thereby increase EV-AAV9 yield. Various EV markers fused to mClover3 were overexpressed in HEK293T stable cell lines, focusing on tetraspanins (e.g., CD63, TSPAN2), single-pass transmembrane proteins (e.g., PTTG1IP, PTGFRN) and cytosolic proteins (e.g., TSG101, ALIX). Results revealed that overexpression of CD63, TSPAN2 and PTTG1IP led to significantly higher EV-AAV9 yield and an increased number of protected viral genomes (Fig 4.5E-F). CD63, a well-characterized tetraspanin involved in EV biogenesis and cargo sorting, was selected for further evaluation due to its robust effect on EV-AAV9 production. A doxycycline-inducible HEK293T stable cell line overexpressing CD63 was generated and resulted in a ten-fold increase in EV-AAV9 yields

compared to wild-type cells (Fig 4.6B). Importantly, this overexpression did not alter the ratio of empty to full AAV capsids (Fig 4.6C) and led to a higher percentage of protected viral particles, with magnetic pull-down experiments confirming that 70% of viral genomes were encapsulated within EVs (Fig 4.5D-E). *In vitro* transduction assays demonstrated that CD63-EV-AAV9s exhibited comparable to EV-AAV9 transduction efficiency in SH-SY5Y-Traffic light cells (Fig 4.7).

When assessed *in vivo*, the CD63EV-AAV9s did not show a significant increase in transduction efficiency compared to wild-type EV-AAV9s, as measured by Nanoluciferase activity (Fig 4.8B). In fact, although not significant the mean expression in EV-AAV9 treated was 4 times higher in the heart, and approximately 2 times higher in the liver and diaphragm in comparison to CD63EV-AAV9 (Fig 4.8B). Despite this, the substantial increase in EV-AAV yield enabled us to administer a ten-fold higher dose, reaching a clinically relevant level of 1×10^{12} viral genomes per mouse (equivalent to $\sim 5 \times 10^{13}$ vg/kg for a 20 g animal), which falls closer to the range of systemic AAV doses used in human clinical trials (e.g., Zolgensma for SMA at 1.1×10^{14} vg/kg)²⁵². This underscored the practical advantage of CD63 overexpression in scaling up EV-AAV9 production for therapeutic applications. In a repeated dosing study designed to evaluate the potential of CD63-EV-AAV9s to overcome pre-existing immunity, mice were administered an initial dose of AAV9 or CD63-EV-AAV9 carrying a Nanoluciferase-eGFP transgene, followed by a second dose with a Nanoluciferase-mScarlet transgene (Fig 4.9A). No significant differences in Nanoluciferase expression were observed between single-dose EV and double-dose EV-EV treatments across the tissues examined (Fig. 4.9B). In the heart, the AAV-EV group had the highest Nanoluciferase and eGFP expression levels compared to other groups, however, only the increase in eGFP expression relative to the single-dose EV group reached

statistical significance. In the liver, the single-dose AAV group showed the highest Nanoluciferase and eGFP expression, with a significant difference observed only for Nanoluciferase expression compared to the EV single-dose group (Fig. 4.9B). Antibody levels were significantly elevated at 4 weeks in the AAV single-dose, AAV-AAV, and AAV-EV groups compared to their 2-week counterparts, indicating a time-dependent increase in humoral immune response. In contrast, EV single-dose, EV-EV, and uninjected groups showed no significant changes in antibody levels between timepoints and overall maintained low anti-AAV9 responses. These results suggest that initial AAV exposure—whether alone or followed by EV-AAV—triggers a robust neutralizing antibody response, whereas EV-AAV alone does not (Fig 4.9C). This finding suggests that EV encapsulation may modulate the immune response, potentially allowing for repeated dosing without eliciting strong humoral immunity. Based on the results presented, it is likely that overexpression of CD63 increased EV-AAV9 yield by promoting EV biogenesis rather than by directly interacting with AAV particles. Previous reports that EVs can modulate immune responses, possibly by sequestering antigens or altering antigen presentation. This property could be leveraged to develop gene therapies capable of repeated dosing, which is critical for treating chronic or progressive diseases. However, further engineering is needed to improve transduction efficiency of CD63EV-AAV9s, as it performed worse than AAV9 *in vivo* (Fig 4.9).

EVs have been shown to exhibit a short half-life *in vivo*, with intravenously injected EVs being cleared from circulation as quickly as ~20 minutes. Strategies to extend their circulation time, such as incorporating additional targeting moieties or immunomodulatory molecules, could enhance the performance of CD63EV-AAV9 *in vivo*. For example, the incorporation of CD47 has been proposed to prevent EVs from being phagocytosed by immune cells, thereby increasing

their circulation time and allowing them more opportunity to reach target tissues effectively^{149,165}.

An additional possible reason as to why CD63EV-AAV9s have decreased transduction *in vivo* is endosomal escape. When AAV9 enters a host cell, it must navigate through multiple cellular compartments before reaching the nucleus, where it can deliver its transgene. AAV9 is internalized through receptor-mediated endocytosis, leading to its encapsulation within endosomal vesicles. The endosomal membrane represents a significant barrier, as the virus must escape this compartment to access the cytoplasm and subsequently the nucleus. EV-AAV9s, which are already encapsulated in a vesicle, may have an extra membrane barrier that the AAV9 must traverse, complicating the transduction process further. As discussed in Chapter 1, during AAV entry into the cell, when the pH of the endosome drops, the AAV undergoes conformational change, which exposes the N-termini of VP1 and VP2. Exposing these domains is important for endosomal escape, and trafficking to the nucleus. There are two cell entry factors that are important for AAV9 intracellular trafficking: GPR108 and AAVR. In chapter 3, we saw that knockout of AAVR impacted the transduction efficiency of EV-AAV9, suggesting that these entry factors may also be important for EV-AAV9. If the AAV9 cargo is not being efficiently released by CD63EV-AAV9s in the cells, one possible way to improve transduction is to engineer the EV with a fusogenic protein.

Adding a fusogenic protein, such as VSVG (Vesicular Stomatitis Virus Glycoprotein), to CD63EV-AAV9s could potentially enhance the transduction efficiency of AAV9s while maintaining their protection from the immune system²⁵³. VSVG is a well-characterized glycoprotein known for its ability to mediate membrane fusion. It undergoes a conformational change in response to the acidic environment of an endosome to promote membrane fusion and

destabilization of endosomal membranes, resulting in the release of its cargo²⁵⁴. A potential limitation of adding VSVG to the EV is that it is a viral protein and can be immunogenic. Once inside the cytoplasm, AAV9 must then locate the nuclear pore complexes to facilitate its entry into the nucleus. This process is critical because the nuclear membrane is not always permeable; it becomes porous during specific phases of the cell cycle, particularly during mitosis when the nuclear envelope breaks down which facilitates quicker entry into the nucleus²⁵⁵. In contrast, in non-dividing cells, which are more prevalent *in vivo*, the nuclear membrane remains intact for longer periods, potentially delaying the access of AAV9 to the nuclear interior and hindering transgene delivery²⁵⁶. This issue may be less pronounced in cultured cells, where the constant division and cycling of cells provide more frequent opportunities for the nuclear membrane to become accessible, thus enhancing transduction efficiency. It may be that with the added barrier for endosomal escape and the less porous nuclear membrane *in vivo*, EV-AAV9 transduction may be slower than AAV9. Perhaps if the experiment was extended to 8 weeks, there would be less variability in the transduction as previous studies have shown that AAV9 expression continues to increase after 2 weeks²⁵⁷.

Engineering the AAV9 capsid with the full-length pX domain from the hepatitis A virus (HAV) significantly increased AAV incorporation into EVs (Fig 4.10C). The pX domain allows HAV to hijack exosomal pathways for viral release²¹⁰. Notably, only the full-length pX, and not the truncated version containing only Alix-interacting domains, enhanced AAV loading into EVs (Fig 4.10F), suggesting that the complete domain is necessary for effective interaction with EV biogenesis machinery. *In vitro*, pX-EV-AAV9 showed improved transduction in a neuronal cell line compared to wild-type EV-AAV9 (Fig 4.11), indicating that enhanced EV incorporation translates to better gene delivery under controlled conditions. However, *in vivo* studies revealed

no significant difference between pX-EV-AAV9 and wild-type EV-AAV9 transduction, with both performing worse than free AAV9 in the heart, liver quadriceps and diaphragm (Fig 4.12). This discrepancy suggests that factors such as biodistribution, immune clearance, or tissue-specific uptake mechanisms may limit the *in vivo* efficacy of EV-AAV9s despite increased EV incorporation. Similar to CD63EV-AAV9s, which also performed poorly *in vivo*, it may be related to increased levels of AAV9s loaded lumenally and the extra challenges that would pose for endosomal escape. Although levels of anti-AAV9 mouse IgG in response to single and repeated dosing was evaluated, it has since been shown that EVs themselves can also activate an antibody response, and future studies will need to evaluate anti-AAV9 and anti-EV NAb. Next, strategies involving engineering the AAV capsid itself were investigated. Mutation of the LXXL motif in the wildtype AAV9 capsid led to highly decreased viral yield, while mutation of the YXXL motifs resulted in increased AAV9 associated per EV (Fig 4.13). YXXL is an L domain found in the capsid of bovine leukemia virus (BLV). If YXXL was acting as an L domain to interact with Alix to load the virus into the EVs a decrease in AAV9 associated per EV would have been expected in the mutated condition²²³. Some studies have shown that mutating the surface-exposed tyrosine (Y) to phenylalanine (F) can prevent phosphorylation of the capsid and subsequent degradation of the virus, which can improve transduction efficiency^{258,259}. Perhaps this decrease in phosphorylation on the capsid surface results in less capsid degradation and consequently more AAV-EV association.

Given the compact and overlapping nature of the AAV genome, extensive capsid engineering is challenging and often compromises viral assembly or yield. To overcome these limitations, we explored incorporating viral domains known to interact with EV biogenesis

pathways and employed directed evolution to identify capsid variants with improved EV incorporation.

To identify capsid variants with better EV incorporation, we generated an AAV9 capsid library incorporating various L domain motifs, sequences that engage the ESCRT machinery during viral replication of membrane-bound virions. Through directed evolution and multiple rounds of selection and sequencing, specific capsid variants exhibiting enhanced EV incorporation were enriched (Fig 4.14 and 4.15). A further top 120 variant screen was performed to refine the list of candidates, leading to the selection of the top five enriched sequences for validation. An additional limitation of this study is that sequencing of the original input library of the cloned RepCap2/9-ITR revealed incomplete coverage of the theoretical diversity. Furthermore, there was a higher prevalence of small inserts compared to large inserts. To mitigate the potential loss of promising sequences amidst this noise, we performed motif enrichment analysis to identify top candidates and then assessed these sequences in a top variant screen, which was conducted in triplicate to improve confidence in the selected sequences. Currently, this data is being utilized by other lab members to develop a machine-learning model aimed at predicting potential top capsid candidates which may not have been covered by this screen, which will be evaluated in future variant screens.

Validation experiments confirmed that these engineered capsid variants resulted in a higher percentage of viral genomes in the media compared to the wild-type control (Fig 4.15B), without significant changes in EV particle size or concentration (Fig 4.15C-D). Notably, the variant LSDRKIEROGNVF showed a significant increase in EV-associated viral genome copies and enhanced protection of viral particles (Fig 4.15E-F), indicating successful encapsulation within EVs.

