Adeno-associated viruses (AAV) rely on helper viruses to transition from latency to lytic infection. Some AAV serotypes are secreted in a pre-lytic manner as free or extracellular vesicle (EV)-associated particles, although mechanisms underlying such are unknown. Here, we discover that the membrane-associated accessory protein (MAAP), expressed from a frameshifted open reading frame in the AAV \textit{cap} gene, is a novel viral egress factor. MAAP contains a highly conserved, cationic amphipathic domain critical for AAV secretion. Wild type or recombinant AAV with a mutated MAAP start site (MAAPΔ) show markedly attenuated secretion and correspondingly, increased intracellular retention. Trans-complementation with MAAP restored secretion of multiple AAV/MAAPΔ serotypes. Further, multiple processing and analytical methods corroborate that one plausible mechanism by which MAAP promotes viral egress is through AAV/EV association. In addition to characterizing a novel viral egress factor, we highlight a prospective engineering platform to modulate secretion of AAV vectors or other EV-associated cargo.
Adeno-associated virus (AAV) is a non-enveloped, single-stranded DNA virus belonging to the Dependoviridae family. Upon co-infection with a helper such as Adenovirus, Herpesvirus or Papillomavirus, AAV undergoes a transition from latent to lytic life cycle, hijacking the host cell machinery. The AAV capsid consists of 60 capsid monomers of VP1, VP2, and VP3 at a ratio of 1:1:10 that packages a 4.7-kb single-stranded genome. The AAV genome encodes replication (rep), capsid (cap), and assembly-activating protein (AAP) open reading frames (ORFs) flanked by inverted terminal repeats (ITRs), which are the sole requirements for genome packaging. As such, the majority of the genome can be replaced by exogenous DNA sequences and packaged inside the AAV capsid to create a recombinant vector both in vitro and in vivo. Some recombinant AAV serotypes appear to be secreted into cell culture media prior to lysis, albeit with variable efficiency. Exactly how AAV exits the cell upon transitioning into this phase of replication remains unclear. Unlike other autonomous paroviruses that undergo a lytic cycle, wild type AAV does not induce marked cytopathic effects (CPE) and therefore, cellular egress of virions is thought to be primarily driven by overexpression of Adenoviral or Herpesvirus proteins.

Multiple studies have demonstrated that a significant fraction of recombinant AAV associate with extracellular vesicles (EVs) and are also released as free particles into the supernatant fraction of the cell culture media. It is well known that cells shed a variety of membrane-bound vesicles varying in size from 20 nm to 1 µm in diameter, which have been termed exosomes, microvesicles or microvesicles (collectively referred to here as extracellular vesicles or EVs). Such EVs can package different macromolecules including proteins, nucleic acids and viruses, thereby making them an attractive therapeutic platform. Despite being non-enveloped viruses, recombinant AAV capsids associated with EVs can enable efficient gene transfer to the retina, the nervous system, the inner ear and appear shielded from anti-AAV neutralizing antibodies. However, the mechanism(s) by which AAV exits the cell or associates with EVs remain to be determined.

Recent work has revealed a novel +1 frameshifted open reading frame (ORF) in the VP1 region of the AAV cap gene that mediates expression of the membrane-associated accessory protein (MAAP) (Fig. 1A), which was postulated to limit AAV secretion in case of MAAPsecretion in case of MAAPΔ particles, which were equally apportioned between EVs and AAV vector production in general.

Results
MAAP is a conserved AAV cap encoded protein with a predicted C-terminal membrane anchoring domain. Conferring that MAAP is a novel viral-encoded protein of unknown function, a BLAST search of multiple AAV cap gene derived MAAP sequences on the National Center for Biotechnology Information (NCBI) website did not return any proteins with significant homology. Amino acid sequence alignment of MAAPs derived from different AAV serotypes revealed conserved N- and C-terminal regions containing hydrophobic and basic amino acid residues interconnected by a threonine/serine (T/S) rich region (Fig. 1B). MAAP 3D structures generated using TrRosetta deep-learning-based modeling predicted the following, (i) a conserved N-terminal hydrophobic motif with both alpha helical and beta strand secondary structure elements; (ii) four T/S rich sequence clusters spanning 7–17 residues in length with last two being separated by a smaller alpha helical interspersed with basic residues and (iii) a C-terminal domain defined by another hydrophobic alpha helical motif merging into a cluster of arginine/lysine (R/K) residues (Fig. 1B, C). Importantly, secondary structure software analysis strongly predicts that the C-terminal domain constitutes a putative membrane binding, cationic amphipathic peptide (residues 96–114). It is noteworthy to mention that the secondary structure of MAAP is strikingly similar to the assembly-activating protein (AAP), which is similarly encoded downstream from a (+1) frameshifted ORF in the cap gene. When combined with phylogenetic analysis using the neighbor-joining tree method, we observed that MAAPs from AAV serotypes 1,6,8,10,11 were tightly clustered, while other sequences, in particular, MAAP2, 5 and 9 showed significant divergence from other serotypes (Fig. 1D).

We then transfected plasmids encoding recombinant MAAPs derived from the VP1 sequences of AAV serotypes 1, 2, 5, 8, and 9 and fused to a C-terminal green fluorescent protein (GFP) in vitro to assess their expression (Fig. 1E) and cellular localization. Fluorescence micrographs confirmed the propensity of MAAP to associate with cell surface membranes as well as subcellular organelles as observed by the punctate patterns throughout the cell (Fig. 1F). Taken together, these data confirm that MAAP is a novel AAV protein predicted to contain a cationic amphipathic C-terminal domain potentially critical for membrane anchoring.

MAAP is essential for extracellular secretion of wild type and recombinant AAV particles. We then sought to determine whether MAAP played a role in the synthesis of (i) (pseudo)wild type AAV serotype 8 (i.e., wtAAV8 packaging AVV2 rep and AAV8 cap flanked by AAV2 inverted terminal repeats [ITRs]) and (ii) recombinant AAV8 (i.e., rAAV8 packaging a chicken beta-actin promoter driven luciferase transgene flanked by AAV2 ITRs) (Fig. 2D). To ablate MAAP expression, we mutated the CTG start codon in the MAAP alternative open reading frame (ORF) without affecting the VP1 ORF in both wtAAV8 and rAAV8 plasmids. Culture media and cell pellets were harvested following co-transfection with an Adenovirus helper plasmid (and an additional ITR flanked luciferase encoding transgene cassette in case of rAAV) on days 3 and 5. Strikingly, quantitative PCR of viral genomes revealed a significantly higher (~1 log) amount of extracellular wtAAV8 particles in contrast to MAAPΔ particles recovered from media on day 3 (Fig. 2B). Further, we observed delayed secretion in case of MAAPΔ particles, which were equally apportioned between extracellular and cell lysate fractions on day 5. Although statistically significant, overall viral titers on day 5 were only minimally altered. Of the total virus produced, nearly 70% of wtAAV8 particles were secreted by day 3, while MAAPΔ particles recovered in the extracellular fraction comprised <10% of total (Fig. 2C). A similar trend was observed in case of rAAV8 particles, with a 4–5-fold higher recovery from media over cell lysate and delayed secretion in case of MAAPΔ particles (Fig. 2E). Of the total virus produced, ~60% of rAAV8 particles were secreted by day 3 in contrast to <10% of MAAPΔ particles (Fig. 2F). Further evaluation of AAV capsid proteins – VP1,2 and 3 by western
blot confirmed these results with undetectable to relatively lower levels in the extracellular fraction on days 3 and 5, respectively, and correspondingly high(er) cellular retention in case of MAAPΔ particles (Fig. 2G, H). No differences were observed when comparing the transduction efficiency of rAAV8 and MAAPΔ particles in vitro (Fig. 2I). Furthermore, MAAPΔ recombinant virus showed similar VP1, VP2, and VP3 expression ratios and overall virus morphology compared to rAAV8
Taken together, these results suggest that encoding MAAP from the alternative ORF in VP1 is essential for efficient cellular egress of AAV particles. Another interesting observation is the relatively low recovery of recombinant AAV serotype 9 (rAAV9) (~15%) and MAAPΔ particles (~5%) from media on day 3 (Supplementary Fig. 2). In contrast to rAAV8, rAAV9 particles appear to display delayed secretion as reported previously20, with only a modest difference in cellular egress efficiency compared to MAAPΔ particles. Thus, MAAP8 has a higher propensity to promote viral egress when compared to MAAP9. When taken together with the differences in sequence homology between AAV8 and AAV9, it is tempting to speculate that the cellular egress efficiency of different AAV capsids could be determined by their cognate MAAPs.