The top enriched sequence is SVPPHHAYVPTG, which has several histidine residues which are positively charged. Histidine residues in other viral capsids, such as blue tongue virus (BTV), which are involved in pH sensing and can assist the virus in penetrating host cell membranes. Additionally, the charged histidine residues can interact with metal cations such as Zinc, to stabilize capsids. PPX_nY sequences have been identified in the capsid of Ebola viruses, and Lassa viruses, where it interacts with NEDD4 family of ubiquitin ligases, leading to ubiquitination of capsid protein and interaction with ESCRT machinery. Its possible that engineered AAV9-SVPPHHAYVPTG variant. The second most enriched variant was LSGGLDILPKLC, which contains a LXXL motif, which has been reported to interact with Alix. LRGDVGGP was the third most enriched insertion, it does not contain any late domain motifs but does contain the residues RGD, which is a motif that has been shown to interact with integrins. Fourth most enriched engineered capsid insertion was GRPLPPVTG, PLPPV is the L domain found in the Gag protein of MMTV. Fifth in the list is LSDRKIERQGNVF, which is similar to the KFERQ sequence, which can interact with LAMP2A to increase protein loading into extracellular vesicles. Further work is needed to determine whether these motifs in the AAV9 capsid would have similar interacting partners.

Future engineering strategies can combine producer cell engineering with AAV capsid engineering. For example, overexpression of the predicted interacting partner with its corresponding engineered capsid could result in increased EV-AAV9 yield. For example, overexpression of LAMP2A during production of LSDRKIERQGNVF-AAV9s could further increase EV-AAV9 yield. Further testing is needed to show the *in vitro* and *in vivo* transduction efficacy of the engineered EV-AAV9s.

In conclusion, these studies provide valuable insights into the potential of EV-based strategies to improve AAV delivery in the presence of neutralizing antibodies. Despite promising *in vitro* results, surface-loaded AAVs on EVs showed limited transduction efficiency *in vivo*, possibly due to immune clearance mechanisms and cellular trafficking barriers. Encapsulating AAVs within the EV lumen significantly enhanced tissue transduction and protection from NAbs, underscoring the advantage of luminal loading over surface attachment.

These findings highlight the complexity and potential of engineering EV-based AAV delivery systems for enhanced transduction efficiency and immune evasion. While *in vitro* results show promising gains in EV-AAV9 production and transduction through methods like CD63 overexpression and capsid engineering, *in vivo* results show that significant challenges remain. Future efforts should prioritize optimizing endosomal escape mechanisms, potentially through the inclusion of fusogenic proteins like VSVG, and leveraging advanced computational methods to refine capsid designs. These strategies could pave the way for scalable, immune-tolerant EV-AAV systems capable of achieving consistent therapeutic efficacy *in vivo*.

5 Chapter 5. Discussion and Conclusion

5.1 Summary of Main Findings

This thesis has provided significant insights into the biology of EV-AAVs and explored multiple engineering strategies to enhance their therapeutic potential. Through systematic characterization and engineering approaches, the research has advanced the understanding of these promising gene therapy vectors while identifying key challenges that must be addressed for clinical translation.

Chapter one outlined advancements in gene therapy, focusing on AAVs and EVs as therapeutic tools. The chapter discussed AAV biology, highlighting their genome structure, life cycle and serotype-specific tropisms that makes them effective in gene therapy while acknowledging limitations such as limited vector capacity and immune responses. EVs were explored for their biocompatibility and potential to deliver therapeutic cargo, particularly in hybrid EV-AAV systems, which combine the advantages of both platforms to enhance gene delivery and address the limitations of conventional AAV therapy.

In Chapter Three, I sought to characterize the properties and biogenesis of EV-AAVs. Characterization studies revealed that AAVs preferentially associate with larger vesicles, with only 1.2-1.6% of the total EV population naturally incorporating AAV9s. This low incorporation rate highlights a critical limitation of current EV-AAV production methods. Importantly, EV-AAVs are enriched in phosphatidylserine but show low levels of tetraspanins. Inhibiting the ectosome pathway led to a non-significant decrease in EV-AAV9 yield, suggesting but not concluding that ectosome pathways are important for EV-AAV9 biogenesis. The dependency on AAVR for efficient EV-AAV9 transduction, demonstrated through knockout and rescue

experiments, indicates that despite EV encapsulation, the native AAV9 intracellular trafficking mechanisms remain crucial for successful gene delivery.

In chapter four, I aimed to address the challenge of low EV-AAV9 yield. To that end, multiple engineering strategies were explored. Surface loading of AAV9s onto EVs using AAVR showed promise *in vitro* with partial protection from NAbs, but this approach proved less effective *in vivo*, likely due to interference with the virus's natural cell entry mechanisms. Overexpression of CD63 in producer cells led to a remarkable 10-fold increase in EV-AAV9 yield, with approximately 70% of viral particles protected within CD63EV-AAV9s compared to 10% in traditional EV-AAV9s. This significant improvement in production efficiency brings the field closer to clinically relevant doses. Engineering of the AAV9 capsid itself through the incorporation of the HAV pX domain and directed evolution yielded promising variants with enhanced EV association. The directed evolution screen identified specific sequences, such as LSGGLDILPKLC and LSDRKIERQGNVF, that significantly improved AAV9 incorporation into EVs. While the pX-engineered variant showed increased EV loading and markedly increased transduction *in vitro*, the *in vivo* experiments did not, highlighting the complexity of translating *in vitro* success to therapeutic efficacy.

A limitation of the animal studies presented in this thesis is that they are underpowered. Due to constraints in EV-AAV9 production, only four mice were injected per condition. For example, when evaluating the pX engineering strategy, and comparing AAV9, pXAAV9, EV-AAV9, and EV-pXAAV9, power calculations were performed using G*Power 3.1.9 with a chosen power of 80%, which corresponds to a 20% chance of missing a true effect. These calculations indicated that detecting a medium effect size (Cohen's $f = 0.25$) with four groups would require 45 mice per group²⁶⁰. However, due to logistical constraints, the experiment was

limited to four mice per group (N = 16 total), resulting in a substantially lower achieved power—approximately 10% for medium effects ($f = 0.25$) and less than 5% for small effects ($f = 0.10$). At this level of power, there is a 90% chance of failing to detect a true medium-sized effect. Therefore, the results presented in this thesis should be considered exploratory, and both significant and non-significant findings should be interpreted with caution.

Improved transduction efficiency *in vivo* of EV-AAVs over AAV controls has been demonstrated in literature^{76,181,261}, however I was not able to convincingly reproduce this result. EV-AAV engineering strategies (CD63EV-AAV9 and EV-pXAAV9) which significantly increased AAV encapsulation performed poorly *in vivo*. A recent study has shown that when wildtype EV-AAV9s from HEK293T cells are depleted of EV-AAV9s which have AAV9 on their surface through anti-AAV9 pulldown and are then injected into the mouse brain (at equal dose to the bulk EV-AAV9s), they show significantly less transduction²⁶². These results suggest that EV-AAV populations with AAVs on the surface have improved transduction perhaps through interaction with AAVR which we showed in Chapter 3 is still important for EV-AAV9 transduction. If this is true then in engineering EV-AAVs (CD63EV-AAV9 and EV-pXAAV9) with more lumenally loaded AAVs, we have made EV-AAVs with poorer *in vivo* transduction. Engineering EV-AAV9s with the potential for alternative receptor interactions for cellular uptake may be beneficial. For example, EVs engineered with the RVG peptide, can bind to the nicotinic acetylcholine receptor, and are then internalized by receptor-mediated endocytosis¹⁵⁴. Adding something similar to the engineered EV-AAV9 variants in this study may help translate the *in vitro* success in the human neuroblastoma cells (SH-SY5Y-TL) to *in vivo*.

In addition to potential cellular uptake challenges, how the EV-AAV9s behave in circulation can also contribute to *in vivo* success. Matsumoto *et al.* compared the circulation time of bulk EVs and phosphatidylserine-EV-depleted (by incubation with TIM-4-magnetic particles) EVs after IV systemic administration in mice²⁶³. It was found that phosphatidylserine EV-depleted EVs remained in circulation longer than the phosphatidylserine-positive bulk EV population. It was also shown that much of the bulk population accumulated in the liver and spleen, whereas the phosphatidylserine-EV-depleted sample was found mostly in the vascular endothelial cells in the lungs. Importantly, authors demonstrated that both bulk EVs and phosphatidylserine-EV depleted samples had similar expression levels of CD47, suggesting that the negative charge of phosphatidylserine may play a role in increased clearance by macrophages²⁶³. Poorer circulation time of phosphatidylserine-positive EVs has potential implications for the *in vivo* efficacy of EV-AAV9s, as in Chapter 3 of this thesis, anti-phosphatidylserine pulldown showed a trend towards increased phosphatidylserine in EV-AAV9s. CD47, already discussed in this thesis, can interact with macrophages to inhibit phagocytosis. Further enhancing CD47 expression on EV-AAV9s—alone or in combination with other anti-phagocytic ligands such as PD-L1 or CD24—may reduce clearance by the mononuclear phagocyte system and thereby extend systemic circulation time¹⁴⁹. Engineering EVs with albumin binding domains have also been shown to increase EV circulation time²⁶⁴. Increasing circulation time increases likelihood that EV-AAVs reach target tissues, before being cleared by the immune system.

5.2 Future of EV-AAVs as therapeutic vectors

The future development of EV-AAVs as therapeutic vectors requires a multi-faceted approach that addresses both fundamental biological understanding and practical implementation challenges. Given the limitations discussed above, future studies should focus on improving EV-AAV9 cellular transduction *in vivo*, by increasing circulation time (e.g., CD47), improving cellular uptake (e.g., RVG), and endosomal escape (e.g., VSVG). Investigation into alternative EV-AAV production cells could provide a better avenue for repeated dosing than repeated dosing of HEK293T-derived EV-AAVs. In addition to improving *in vivo* efficacy, further improving EV-AAV yields through either combination engineering or through the development of scalable production and isolation methods would be helpful.

Further investigation into the cellular trafficking of EV-AAVs is needed to comprehend the mechanisms behind successful versus unsuccessful delivery. Advanced imaging techniques could monitor the intracellular fate of EV-AAVs, providing insights into trafficking bottlenecks and informing engineering strategies. Understanding the timing and location of AAV release from EVs could aid the development of more effective delivery vehicles. This understanding is particularly important for addressing the poor *in vivo* performance compared to *in vitro* success.

Combining multiple engineering strategies presents a logical path forward to further increase EV-AAV yield. The successful outcomes from individual approaches—CD63 overexpression, pX domain incorporation and evolved capsid variants—suggest that combining these modifications could yield synergistic improvements. For instance, producing pX-modified AAVs in CD63-overexpressing cells may enhance both production yield and functional delivery. Similarly, evolved capsid variants could be integrated with producer cell engineering to further optimize EV-AAV9 generation. Recently, metabolic engineering of the producer cell resulted in

increased EV-AAV yield, a strategy that could also be combined with capsid engineering²⁴⁰. It was found that knocking out phosphatidylserine synthase led to a decrease in total EV-AAV, however these EV-AAVs showed a 3-fold increase in full saturated phosphatidylserine, and a significant increase in transduction compared to wild type EV-AAV²⁶⁵. As discussed above phosphatidylserine can also decrease EV circulation time. Therefore, a potential strategy could involve knocking out phosphatidylserine synthase in the doxycycline-inducible CD63-overexpressing HEK293T cell line and using it to produce CD63-EVAAV9s with theoretically improved transduction efficiency. This strategy is speculative and would require experimental validation.

The high-throughput screening approach used in capsid evolution could be expanded through machine learning-guided optimization. The sequence data generated from evolution experiments could train algorithms to predict more effective capsid variants, potentially identifying optimal combinations of motifs and linker regions not covered in the initial libraries. This computational approach could accelerate the development of next-generation EV-AAV vectors. Notably, machine learning frameworks such as CAPSID and AAV-Perturb-seq have been successfully applied to predict functional AAV variants and improve muscle or CNS targeting^{266–269}. However, predictive accuracy may be limited by the inherent biological variability of in vivo environments. Moreover, while numerous datasets exist for conventional AAVs, there is currently a lack of comprehensive datasets for EV-AAVs, which may constrain the applicability of machine learning approaches in this context.

To enable higher-powered studies, scalable production of EV-AAV9 is also essential. Currently, the lack of efficient large-scale production methods poses a significant barrier to the clinical translation of EV-AAV9 therapies. While large bioreactors and suspension cultures of

HEK293T cells offer a potential route for scale-up, the inherent heterogeneity of EV populations remains a major challenge, necessitating thorough characterization of the resulting material. In this and other studies, iodixanol gradient ultracentrifugation was employed to separate free AAVs from EV-AAVs however, this method is not amenable to large-scale manufacturing, and alternative purification strategies will need to be developed. One of the key limitations of AAV-based gene therapy is the high cost of vector production²⁷⁰. Given that only approximately 2–4% of secreted vector genomes are present in EV-rich fractions (fractions 4–6), EV-AAV production yields are even lower than those of conventional AAV. As a result, the cost of EV-AAV-based therapies may represent an even greater barrier to clinical implementation than current AAV-based approaches. Additionally, considerable translational concern arises from utilizing HEK293T cells as the producer cell line. These cells, derived from human embryonic kidney tissue, were immortalized using adenoviral E1A and E1B genes and further modified with the SV40 large T antigen. EVs from HEK293T cells were not harmful in mice; however, characterization of the protein, mRNA and miRNA components of HEK293T-derived EVs has identified some potentially immunogenic molecules²⁷¹. These include heat shock proteins (e.g., HSP70, HSP90), residual SV40 large T antigen, and pro-inflammatory miRNAs such as miR-21 and members of the miR-17-92 cluster. Such molecules may activate innate immune responses or pose safety risks if used clinically^{272,273}. Exploring alternative producer cell lines such as mesenchymal stem cells which have immunomodulatory benefits and are already being used in clinic but may be challenging to adapt to producing AAVs^{142,274,275}. Developing safer, stable cell lines may be necessary to mitigate these risks before clinical translation. In addition to checking for potentially harmful biomolecules that could be co-loaded into EVs, a critical quality control challenge unique to EV-AAVs is the inability to separate empty from full capsids once they are

incorporated into EVs. While traditional AAV manufacturing can employ density-based separation to remove empty capsids, thereby reducing immunogenicity and improving therapeutic index, this approach is not feasible with EV-encapsulated AAVs. This limitation necessitates upstream optimization of transfection conditions to minimize empty capsid production during the manufacturing process²⁷⁶.

Anti-EV immunity was not investigated in this study but represents an important consideration for the clinical translation of EV-AAV9 therapies. One proposed advantage of EV-AAVs over conventional AAVs is the potential for repeated dosing. However, recent studies have shown that repeated intravenous administration of EVs—particularly those from non-autologous sources—can elicit adaptive immune responses, including the production of EV-specific IgG, which leads to accelerated blood clearance upon subsequent doses²⁷⁷. A non-human primate (NHP) study demonstrated that repeated dosing of human-derived EVs significantly reduced their circulation time and correlated with increased levels of EV-specific IgG, despite unchanged total IgG and cytokine levels^{277,278}. Notably, this study also reported a striking difference in EV pharmacokinetics between species: EVs in mice exhibit short plasma half-lives (~5–10 minutes), while in NHPs, the half-life extends to ~40 minutes, with detectable signal up to 24 hours post-injection²⁷⁷. These interspecies differences underscore the importance of evaluating EV-AAV pharmacokinetics and efficacy in larger animal models. Following IV administration, EVs primarily accumulate in the liver and spleen in both mice and macaques, with additional uptake observed in the lungs, kidneys, heart, and colon. Given this hepatic accumulation, the liver represents a promising therapeutic target for EV-AAV-mediated gene delivery, particularly for monogenic liver diseases, for example delivery of Factor IX to treat hemophilia B^{181,191}. Given emerging evidence that B cells may preferentially internalize EVs,

EV-AAV9s could also be explored as a targeted gene delivery platform for B cell-related diseases²⁷⁹. In conclusion, future studies should employ NHPs to better model the biodistribution of EVs in larger animals, and targeting tissues where EVs naturally accumulates, such as the liver and B cells, may represent a promising therapeutic target for EV-AAVs.

Ultimately, the successful clinical implementation of EV-AAVs will require balancing multiple factors, including production efficiency, therapeutic efficacy, and safety considerations. While significant challenges persist, the ongoing development of this platform promises to advance the field of gene therapy.

5.3 *Concluding Remarks*

The therapeutic potential of AAVs and EVs has been well documented, and advancements in their combined application, such as EV-AAVs, could be promising. Findings in this thesis addressed key challenges in the EV-AAV field, including the low incorporation efficiency of AAVs into EVs and the limited understanding of their biogenesis. Novel engineering strategies and optimization approaches described in this work provided critical insights into enhancing EV-AAV yield and functionality. A deeper understanding of the molecular mechanisms driving EV-AAV formation and the interplay between EVs and AAVs will enable more precise engineering of these hybrid systems. This, in turn, lays a foundation for developing EV-AAV-based therapeutics, offering innovative solutions to overcome current limitations in gene therapy and advancing their clinical application.

6 Appendix

1. Papers

Hanson B, Vorobieva I, Zheng W, Conceição M, Lomonosova Y, Mäger I, Puri PL, El Andaloussi S, Wood MJA, Roberts TC. EV-mediated promotion of myogenic differentiation is dependent on dose, collection medium, and isolation method. *Mol Ther Nucleic Acids*. 2023 Jul 15;33:511-528. doi: 10.1016/j.omtn.2023.07.005. PMID: 37602275; PMCID: PMC10432918.

2. Conference Presentations

6.1.1 Poster Presentations

“Enhancing EV-AAV incorporation: Insights into natural loading and exogenous binding strategies for improved gene therapy vector delivery” International Society for Cell and Gene Therapy (ISGCT) 2024

“Enhancing EV-AAV incorporation: Insights into natural loading and exogenous binding strategies for improved gene therapy vector delivery” European Society of Cell and Gene Therapy (ESGCT) 2023

“Characterisation of extracellular vesicles that incorporate adeno-associated viruses” European Society of Cell and Gene Therapy (ESGCT) 2022

6.1.2 Oral Presentations

“Encapsulating AAV (Adeno-associated virus) in Extracellular Vesicles for Enhanced Gene Delivery” Oxosome 2023

“Enhancing EV-AAV incorporation: Insights into natural loading and exogenous binding strategies for improved gene therapy vector delivery” UK Society for Extracellular Vesicles (UKEV) 2023

7 References

1. Jackson, D. A., Symons, R. H. & Berg, P. Biochemical method for inserting new genetic information into DNA of Simian Virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose operon of Escherichia coli. *Proc Natl Acad Sci U S A* 69, 2904–2909 (1972).
2. Liu, F., Li, R., Zhu, Z., Yang, Y. & Lu, F. Current developments of gene therapy in human diseases. *MedComm* vol. 5 Preprint at <https://doi.org/10.1002/mco2.645> (2024).
3. Russell, S. *et al.* Efficacy and safety of voretigene neparvovec (AAV2-hRPE65v2) in patients with RPE65-mediated inherited retinal dystrophy: a randomised, controlled, open-label, phase 3 trial. *The Lancet* 390, 849–860 (2017).
4. Loo, J. C. M. van der & Wright, J. F. Progress and Challenges in Viral Vector Manufacturing. *Hum Mol Genet* 25, R42–R52 (2015).
5. Colella, P., Ronzitti, G. & Mingozzi, F. Emerging Issues in AAV-Mediated In Vivo Gene Therapy. *Molecular Therapy - Methods and Clinical Development* vol. 8 87–104 Preprint at <https://doi.org/10.1016/j.omtm.2017.11.007> (2018).
6. Gross, D.-A., Tedesco, N., Leborgne, C. & Ronzitti, G. Overcoming the Challenges Imposed by Humoral Immunity to AAV Vectors to Achieve Safe and Efficient Gene Transfer in Seropositive Patients. *Front Immunol* 13, (2022).
7. Yoon, Y. J., Kim, O. Y. & Gho, Y. S. Extracellular Vesicles as Emerging Intercellular Communicasomes. *BMB Rep* (2014) doi:10.5483/bmbrep.2014.47.10.164.
8. Ovchinnikova, L. A. *et al.* Reprogramming Extracellular Vesicles for Protein Therapeutics Delivery. *Pharmaceutics* (2021) doi:10.3390/pharmaceutics13060768.
9. Yu, B. *et al.* Extracellular Vesicles Engineering by Silicates-Activated Endothelial Progenitor Cells for Myocardial Infarction Treatment in Male Mice. *Nat Commun* 14, (2023).
10. Molina, E. *et al.* Insights in AAV-mediated antigen-specific immunity and a strategy for AAV vaccine dose reduction through AAV-extracellular vesicle association. *Mol Ther Methods Clin Dev* 32, 101358 (2024).
11. György, B., Fitzpatrick, Z., Crommentuijn, M. H. W., Mu, D. & Maguire, C. A. Naturally enveloped AAV vectors for shielding neutralizing antibodies and robust gene delivery invivo. *Biomaterials* 35, 7598–7609 (2014).
12. Hoggan, M. D., Blacklow, N. R. & Rowe, W. P. Studies of small DNA viruses found in various adenovirus preparations: physical, biological, and immunological characteristics. *Proc Natl Acad Sci U S A* 55, 1467–1474 (1966).
13. Atchison, R. W., Casto, B. C. & Hammon, W. M. D. Adenovirus-associated defective virus particles. *Science* (1979) 149, 754–756 (1965).
14. Xiao, X., Juan, L. & Jude, S. R. Production of High-Titer Recombinant Adeno-Associated Virus Vectors in the Absence of Helper Adenovirus. *J Virol* 72, 2224–2232 (1998).
15. Galli, A. & Cervelli, T. Inverted Terminal Repeats of Adeno-Associated Virus Decrease Random Integration of a Gene Targeting Fragment in *Saccharomyces Cerevisiae*. *BMC Mol Biol* 15, 5 (2014).
16. Savy, A. *et al.* Impact of Inverted Terminal Repeat Integrity on rAAV8 Production Using the Baculovirus/Sf9 Cells System. *Hum Gene Ther Methods* 28, 277–289 (2017).