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Helical and cationic domains are indispensable for MAAP expression and supporting AAV secretion. We next sought to determine the regions and/or domains of MAAP necessary to promote AAV viral egress. We first inserted a 3X-FLAG tag onto the C-terminus of MAAP8 under the control of its endogenous promoter and verified its expression and function (Supplementary Fig. 3). At the primary structure level, MAAP can be separated into multiple regions including N-terminus, Linker, C-terminus, hydrophobic regions 1 and 2, threonine/serine-rich regions 1–4, (T/S), and the basic region. Taking a structure–function approach, we generated multiple MAAP8 deletion mutants to systematically dissect the regions of MAAP critical for AAV viral egress (Fig. 3A). We discovered that deletions of the N or C-terminus severely diminished MAAP

Fig. 2 Ablation of MAAP expression results in a significant delay in the extracellular secretion of wildtype and recombinant AAV8 particles. A Schematic of WT AAV8 MAAPΔ mutant. B AAV8 ssCBA-Luc vectors produced with WT Cap or MAAPΔ Cap. Total vector genomes collected from the cells and media and the proportion of virus found in each media harvest or associated with the cells C are shown. Data are presented as mean values ± SD. Significance was determined by two-way ANOVA, with Sidak’s post-test. (Media (D3) c Media (D5) ***p < 0.0001; Cells (D5) ***p < 0.0001, Total ***p = 0.0004). D Schematic of rAAV8 MAAPΔ mutant. E AAV8 ssCBA-Luc vectors produced with recombinant Cap or MAAPΔ Cap. Total vector genomes collected from the cells and media and the proportion of virus found in each media harvest or associated with the cells (F) are shown. Data are presented as mean values ± SD. Significance was determined by two-way ANOVA, with Sidak’s post-test. (Media (D3) **p = 0.0081; Media (D5) **p = 0.0036; Cells (D5) ***p < 0.0001, Total ****p < 0.0001). Recombinant AAV8 and AAV8 MAAPΔ viruses were analyzed from the media and pellet of HEK293 producing cells at days 3 (G) and 5 (H) post transfection. Capsid proteins were analyzed by SDS-PAGE under reducing conditions and probed with a capsid (B1)-specific antibody. Immunoblots are representative images of two independent experiments. I Luciferase assay analyzing transduction of HEK293 cells by AAV8 and AAV8 MAAPΔ mutant virus at MOIs of 10,000 and 50,000 vg/cell. Each bar is a representation of three experiments that are biological replicates.
expression (Fig. 3B, C). MAAP deletions involving alpha helical domains or amphipathic domains rich in basic residues demonstrated the most deleterious effects on protein expression at steady state. Furthermore, of the MAAP8 deletion mutants that expressed, only T/S-rich region 2 and T/S-rich region 4 (MAAP8Δ3 and MAAP8Δ5, respectively) were found to be dispensable for MAAP function as measured through AAV secretion into the media (Fig. 3D–G). Taken together, these data identify critical secondary structure elements in MAAP that are likely essential for expression and function.
MAAP transcomplementation promotes extracellular secretion of diverse AAV serotypes. To determine whether MAAP expression regulates the secretion of other AAV serotypes, we mutated the CTG start codon in the MAAP ORF for rAAV serotypes 1, 2, 8, and 9, and compared viral titers in extracellular and cellular fractions at day 3 as described earlier. In parallel, we also evaluated whether MAAP transcomplementation could rescue the extracellular secretion of MAAPΔ rAAV particles. To achieve the latter, we expressed MAAP alone from the AAV helper plasmid containing rep and cap genes by mutating the start codons in the VP1,2,3 as well as AAV ORFs. Strikingly, viral titers associated with the cellular fraction were markedly increased for rAAV1, rAAV8 and rAAV9 (~4 to 7-fold), but not rAAV2 (Fig. 4A, C, E, G). In addition, overall recovered titers were increased moderately for the same serotypes (up to 2-fold). In corollary, we observed a striking impact of ablating or supplementing MAAP expression on extracellular vs cell-associated fractions of different AAV serotypes. Specifically, in case of rAAV1, this percentage was reversed from 60:40 to 20:80 upon MAAP ablation and restored to normal upon MAAP expression (Fig. 4B). A similar trend was observed for rAAV8 (~80:20 to 20:80 followed by restoration to normal upon supplementation) (Fig. 4F). Both rAAV2 and rAAV9 showed decreased secretion in general (~35:65) compared to rAAV1/8 with MAAP ablation further reducing extracellular viral titers to 15% and 20%, respectively (Fig. 4D, H). Another important observation is that MAAP8 trans-complementation not only fully rescued the extracellular secretion of rAAV1, rAAV2, and rAAV8 particles, but also doubled the recovery of rAAV9 MAAPΔ particles from media compared to rAAV9 particles (from 40% to 80%) (Fig. 4H). These results confirm the critical role played by MAAP in enabling extracellular secretion of AAV particles in a serotype-independent manner, albeit with different efficiencies. Further, our results demonstrate that trans-complementation of MAAP derived from AAV8 can not only rescue secretion of different AAV serotypes, but also potentially enhance the kinetics of secretion.