17. Stracker, T. H. *et al.* The Rep Protein of Adeno-Associated Virus Type 2 Interacts with Single-Stranded DNA-Binding Proteins That Enhance Viral Replication. *J Virol* 78, 441–453 (2004).
18. Philpott, N. J., Gomos, J. B., Berns, K. I. & Falck-Pedersen, E. A P5 Integration Efficiency Element Mediates Rep-Dependent Integration Into AAVS1 at Chromosome 19. *Proceedings of the National Academy of Sciences* 99, 12381–12385 (2002).
19. Hüser, D. *et al.* Integration Preferences of Wildtype AAV-2 for Consensus Rep-Binding Sites at Numerous Loci in the Human Genome. *PLoS Pathog* 6, e1000985 (2010).
20. Bardelli, M., Zarate-Perez, F., Agúndez, L., Escalante, C. & Henckaerts, E. Identification of a Functionally Relevant Adeno-Associated Virus Rep68 Oligomeric Interface. *J Virol* 90, 6612–6624 (2016).
21. Earley, L. F. *et al.* Adeno-Associated Virus (AAV) Assembly-Activating Protein Is Not an Essential Requirement for Capsid Assembly of AAV Serotypes 4, 5, and 11. *J Virol* (2017) doi:10.1128/jvi.01980-16.
22. Wörner, T. P. *et al.* Adeno-associated virus capsid assembly is divergent and stochastic. *Nature Communications* 2021 12:1 12, 1–9 (2021).
23. Galibert, L. *et al.* Functional roles of the membrane-associated AAV protein MAAP. *Sci Rep* 11, (2021).
24. Hermonat, P. L. *et al.* Chromosomal latency and expression at map unit 96 of a wild-type plus adeno-associated virus (AAV)/Neo vector and identification of p81, a new AAV transcriptional promoter. *J Hum Virol* 2, 359–368 (1999).
25. Cao, M., You, H. & Hermonat, P. L. The X gene of Adeno-Associated Virus 2 (AAV2) is involved in viral DNA replication. *PLoS One* 9, (2014).
26. Kotin, R. M. Prospects for the use of adeno-associated virus as a vector for human gene therapy. *Hum Gene Ther* 5, 793–801 (1994).
27. D L Caspar & A Klug. Physical principles in the construction of regular viruses. *Cold Spring Harbor Symp Quant Biol.* (1962) doi:10.1101/sqb.1962.027.001.005.
28. Govindasamy, L. *et al.* Structural Insights Into Adeno-Associated Virus Serotype 5. *J Virol* 87, 11187–11199 (2013).
29. Stahnke, S. *et al.* Intrinsic phospholipase A2 activity of adeno-associated virus is involved in endosomal escape of incoming particles. *Virology* 409, 77–83 (2011).
30. Kelich, J. M. *et al.* Super-resolution imaging of nuclear import of adeno-associated virus in live cells. *Mol Ther Methods Clin Dev* 2, (2015).
31. Junod, S. L., Saredy, J. & Yang, W. Nuclear import of adeno-associated viruses imaged by high-speed single-molecule microscopy. *Viruses* vol. 13 Preprint at <https://doi.org/10.3390/v13020167> (2021).
32. Popa-Wagner, R. *et al.* Impact of VP1-Specific Protein Sequence Motifs on Adeno-Associated Virus Type 2 Intracellular Trafficking and Nuclear Entry. *J Virol* 86, 9163–9174 (2012).
33. DiMattia, M. A. *et al.* Structural Insight into the Unique Properties of Adeno-Associated Virus Serotype 9. *J Virol* 86, 6947–6958 (2012).
34. Meyer, N. *et al.* Structure of the Gene Therapy Vector, Adeno-Associated Virus With Its Cell Receptor, AAVR. *Elife* (2019) doi:10.7554/elife.44707.
35. Xie, Q. *et al.* The Atomic Structure of Adeno-Associated Virus (AAV-2), a Vector for Human Gene Therapy. *Proceedings of the National Academy of Sciences* 99, 10405–10410 (2002).

36. Xie, Q. *et al.* The 2.8 Å Electron Microscopy Structure of Adeno-Associated Virus-DJ Bound by a Heparinoid Pentasaccharide. *Mol Ther Methods Clin Dev* (2017) doi:10.1016/j.omtm.2017.02.004.
37. Santosh, V. *et al.* The Cryo-Em Structure of AAV2 Rep68 in Complex With ssDNA Reveals a Malleable AAA+ Machine That Can Switch Between Oligomeric States. *Nucleic Acids Res* 48, 12983–12999 (2020).
38. C, G. J., Stephen, S. & J, S. R. Separate Basic Region Motifs within the Adeno-Associated Virus Capsid Proteins Are Essential for Infectivity and Assembly. *J Virol* 80, 5199–5210 (2006).
39. Summerford, C. & Samulski, R. J. *Membrane-Associated Heparan Sulfate Proteoglycan Is a Receptor for Adeno-Associated Virus Type 2 Virions.* *JOURNAL OF VIROLOGY* vol. 72 (1998).
40. Mietzsch, M., Broecker, F., Reinhardt, A., Seeberger, P. H. & Heilbronn, R. Differential Adeno-Associated Virus Serotype-Specific Interaction Patterns With Synthetic Heparins and Other Glycans. *J Virol* (2014) doi:10.1128/jvi.03371-13.
41. Vandenberghe, L. H. *et al.* Efficient serotype-dependent release of functional vector into the culture medium during adeno-associated virus manufacturing. *Hum Gene Ther* 21, 1251–1257 (2010).
42. Hüser, D., Weger, S. & Heilbronn, R. Kinetics and Frequency of Adeno-Associated Virus Site-Specific Integration Into Human Chromosome 19 Monitored by Quantitative Real-Time PCR. *J Virol* 76, 7554–7559 (2002).
43. Slanina, H., Weger, S., Stow, N. D., Kuhrs, A. & Heilbronn, R. Role of the Herpes Simplex Virus Helicase-Primase Complex During Adeno-Associated Virus DNA Replication. *J Virol* 80, 5241–5250 (2006).
44. Young, S., McCarty, D. M., Degtyareva, N. & Samulski, R. J. Roles of Adeno-Associated Virus Rep Protein and Human Chromosome 19 in Site-Specific Recombination. *J Virol* 74, 3953–3966 (2000).
45. Wang, L. *et al.* Productive life cycle of adeno-associated virus serotype 2 in the complete absence of a conventional polyadenylation signal. *Journal of General Virology* 96, 2780–2787 (2015).
46. Akache, B. *et al.* The 37/67-Kilodalton Laminin Receptor Is a Receptor for Adeno-Associated Virus Serotypes 8, 2, 3, and 9. *J Virol* 80, 9831–9836 (2006).
47. Pillay, S. *et al.* Adeno-Associated Virus (AAV) Serotypes Have Distinctive Interactions With Domains of the Cellular AAV Receptor. *J Virol* (2017) doi:10.1128/jvi.00391-17.
48. Dudek, A. M. *et al.* GPR108 Is a Highly Conserved AAV Entry Factor. *Molecular Therapy* 28, 367–381 (2020).
49. Uhrig, S. *et al.* Successful Target Cell Transduction of Capsid-Engineered rAAV Vectors Requires Clathrin-Dependent Endocytosis. *Gene Ther* 19, 210–218 (2011).
50. Jin, L. *et al.* Ultrasound Targeted Microbubble Destruction Stimulates Cellular Endocytosis in Facilitation of Adeno-Associated Virus Delivery. *Int J Mol Sci* 14, 9737–9750 (2013).
51. Castle, M. J., Perlson, E., Holzbaur, E. L. F. & Wolfe, J. H. Long-distance axonal transport of AAV9 is driven by dynein and kinesin-2 and is trafficked in a highly motile Rab7-positive compartment. *Molecular Therapy* 22, 554–566 (2014).
52. Dongsheng, D. *et al.* Dynamin Is Required for Recombinant Adeno-Associated Virus Type 2 Infection. *J Virol* 73, 10371–10376 (1999).

53. Nonnenmacher, M. & Weber, T. Adeno-Associated Virus 2 Infection Requires Endocytosis Through the CLIC/GEEC Pathway. *Cell Host Microbe* (2011) doi:10.1016/j.chom.2011.10.014.
54. Nonnenmacher, M. & Weber, T. Intracellular transport of recombinant adeno-associated virus vectors. *Gene Ther* 19, 649–658 (2012).
55. Florian, S., Svenja, B., Barbara, L., Roger, F. & A, K. J. Adeno-Associated Virus Type 2 Capsids with Externalized VP1/VP2 Trafficking Domains Are Generated prior to Passage through the Cytoplasm and Are Maintained until Uncoating Occurs in the Nucleus. *J Virol* 80, 11040–11054 (2006).
56. Choi, V. W., McCarty, D. M. & Samulski, R. J. Host Cell DNA Repair Pathways in Adeno-Associated Viral Genome Processing. *J Virol* 80, 10346–10356 (2006).
57. Hauck, B., Zhao, W., High, K. A. & Xiao, W. Intracellular Viral Processing, Not Single-Stranded DNA Accumulation, Is Crucial for Recombinant Adeno-Associated Virus Transduction. *J Virol* 78, 13678–13686 (2004).
58. Li, Z., Brister, J. R., Im, D. S. & Muzyczka, N. Characterization of the adenoassociated virus Rep protein complex formed on the viral origin of DNA replication. *Virology* 313, 364–376 (2003).
59. Yamamoto, N. *et al.* Adeno-Associated Virus Site-Specific Integration Is Regulated by TRP-185. *J Virol* 81, 1990–2001 (2007).
60. Ward, P. & Elias, P. Rescue of the Adeno-Associated Virus Genome From a Plasmid Vector: Evidence for Rescue by Replication. *J Virol* 77, 11480–11490 (2003).
61. Henckaerts, E. & Linden, R. J. Adeno-Associated Virus: A Key to the Human Genome? *Future Virol* 5, 555–574 (2010).
62. Matsushita, T. *et al.* Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther* 5, 938–945 (1998).
63. Choi, Y.-H., Nash, K., Byrne, B. J., Muzyczka, N. & Song, S. The Effect of DNA-Dependent Protein Kinase on Adeno-Associated Virus Replication. *PLoS One* 5, e15073 (2010).
64. Wu, Z., Asokan, A. & Samulski, R. J. Adeno-associated Virus Serotypes: Vector Toolkit for Human Gene Therapy. *Molecular Therapy* 14, 316–327 (2006).
65. Samulski, R. J., Berns, K. I., Tan, M. & Muzyczka, N. Cloning of adeno-associated virus into pBR322: Rescue of intact virus from the recombinant plasmid in human cells. *Proc Natl Acad Sci U S A* 79, 2077–2081 (1982).
66. Grimm, D. & Kay, M. A. From Virus Evolution to Vector Revolution: Use of Naturally Occurring Serotypes of Adeno-Associated Virus (AAV) as Novel Vectors for Human Gene Therapy. *Curr Gene Ther* 3, 281–304 (2003).
67. Wagner, J. A. *et al.* Safety and Biological Efficacy of an Adeno-Associated Virus Vector–Cystic Fibrosis Transmembrane Regulator (AAV-CFTR) in the Cystic Fibrosis Maxillary Sinus. *Laryngoscope* 109, 266–274 (1999).
68. Ylä-Herttuala, S. Endgame: Glybera finally recommended for approval as the first gene therapy drug in the European union. *Molecular Therapy* vol. 20 1831–1832 Preprint at <https://doi.org/10.1038/mt.2012.194> (2012).
69. FDA.gov. FDA Approves First Gene Therapy for Treatment of Aromatic L-amino Acid Decarboxylase Deficiency. https://www.fda.gov/news-events/press-announcements/fda-approves-first-gene-therapy-treatment-aromatic-l-amino-acid-decarboxylase-deficiency?utm_source=chatgpt.com (2024).

70. Wang, J.-H., Gessler, D. J., Zhan, W., Gallagher, T. L. & Gao, G. Adeno-associated virus as a delivery vector for gene therapy of human diseases. *Signal Transduct Target Ther* 9, 78 (2024).
71. Warrington, K. H. & Herzog, R. W. Treatment of Human Disease by Adeno-Associated Viral Gene Transfer. *Hum Genet* 119, 571–603 (2006).
72. Tenenbaum, L., Lehtonen, E. & Monahan, P. E. Evaluation of Risks Related to the Use of Adeno-Associated Virus-Based Vectors. *Curr Gene Ther* 3, 545–565 (2003).
73. Dismuke, D. J., Tenenbaum, L. & Samulski, R. J. Biosafety of Recombinant Adeno-Associated Virus Vectors. *Curr Gene Ther* 13, 434–452 (2014).
74. Penaud-Budloo, M. *et al.* Adeno-Associated Virus Vector Genomes Persist as Episomal Chromatin in Primate Muscle. *J Virol* 82, 7875–7885 (2008).
75. Dongsheng, D. *et al.* Circular Intermediates of Recombinant Adeno-Associated Virus Have Defined Structural Characteristics Responsible for Long-Term Episomal Persistence in Muscle Tissue. *J Virol* 72, 8568–8577 (1998).
76. Wang, Z. *et al.* Adeno-Associated Virus Serotype 8 Efficiently Delivers Genes to Muscle and Heart. *Nat Biotechnol* 23, 321–328 (2005).
77. Flotte, T. R. Gene Therapy Progress and Prospects: Recombinant Adeno-Associated Virus (rAAV) Vectors. *Gene Ther* 11, 805–810 (2004).
78. Koerber, J. T., Jang, J. H. & Schaffer, D. V. DNA shuffling of adeno-associated virus yields functionally diverse viral progeny. *Molecular Therapy* 16, 1703–1709 (2008).
79. Han, J. *et al.* Rational engineering of adeno-associated virus capsid enhances human hepatocyte tropism and reduces immunogenicity. *Cell Prolif* 55, (2022).
80. Bartel, M. A., Weinstein, J. R. & Schaffer, D. V. Directed evolution of novel adeno-associated viruses for therapeutic gene delivery. *Gene Therapy* vol. 19 694–700 Preprint at <https://doi.org/10.1038/gt.2012.20> (2012).
81. Hoffmann, M. D., Gallant, J. P., LeBeau, A. M. & Schmidt, D. Unlocking precision gene therapy: harnessing AAV tropism with nanobody swapping at capsid hotspots. *NAR Molecular Medicine* 1, (2024).
82. Büning, H. & Srivastava, A. Capsid Modifications for Targeting and Improving the Efficacy of AAV Vectors. *Molecular Therapy - Methods and Clinical Development* vol. 12 248–265 Preprint at <https://doi.org/10.1016/j.omtm.2019.01.008> (2019).
83. Varadi, K. *et al.* Novel random peptide libraries displayed on AAV serotype 9 for selection of endothelial cell-directed gene transfer vectors. *Gene Ther* 19, 800–809 (2012).
84. Girod, A. *et al.* Genetic capsid modifications allow efficient re-targeting of adeno-associated virus type 2. *Nat Med* 5, 1052–1056 (1999).
85. Hamann, M. V *et al.* Improved Targeting of Human CD4+ T Cells by Nanobody-Modified AAV2 Gene Therapy Vectors. *PLoS One* 16, e0261269 (2021).
86. Chen, Y. A Comprehensive Study of the Effects by Sequence Truncation Within Inverted Terminal Repeats (ITRs) on the Productivity, Genome Packaging, and Potency of AAV Vectors. *Microorganisms* 12, 310 (2024).
87. Faust, S. M. *et al.* CpG-depleted adeno-associated virus vectors evade immune detection. *J Clin Invest* 123, 2994–3001 (2013).
88. Pan, X. *et al.* Rational engineering of a functional CpG-free ITR for AAV gene therapy. *Gene Ther* 29, 333–345 (2022).
89. Song, L. *et al.* AAV vector transduction restriction and attenuated toxicity in hESCs via a rationally designed inverted terminal repeat. *Nucleic Acids Res* 53, gkaf013 (2025).

90. Yuan, Y. Stabilization of a Single-Stranded DNA of Adeno-Associated Virus by Inverted Terminal Repeats. (2024) doi:10.21203/rs.3.rs-4126250/v1.
91. Foust, K. D. *et al.* Intravascular AAV9 Preferentially Targets Neonatal Neurons and Adult Astrocytes. *Nat Biotechnol* 27, 59–65 (2008).
92. Liu, J., Koay, T. W., Maiakovska, O., Zayas, M. & Grimm, D. Progress in Bioengineering of Myotropic Adeno-Associated Viral Gene Therapy Vectors. *Human Gene Therapy* vol. 34 350–364 Preprint at <https://doi.org/10.1089/hum.2023.057> (2023).
93. Han, Z. *et al.* Computer-Aided Directed Evolution Generates Novel AAV Variants with High Transduction Efficiency. *Viruses* 15, (2023).
94. Westhaus, A. *et al.* AAV capsid bioengineering in primary human retina models. *Sci Rep* 13, (2023).
95. Goedeker, N. L. *et al.* Evaluation of rAAVrh74 Gene Therapy Vector Seroprevalence by Measurement of Total Binding Antibodies in Patients With Duchenne Muscular Dystrophy. *Ther Adv Neurol Disord* 16, (2023).
96. Barnes, C. R., Scheideler, O. J. & Schaffer, D. V. Engineering the AAV Capsid to Evade Immune Responses. *Curr Opin Biotechnol* 60, 99–103 (2019).
97. Frontino, G., Stancampiano, M. R. & Aiuti, A. Potentialities of Gene Therapy in Pediatric Endocrinology. *Horm Res Paediatr* 96, 646–657 (2021).
98. Duan, D. Lethal immunotoxicity in high-dose systemic AAV therapy. *Molecular Therapy* 31, 3123–3126 (2023).
99. Neurogene Provides Update on NGN-401 Gene Therapy Clinical Trial for Rett Syndrome | Neurogene Inc. <https://ir.neurogene.com/news-releases/news-release-details/neurogene-provides-update-ngn-401-gene-therapy-clinical-trial>.
100. Lek, A. *et al.* Death after High-Dose rAAV9 Gene Therapy in a Patient with Duchenne's Muscular Dystrophy. *New England Journal of Medicine* 389, 1203–1210 (2023).
101. Rasko, P. J. *et al.* Global Seroprevalence of Neutralizing Antibodies Against Adeno-Associated Virus (AAV) Serotypes of Relevance to Gene Therapy. *Blood* 140, 10668–10670 (2022).
102. Hamilton, B. A. & Wright, J. F. Challenges Posed by Immune Responses to AAV Vectors: Addressing Root Causes. *Front Immunol* 12, (2021).
103. Mingozzi, F. & High, K. A. Overcoming the Host Immune Response to Adeno-Associated Virus Gene Delivery Vectors: The Race between Clearance, Tolerance, Neutralization, and Escape. *Annu Rev Virol* 4, 511–534 (2017).
104. Herzog, R. W. Complexity of immune responses to AAV transgene products – Example of factor IX. *Cellular Immunology* vol. 342 Preprint at <https://doi.org/10.1016/j.cellimm.2017.05.006> (2019).
105. Su, W. AAV Production in Stable Packaging Cells Requires Expression of Adenovirus 22/33k Protein to Allow Episomal Amplification of Integrated Rep/Cap Genes. *Sci Rep* 13, (2023).
106. Merten, O. W. Development of Stable Packaging and Producer Cell Lines for the Production of AAV Vectors. *Microorganisms* vol. 12 Preprint at <https://doi.org/10.3390/microorganisms12020384> (2024).
107. Yuan, Z., Qiao, C., Hu, P., Li, J. & Xiao, X. A versatile adeno-associated virus vector producer cell line method for scalable vector production of different serotypes. *Hum Gene Ther* 22, 613–624 (2011).

108. Chargaff, E. & West, R. The biological significance of the thromboplastic protein of Wood. *Journal of Biological Chemistry* 166, 189–197 (1946).
109. Wolf, P. The Nature and Significance of Platelet Products in Human Plasma. *Br J Haematol* 13, 269–288 (1967).
110. Pan, B. T. & Johnstone, R. M. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: Selective externalization of the receptor. *Cell* 33, 967–978 (1983).
111. Harding, C., Heuser, J. & Stahl, P. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J Cell Biol* 97, 329–339 (1983).
112. Raposo, G. *et al.* B lymphocytes secrete antigen-presenting vesicles. *Journal of Experimental Medicine* 183, 1161–1172 (1996).
113. Crescitelli, R. *et al.* Distinct RNA Profiles in Subpopulations of Extracellular Vesicles: Apoptotic Bodies, Microvesicles and Exosomes. *J Extracell Vesicles* (2013) doi:10.3402/jev.v2i0.20677.
114. Valadi, H. *et al.* Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 9, 654–659 (2007).
115. Théry, C. *et al.* Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018): A Position Statement of the International Society for Extracellular Vesicles and Update of the MISEV2014 Guidelines. *J Extracell Vesicles* (2018) doi:10.1080/20013078.2018.1535750.
116. Raposo, G. & Stoorvogel, W. Extracellular vesicles: Exosomes, microvesicles, and friends. *Journal of Cell Biology* vol. 200 373–383 Preprint at <https://doi.org/10.1083/jcb.201211138> (2013).
117. Hurley, J. H. The ESCRT complexes. *Critical Reviews in Biochemistry and Molecular Biology* vol. 45 463–487 Preprint at <https://doi.org/10.3109/10409238.2010.502516> (2010).
118. Hurley, J. H. & Hanson, P. I. Membrane budding and scission by the ESCRT machinery: It's all in the neck. *Nature Reviews Molecular Cell Biology* vol. 11 556–566 Preprint at <https://doi.org/10.1038/nrm2937> (2010).
119. Katzmann, D. J., Odorizzi, G. & Emr, S. D. Receptor downregulation and multivesicular-body sorting. *Nat Rev Mol Cell Biol* 3, 893–905 (2002).
120. Trajkovic, K. *et al.* Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science* (1979) 319, 1244–1247 (2008).
121. Wei, D. *et al.* RAB31 marks and controls an ESCRT-independent exosome pathway. *Cell Res* 31, 157–177 (2021).
122. Stenmark, H. *et al.* Inhibition of rab5 GTPase activity stimulates membrane fusion in endocytosis. *EMBO Journal* 13, 1287–1296 (1994).
123. Ostrowski, M. *et al.* Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nat Cell Biol* 12, 19–30 (2010).
124. Savina, A., Fader, C. M., Damiani, M. T. & Colombo, M. I. Rab11 promotes docking and fusion of multivesicular bodies in a calcium-dependent manner. *Traffic* 6, 131–143 (2005).
125. Taylor, J. & Bebawy, M. Proteins Regulating Microvesicle Biogenesis and Multidrug Resistance in Cancer. *Proteomics* vol. 19 Preprint at <https://doi.org/10.1002/pmic.201800165> (2019).

126. Mathieu, M. *et al.* Specificities of exosome versus small ectosome secretion revealed by live intracellular tracking of CD63 and CD9. *Nature Communications* 2021 12:1 12, 1–18 (2021).
127. Choi, D.-S., Kim, D.-K., Kim, E. C. & Gho, Y. S. Proteomics, Transcriptomics and Lipidomics of Exosomes and Ectosomes. *Proteomics* 13, 1554–1571 (2013).
128. Muralidharan-Chari, V., Clancy, J. W., Sedgwick, A. & D'Souza-Schorey, C. Microvesicles: Mediators of extracellular communication during cancer progression. *Journal of Cell Science* vol. 123 1603–1611 Preprint at <https://doi.org/10.1242/jcs.064386> (2010).
129. Sedgwick, A., Clancy, J., Balmert, M. O. & D'Souza-Schorey, C. Extracellular Microvesicles and Invadopodia Mediate Non-Overlapping Modes of Tumor Cell Invasion. *Sci Rep* (2015) doi:10.1038/srep14748.
130. Hurley, J. H. ESCRT s are everywhere . *EMBO J* 34, 2398–2407 (2015).
131. Bernardi, S. & Balbi, C. Extracellular Vesicles: From Biomarkers to Therapeutic Tools. *Biology (Basel)* (2020) doi:10.3390/biology9090258.
132. Wen, J. Comparison of Immunotherapy Mediated by Apoptotic Bodies, Microvesicles and Exosomes: Apoptotic Bodies' Unique Anti-Inflammatory Potential. *J Transl Med* (2023) doi:10.1186/s12967-023-04342-w.
133. Bartel, S., Deshane, J., Wilkinson, T. & Gabrielsson, S. Extracellular Vesicles as Mediators of Cellular Cross Talk in the Lung Microenvironment. *Front Med (Lausanne)* 7, (2020).
134. Osteikoetxea, X. *et al.* Improved Characterization of EV Preparations Based on Protein to Lipid Ratio and Lipid Properties. *PLoS One* (2015) doi:10.1371/journal.pone.0121184.
135. Alfsen, A. & Tatischeff, I. The Lipid Bilayer of Biological Vesicles: A Liquid-Crystalline Material as Nanovehicles of Information. *J Biomater Nanobiotechnol* 05, 105–115 (2014).
136. Pedrioli, G., Piovesana, E., Vacchi, E. & Balbi, C. Extracellular Vesicles as Promising Carriers in Drug Delivery: Considerations From a Cell Biologist's Perspective. *Biology (Basel)* 10, 376 (2021).
137. Kordelas, L. *et al.* MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. *Leukemia* 28, 970–973 (2014).
138. Lai, R. C. *et al.* MSC Secretes at Least 3 EV Types Each With a Unique Permutation of Membrane Lipid, Protein and RNA. *J Extracell Vesicles* (2016) doi:10.3402/jev.v5.29828.
139. Penault-Llorca, F. & Radosevich-Robin, N. Biomarkers of residual disease after neoadjuvant therapy for breast cancer. *Nat Rev Clin Oncol* 13, 487–503 (2016).
140. Butreddy, A., Kommineni, N., Dudhipala, N., Guerrero-Martínez, A. & Valpuesta, J. M. Exosomes as Naturally Occurring Vehicles for Delivery of Biopharmaceuticals: Insights from Drug Delivery to Clinical Perspectives. (2021) doi:10.3390/nano11061481.
141. Lv, P. *et al.* Genetically Engineered Cell Membrane Nanovesicles for Oncolytic Adenovirus Delivery: A Versatile Platform for Cancer Virotherapy. *Nano Lett* 19, 2993–3001 (2019).
142. Hu, J.-C., Zheng, C.-X., Sui, B.-D., Liu, W.-J. & Jin, Y. Mesenchymal stem cell-derived exosomes: A novel and potential remedy for cutaneous wound healing and regeneration. *World J Stem Cells* 14, 318–329 (2022).
143. Golan, M. E. & Stice, S. L. Extracellular vesicle lyophilization for enhanced distribution to the point of care. *Extracellular Vesicle* 3, 100041 (2024).

144. Bader, J., Brigger, F. & Leroux, J. C. Extracellular vesicles versus lipid nanoparticles for the delivery of nucleic acids. *Advanced Drug Delivery Reviews* vol. 215 Preprint at <https://doi.org/10.1016/j.addr.2024.115461> (2024).
145. Murphy, D. E. *et al.* Natural or synthetic RNA delivery: A stoichiometric comparison of extracellular vesicles and synthetic nanoparticles. *Nano Lett* 21, 1888–1895 (2021).
146. Wiklander, O. P. B. *et al.* Antibody-displaying extracellular vesicles for targeted cancer therapy. *Nat Biomed Eng* (2024) doi:10.1038/s41551-024-01214-6.
147. Liang, X. *et al.* Multimodal Engineering of Extracellular Vesicles for Efficient Intracellular Protein Delivery. (2023) doi:10.1101/2023.04.30.535834.
148. Ramos-Zaldívar, H. M. *et al.* Extracellular Vesicles Through the Blood–brain Barrier: A Review. *Fluids Barriers CNS* 19, (2022).
149. Parada, N., Romero-Trujillo, A., Georges, N. & Alcayaga-Miranda, F. Camouflage strategies for therapeutic exosomes evasion from phagocytosis. *Journal of Advanced Research* vol. 31 61–74 Preprint at <https://doi.org/10.1016/j.jare.2021.01.001> (2021).
150. Hosseini-Kharat, M., Bremmell, K. E. & Prestidge, C. A. Why do lipid nanoparticles target the liver? Understanding of biodistribution and liver-specific tropism. *Molecular Therapy Methods and Clinical Development* vol. 33 Preprint at <https://doi.org/10.1016/j.omtm.2025.101436> (2025).
151. Cheng, M. H. Y. *et al.* Liposomal lipid nanoparticles for extrahepatic delivery of mRNA. *Nat Commun* 16, 4135 (2025).
152. Lamichhane, T. N. *et al.* Oncogene Knockdown via Active Loading of Small RNAs into Extracellular Vesicles by Sonication. *Cell Mol Bioeng* 9, 315–324 (2016).
153. El-Andaloussi, S. *et al.* Exosome-mediated delivery of siRNA in vitro and in vivo. *Nat Protoc* 7, 2112–2126 (2012).
154. Alvarez-Erviti, L. *et al.* Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat Biotechnol* 29, 341–345 (2011).
155. Xu, G. *et al.* Structural Basis for the Neurotropic AAV9 and the Engineered AAVPHP.eB Recognition With Cellular Receptors. *Mol Ther Methods Clin Dev* (2022) doi:10.1016/j.omtm.2022.05.009.
156. Lukashchuk, V., Lewis, K. E., Coldicott, I., Grierson, A. J. & Azzouz, M. AAV9-mediated Central Nervous System–targeted Gene Delivery via Cisterna Magna Route in Mice. *Mol Ther Methods Clin Dev* (2016) doi:10.1038/mtm.2015.55.
157. Huang, Q. *et al.* Delivering genes across the blood-brain barrier: LY6A, a novel cellular receptor for AAV-PHP.B capsids. *PLoS One* 14, e0225206- (2019).
158. Chan, K. Y. *et al.* Engineered AAVs for Efficient Noninvasive Gene Delivery to the Central and Peripheral Nervous Systems. *Nat Neurosci* (2017) doi:10.1038/nn.4593.
159. Nolte-‘t Hoen, E., Cremer, T., Gallo, R. C. & Margolis, L. B. Extracellular vesicles and viruses: Are they close relatives? *Proceedings of the National Academy of Sciences* 113, 9155–9161 (2016).
160. Votteler, J. & Sundquist, W. I. Virus Budding and the ESCRT Pathway. *Cell Host Microbe* (2013) doi:10.1016/j.chom.2013.08.012.
161. Poletti, V. & Mavilio, F. Designing Lentiviral Vectors for Gene Therapy of Genetic Diseases. *Viruses* 13, (2021).
162. Annoni, A., Gregori, S., Naldini, L. & Cantore, A. Modulation of immune responses in lentiviral vector-mediated gene transfer. *Cell Immunol* 342, 103802 (2019).

163. Faserl, K., Chetwynd, A. J., Lynch, I., Thorn, J. A. & Lindner, H. Corona Isolation Method Matters: Capillary Electrophoresis Mass Spectrometry Based Comparison of Protein Corona Compositions Following on-Particle Versus in-Solution or in-Gel Digestion. *Nanomaterials* (2019) doi:10.3390/nano9060898.
164. Costa-Silva, B. *et al.* Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. *Nat Cell Biol* 17, 816–826 (2015).
165. Walz, S. *et al.* MYC regulates the antitumor immune response through CD47 and PD-L1. *Science* (1979) 352, 227–231 (2016).
166. Gečys, D. *et al.* Internalisation of RGD-Engineered Extracellular Vesicles by Glioblastoma Cells. *Biology (Basel)* 11, (2022).
167. Zheng, W. *et al.* Identification of scaffold proteins for improved endogenous engineering of extracellular vesicles. *Nat Commun* 14, (2023).
168. Martin Perez, C. *et al.* An extracellular vesicle delivery platform based on the PTTG1IP protein. *Extracellular Vesicle* 4, 100054 (2024).
169. Haraszti, R. A., Coles, A. H., Aronin, N., Khvorova, A. & Didiot, M.-C. Loading of Extracellular Vesicles With Chemically Stabilized Hydrophobic siRNAs for the Treatment of Disease in the Central Nervous System. *Bio Protoc* 7, (2017).
170. Zhu, L. *et al.* Aptamer-based Extracellular Vesicle Isolation, Analysis and Therapeutics. *Interdisciplinary Medicine* 1, (2023).
171. Wang, L., Yu, X., Zhou, J. & Su, C. Extracellular Vesicles for Drug Delivery in Cancer Treatment. *Biol Proced Online* 25, 28 (2023).
172. Dooley, K. *et al.* A versatile platform for generating engineered extracellular vesicles with defined therapeutic properties. *Molecular Therapy* 29, 1729–1743 (2021).
173. Feng, Z., Hirai-Yuki, A., McKnight, K. L. & Lemon, S. M. Naked viruses that aren't always naked: Quasi-enveloped agents of acute hepatitis. *Annu Rev Virol* 1, 539–560 (2014).
174. Jiang, W. *et al.* Hepatitis A virus structural protein pX interacts with ALIX and promotes the secretion of virions and foreign proteins through exosome-like vesicles. *J Extracell Vesicles* 9, (2020).
175. Feng, Z. *et al.* A pathogenic picornavirus acquires an envelope by hijacking cellular membranes. *Nature* 496, 367–371 (2013).
176. Chen, Y. H. *et al.* Phosphatidylserine Vesicles Enable Efficient en Bloc Transmission of Enteroviruses. *Cell* (2015) doi:10.1016/j.cell.2015.01.032.
177. Maguire, C. A. *et al.* Microvesicle-associated AAV vector as a novel gene delivery system. *Molecular Therapy* 20, 960–971 (2012).
178. Cheng, M. *et al.* Neutralizing Antibody Evasion and Transduction with Purified Extracellular Vesicle-Enveloped Adeno-Associated Virus Vectors. *Hum Gene Ther* 32, 1457–1470 (2021).
179. György, B. *et al.* Rescue of Hearing by Gene Delivery to Inner-Ear Hair Cells Using Exosome-Associated AAV. *Molecular Therapy* 25, 379–391 (2017).
180. Wassmer, S. J., Carvalho, L. S., György, B., Vandenberghe, L. H. & Maguire, C. A. Exosome-associated AAV2 vector mediates robust gene delivery into the murine retina upon intravitreal injection. *Sci Rep* 7, (2017).
181. Meliani, A. *et al.* Enhanced liver gene transfer and evasion of preexisting humoral immunity with exosome-enveloped AAV vectors. *Blood Adv* 1, 2019–2031 (2017).

182. Haar, J., Blazevic, D., Strobel, B., Kreuz, S. & Michelfelder, S. MSD-based assays facilitate a rapid and quantitative serostatus profiling for the presence of anti-AAV antibodies. *Mol Ther Methods Clin Dev* 25, 360–369 (2022).
183. Moggridge, S., Sorensen, P. H., Morin, G. B. & Hughes, C. S. Extending the Compatibility of the SP3 Paramagnetic Bead Processing Approach for Proteomics. *J Proteome Res* 17, 1730–1740 (2018).
184. Kim, S. & Pevzner, P. A. MS-GF+ makes progress towards a universal database search tool for proteomics. *Nat Commun* 5, (2014).
185. Savitski, M. M., Wilhelm, M., Hahne, H., Kuster, B. & Bantscheff, M. A scalable approach for protein false discovery rate estimation in large proteomic data sets. *Molecular and Cellular Proteomics* 14, 2394–2404 (2015).
186. Bardou, P., Mariette, J., Escudié, F., Djemiel, C. & Klopp, C. Jvenn: An interactive Venn diagram viewer. *BMC Bioinformatics* 15, (2014).
187. Metsalu, T. & Vilo, J. ClustVis: A web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. *Nucleic Acids Res* 43, W566–W570 (2015).
188. Tyanova, S. *et al.* The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat Methods* 13, 731–740 (2016).
189. Martin, M. *Cutadapt Removes Adapter Sequences from High-Throughput Sequencing Reads*. <http://www-huber.embl.de/users/an/> (2011).
190. Mendell, J. R. *et al.* Current Clinical Applications of In Vivo Gene Therapy with AAVs. *Molecular Therapy* 29, 464 (2021).
191. Manno, C. S. *et al.* Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med* 12, 342–347 (2006).
192. Guo, P. *et al.* Rapid AAV-Neutralizing Antibody Determination with a Cell-Binding Assay. *Mol Ther Methods Clin Dev* 13, 40–46 (2019).
193. Kuzmin, D. A. *et al.* The clinical landscape for AAV gene therapies. *Nat Rev Drug Discov* 20, 173–174 (2021).
194. High-dose AAV gene therapy deaths. *Nature biotechnology* vol. 38 910 Preprint at <https://doi.org/10.1038/s41587-020-0642-9> (2020).
195. Wollert, T. & Hurley, J. H. Molecular mechanism of multivesicular body biogenesis by ESCRT complexes. *Nature* 464, 864–869 (2010).
196. Henne, W. M., Buchkovich, N. J. & Emr, S. D. The ESCRT Pathway. *Developmental Cell* vol. 21 77–91 Preprint at <https://doi.org/10.1016/j.devcel.2011.05.015> (2011).
197. Colombo, M. *et al.* Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *J Cell Sci* 126, 5553–5565 (2013).
198. Caruso, S. & Poon, I. K. H. Apoptotic cell-derived extracellular vesicles: More than just debris. *Frontiers in Immunology* vol. 9 Preprint at <https://doi.org/10.3389/fimmu.2018.01486> (2018).
199. Prasad, K.-M. R., Xu, Y., Yang, Z., Acton, S. T. & French, B. A. Robust Cardiomyocyte-Specific Gene Expression Following Systemic Injection of AAV: In Vivo Gene Delivery Follows a Poisson Distribution. *Gene Ther* (2010) doi:10.1038/gt.2010.105.
200. Fitzpatrick, Z., György, B., Skog, J. & Maguire, C. A. Extracellular vesicles as enhancers of virus vector-mediated gene delivery. *Hum Gene Ther* 25, 785–786 (2014).

201. Jimenez, D. E. Comparison of Four Purification Methods on Serum Extracellular Vesicle Recovery, Size Distribution, and Proteomics. *Proteomes* (2023) doi:10.3390/proteomes11030023.
202. Willms, E. *et al.* Cells Release Subpopulations of Exosomes With Distinct Molecular and Biological Properties. *Sci Rep* (2016) doi:10.1038/srep22519.
203. Willms, E., Cabañas, C., Mäger, I., Wood, M. J. A. & Vader, P. Extracellular vesicle heterogeneity: Subpopulations, isolation techniques, and diverse functions in cancer progression. *Frontiers in Immunology* vol. 9 Preprint at <https://doi.org/10.3389/fimmu.2018.00738> (2018).
204. Théry, C., Amigorena, S., Raposo, G. & Clayton, A. Isolation and Characterization of Exosomes from Cell Culture Supernatants and Biological Fluids. *Curr Protoc Cell Biol* 30, 3.22.1-3.22.29 (2006).
205. Bajar, B. T. *et al.* Improving brightness and photostability of green and red fluorescent proteins for live cell imaging and FRET reporting OPEN. (2016) doi:10.1038/srep20889.
206. Cashikar Anil G. AND Hanson, P. I. A cell-based assay for CD63-containing extracellular vesicles. *PLoS One* 14, 1–19 (2019).
207. Brassart, B. *et al.* Tumour cell blebbing and extracellular vesicle shedding: key role of matrikines and ribosomal protein SA. *Br J Cancer* 120, 453–465 (2019).
208. Liu, B. *et al.* AAV-Containing Exosomes as a Novel Vector for Improved Gene Delivery to Lung Cancer Cells. *Front Cell Dev Biol* (2021) doi:10.3389/fcell.2021.707607.
209. Henriques, C. Isolation of Biologically Active Extracellular Vesicles-Associated AAVs for Gene Delivery to the Brain by Size Exclusion Chromatography. (2023) doi:10.1101/2023.05.30.542901.
210. György, B. & Maguire, C. A. Extracellular vesicles: nature's nanoparticles for improving gene transfer with adeno-associated virus vectors. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology* vol. 10 Preprint at <https://doi.org/10.1002/wnan.1488> (2018).
211. Hudry, E. *et al.* Exosome-associated AAV vector as a robust and convenient neuroscience tool. *Gene Ther* 23, 380–392 (2016).
212. de Mello, R. G. *et al.* Zika Virus-Like Particles (VLPs) Produced in Insect Cells. *Front Pharmacol* (2023) doi:10.3389/fphar.2023.1181566.
213. Kovacovicova, K. & Vinciguerra, M. Isolation of Senescent Cells by Iodixanol (OptiPrep) Density Gradient-based Separation. *Cell Prolif* (2019) doi:10.1111/cpr.12674.
214. Zicari, S. *et al.* Human Cytomegalovirus-Infected Cells Release Extracellular Vesicles That Carry Viral Surface Proteins. *Virology* (2018) doi:10.1016/j.virol.2018.08.008.
215. Elmore, Z. C., Havlik, L. P., Oh, D., A. Vincent, H. & Asokan, A. The Membrane Associated Accessory Protein Is an Adeno-Associated Viral Egress Factor. (2021) doi:10.1101/2021.06.17.448857.
216. Leventis, P. A. & Grinstein, S. The Distribution and Function of Phosphatidylserine in Cellular Membranes. *Annual Review of Biophysics* Preprint at <https://doi.org/10.1146/annurev.biophys.093008.131234> (2010).
217. Nakai, W. *et al.* A Novel Affinity-Based Method for the Isolation of Highly Purified Extracellular Vesicles. *Sci Rep* (2016) doi:10.1038/srep33935.
218. Pillay, S. *et al.* An essential receptor for adeno-associated virus infection. *Nature* 530, 108–112 (2016).

219. Havlik, L. P. *et al.* Receptor Switching in Newly Evolved Adeno-Associated Viruses. *J Virol* (2021) doi:10.1128/jvi.00587-21.
220. Zhang, R. *et al.* Divergent Engagements Between Adeno-Associated Viruses With Their Cellular Receptor AAVR. *Nat Commun* (2019) doi:10.1038/s41467-019-11668-x.
221. Hamilton, B. A., Li, X., Pezzulo, A. A., Abou Alaiwa, M. H. & Zabner, J. Polarized AAVR Expression Determines Infectivity by AAV Gene Therapy Vectors. *Gene Ther* (2019) doi:10.1038/s41434-019-0078-3.
222. Zheng, Z. *et al.* Enhanced Sensitivity of Neutralizing Antibody Detection for Different AAV Serotypes Using HeLa Cells With Overexpressed AAVR. *Front Pharmacol* (2023) doi:10.3389/fphar.2023.1188290.
223. Xu, G. *et al.* Structural Basis for the Neurotropic AAV9 and the Engineered AAVPHP.eB Recognition With Cellular Receptors. (2022) doi:10.1101/2022.01.23.477411.
224. Stanford, S. N. *et al.* Adenovirus-associated Antibodies in UK Cohort of Hemophilia Patients: A Seroprevalence Study of the Presence of Adenovirus-associated Virus Vector–serotypes AAV5 and AAV8 Neutralizing Activity and Antibodies in Patients With Hemophilia A. *Research and Practice in Thrombosis and Haemostasis* Preprint at <https://doi.org/10.1002/rth2.12177> (2019).
225. Lisowski, L. *et al.* Selection and evaluation of clinically relevant AAV variants in a xenograft liver model. *Nature* 506, 382–386 (2014).
226. Kotterman, M. A. & Schaffer, D. V. Engineering adeno-associated viruses for clinical gene therapy. *Nat Rev Genet* 15, 445–451 (2014).
227. Monteilhet, V. *et al.* A 10 patient case report on AAV vector administration after plasmapheresis. *Molecular Therapy* 19, 2084–2091 (2011).
228. Meliani, A. *et al.* Antigen-selective modulation of AAV immunogenicity with tolerogenic rapamycin nanoparticles enables successful vector re-administration. *Nat Commun* 9, 4098 (2018).
229. Mingozzi, F. & High, K. A. Immune responses to AAV vectors: overcoming barriers to successful gene therapy. *Blood* 122, 23–36 (2013).
230. Andreu, Z. & Yáñez-Mó, M. Tetraspanins in extracellular vesicle formation and function. *Front Immunol* 5, 442 (2014).
231. Welker, L., Paillart, J. C. & Bernacchi, S. Importance of viral late domains in budding and release of enveloped rna viruses. *Viruses* vol. 13 Preprint at <https://doi.org/10.3390/v13081559> (2021).
232. Ariumi, Y. *et al.* The ESCRT System Is Required for Hepatitis C Virus Production. *PLoS One* (2011) doi:10.1371/journal.pone.0014517.
233. Dolnik, O. *et al.* Interaction With Tsg101 Is Necessary for the Efficient Transport and Release of Nucleocapsids in Marburg Virus-Infected Cells. *PLoS Pathog* (2014) doi:10.1371/journal.ppat.1004463.
234. Zhai, Q., Landesman, M. B., Robinson, H., Sundquist, W. I. & Hill, C. P. Identification and Structural Characterization of the ALIX-Binding Late Domains of Simian Immunodeficiency Virus SIV _{mac239} and SIV _{agmTan-1}. *J Virol* (2011) doi:10.1128/jvi.01683-10.
235. Shirasaki, T. *et al.* Nonlytic Cellular Release of Hepatitis a Virus Requires Dual Capsid Recruitment of the ESCRT-associated Bro1 Domain Proteins HD-PTP and ALIX. *PLoS Pathog* (2022) doi:10.1371/journal.ppat.1010543.

236. Maaroufi, H. SARS-CoV-2 Encodes a PPxY Late Domain Motif Known to Enhance Budding and Spread in Enveloped RNA Viruses. (2020) doi:10.1101/2020.04.20.052217.
237. Baillet, N. *et al.* E3 Ligase ITCH Interacts With the Z Matrix Protein of Lassa and Mopeia Viruses and Is Required for the Release of Infectious Particles. *Viruses* (2019) doi:10.3390/v12010049.
238. Vecchio, C. Del *et al.* Alix-Mediated Rescue of Feline Immunodeficiency Virus Budding Differs from That Observed with Human Immunodeficiency Virus. *J Virol* 94, 10.1128/jvi.02019-19 (2020).
239. Coren, L. V, Nagashima, K. & Ott, D. E. A PLPPV sequence in the p8 region of Gag provides late domain function for mouse mammary tumor virus. *Virology* 535, 272–278 (2019).
240. Schmitt, A. P., Leser, G. P., Morita, E., Sundquist, W. I. & Lamb, R. A. Evidence for a New Viral Late-Domain Core Sequence, FPIV, Necessary for Budding of a Paramyxovirus. *J Virol* 79, 2988–2997 (2005).
241. Pei, Y. *et al.* Mutation of Phenylalanine 23 of Newcastle Disease Virus Matrix Protein Inhibits Virus Release by Disrupting the Interaction between the FPIV L-Domain and Charged Multivesicular Body Protein 4B. *Microbiol Spectr* 11, e04116-22 (2023).
242. Matsuura, R. *et al.* Three YXXL Sequences of a Bovine Leukemia Virus Transmembrane Protein are Independently Required for Fusion Activity by Controlling Expression on the Cell Membrane. *Viruses* 11, (2019).
243. Ferreira, J. V. *et al.* LAMP2A regulates the loading of proteins into exosomes. *Sci Adv* 8, 1140 (2022).
244. Dalkara, D. *et al.* In vivo-directed evolution of a new adeno-associated virus for therapeutic outer retinal gene delivery from the vitreous. *Sci Transl Med* 5, (2013).
245. Eichhoff, A. M. *et al.* Nanobody-Enhanced Targeting of AAV Gene Therapy Vectors. *Mol Ther Methods Clin Dev* (2019) doi:10.1016/j.omtm.2019.09.003.
246. Wan, S. *et al.* The “Sweet” Side of the Protein Corona: Effects of Glycosylation on Nanoparticle–Cell Interactions. *ACS Nano* (2015) doi:10.1021/nn506060q.
247. Heidarzadeh, M., Zarebkohan, A., Rahbarghazi, R. & Sokullu, E. Protein corona and exosomes: new challenges and prospects. *Cell Communication and Signaling* vol. 21 Preprint at <https://doi.org/10.1186/s12964-023-01089-1> (2023).
248. Xiao, C., Xue, S., Pan, Y., Liu, X. & Huang, M. Overexpression of genes by stress-responsive promoters increases protein secretion in *Saccharomyces cerevisiae*. *World J Microbiol Biotechnol* 39, 203 (2023).
249. Gossen, M. & Bujard, H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences* 89, 5547–5551 (1992).
250. Warrington, K. H. *et al.* Adeno-Associated Virus Type 2 VP2 Capsid Protein Is Nonessential and Can Tolerate Large Peptide Insertions at Its N Terminus. *J Virol* 78, 6595–6609 (2004).
251. Schiller, L. T., Lemus-Diaz, N., Rinaldi Ferreira, R., Böker, K. O. & Gruber, J. Enhanced Production of Exosome-Associated AAV by Overexpression of the Tetraspanin CD9. *Mol Ther Methods Clin Dev* 9, 278–287 (2018).
252. Mendell, J. R. *et al.* Five-Year Extension Results of the Phase 1 START Trial of Onasemnogene Apeparvovec in Spinal Muscular Atrophy. *JAMA Neurol* 78, 834–841 (2021).

253. Meyer, C. *et al.* Pseudotyping exosomes for enhanced protein delivery in mammalian cells. *Int J Nanomedicine* 12, 3153–3170 (2017).
254. Wang, T., Yang, S., Petrenko, V. A. & Torchilin, V. P. Cytoplasmic Delivery of Liposomes Into McF-7 Breast Cancer Cells Mediated by Cell-Specific Phage Fusion Coat Protein. *Mol Pharm* (2010) doi:10.1021/mp1000229.
255. Xiang, Y. S. & Hao, G. G. Biophysical characterization of adeno-associated virus capsid through the viral transduction life cycle. *Journal of Genetic Engineering and Biotechnology* vol. 21 Preprint at <https://doi.org/10.1186/s43141-023-00518-5> (2023).
256. Ellis, B. L. *et al.* A survey of ex vivo/in vitro transduction efficiency of mammalian primary cells and cell lines with Nine natural adeno-associated virus (AAV1-9) and one engineered adeno-associated virus serotype. *Virology Journal* vol. 10 Preprint at <https://doi.org/10.1186/1743-422X-10-74> (2013).
257. Zincarelli, C., Soltys, S., Rengo, G. & Rabinowitz, J. E. Analysis of AAV serotypes 1-9 mediated gene expression and tropism in mice after systemic injection. *Molecular Therapy* 16, 1073–1080 (2008).
258. Nakahama, R. *et al.* The tyrosine capsid mutations on retrograde adeno-associated virus accelerates gene transduction efficiency. *Mol Brain* 15, (2022).
259. Martini, S. V. *et al.* Tyrosine mutation in AAV9 capsid improves gene transfer to the mouse lung. *Cellular Physiology and Biochemistry* 39, 544–553 (2016).
260. Cohen, J. *Statistical Power Analysis for the Behavioral Sciences Second Edition*.
261. Li, X. Extracellular Vesicle–Encapsulated Adeno-Associated Viruses for Therapeutic Gene Delivery to the Heart. *Circulation* (2023) doi:10.1161/circulationaha.122.063759.
262. Cheng, M. *et al.* Probing aspects of extracellular vesicle associated AAV allows increased vector yield and insight into its transduction and immune-evasive properties. *Mol Ther Methods Clin Dev* 33, (2025).
263. Matsumoto, A. *et al.* Phosphatidylserine-deficient small extracellular vesicle is a major somatic cell-derived sEV subpopulation in blood. *iScience* 24, (2021).
264. Liang, X. *et al.* Extracellular vesicles engineered to bind albumin demonstrate extended circulation time and lymph node accumulation in mouse models. *J Extracell Vesicles* 11, e12248 (2022).
265. Espinoza, P. *et al.* Metabolic engineering improves transduction efficiency and downstream vector isolation by altering the lipid composition of extracellular vesicle-enclosed AAV. *Metab Eng* 88, 40–49 (2025).
266. Li, C., Samulski, R. J. & *et al.* Machine learning–guided design of a novel adeno-associated virus capsid for efficient gene delivery. *Nat Commun* 13, 1–12 (2022).
267. Tervo, D. G. R., Hwang, B. K., Viswanathan, S. & *et al.* A designer AAV variant permits efficient retrograde access to projection neurons. *Neuron* 92, 372–382 (2016).
268. Bryant, D. H. *et al.* Deep diversification of an AAV capsid protein by machine learning. *Nat Biotechnol* 39, 691–696 (2021).
269. Davidsson, M. *et al.* A systematic capsid evolution approach performed in vivo for the design of AAV vectors with tailored properties and tropism. *Nat Protoc* 14, 316–349 (2019).
270. Collins, L. T., Ponnazhagan, S. & Curiel, D. T. Synthetic Biology Design as a Paradigm Shift toward Manufacturing Affordable Adeno-Associated Virus Gene Therapies. *ACS Synth Biol* 12, 17–26 (2023).

271. Zhu, X. *et al.* Comprehensive toxicity and immunogenicity studies reveal minimal effects in mice following sustained dosing of extracellular vesicles derived from HEK293T cells. *J Extracell Vesicles* 6, (2017).
272. Kalluri, R. & LeBleu, V. S. The biology, function, and biomedical applications of exosomes. *Science (1979)* 367, eaau6977 (2020).
273. McCann, J. V, Khan, N. S. & Wright, H. L. Oncogenic potential of extracellular vesicles: a systematic review of EV-mediated tumorigenesis. *Int J Mol Sci* 23, 2053 (2022).
274. Ren, Z., Yao, Q., Sun, S., Tao, Y. & Shi, R. Mesenchymal Stem Cell-Derived Exosomes: Hope for Spinal Cord Injury Repair. *Stem Cells Dev* 29, 1467–1478 (2020).
275. Feng, Y. *et al.* Mesenchymal-Stem-Cell–Derived Extracellular Vesicles Mitigate Trained Immunity in the Brain. *Front Bioeng Biotechnol* 8, (2020).
276. Park, S. Enhancing the Production of Adeno-associated Virus (AAV)2 and AAV9 With High Full Capsid Ratio in HEK293 Cells Through Design-of-experiment Optimization of Triple Plasmid Ratio. *Biotechnol J* (2024) doi:10.1002/biot.202300667.
277. Driedonks, T. *et al.* Pharmacokinetics and biodistribution of extracellular vesicles administered intravenously and intranasally to *Macaca nemestrina*. *Journal of Extracellular Biology* 1, (2022).
278. Haney, M. J. *et al.* Biodistribution of Biomimetic Drug Carriers, Mononuclear Cells, and Extracellular Vesicles, in Nonhuman Primates. *Adv Biol* 6, (2022).
279. Rodriguez, B. V. *et al.* An ex vivo model of interactions between extracellular vesicles and peripheral mononuclear blood cells in whole blood. *J Extracell Vesicles* 12, (2023).