MAAP promotes association between AAV and extracellular vesicles. To further explore the biology of the MAAP-dependent AAV secretory process, we adopted a gradient centrifugation method to purify EVs from large volumes of cell culture supernatant. Serum-free cell culture medium from suspension adapted HEK293 cells transfected to generate rAAV8/MAAPΔ particles (complemented in trans with MAAP8-HA or HA alone) was processed by successive filtration and centrifugation steps to generate a crude EV pellet that was then separated on an iodixanol gradient (Fig. 5A). The gradient was split into 18 different fractions and probed for EV markers (CD9, CD63, CD81, MAAP (HA tag) and AAV capsid protein using specific antibodies. Using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), immunoblot analysis and qPCR, we found that MAAP-associated EV fractions co-eluted with genome packaging AAV particles (Fig. 5B–D). Furthermore, overexpression of MAAP8-HA or MAAP8-GFP, but not just HA/GFP alone, was sufficient to generate a higher density MAAP-associated sub-population of EVs (Supplementary Fig. 4).

To further understand this interaction between MAAP and EVs, we utilized size exclusion chromatography (SEC) to probe different vesicular/particulate sub-populations (Fig. 5E). Serum-free cell culture medium from suspension adapted HEK293 cells was processed by centrifugation and tangential flow filtration and then resolved on a CL-2B sepharose column. Of the total 30 different fractions collected and probed with EV and MAAP-HA tag-specific antibodies, we determined that MAAP selectively induced a shift in size and was enriched in larger CD63 + vesicular fractions (Fig. 5F, G). Interestingly, analysis of vector genome titers revealed a corresponding shift in rAAV particles confirming that MAAP enables AAV association with CD63 + EVs (Fig. 5H). Taken together, these data indicate that MAAP is important in promoting the association between AAV and EVs, which likely plays a partial, yet significant role in AAV egress.

MAAP is loaded onto the surface of extracellular vesicles. To understand how MAAP interacts with EVs, we first carried out quantitative confocal fluorescence microscopy of cells over-expressing hemagglutinin (HA) tagged MAAP8 (confirmed by western blot in Supplementary Fig. 5). MAAP8-HA colocalized significantly more with the exosomal biogenesis pathway marker Rab1123–25, than the late endo/lysosomal pathway marker Rab26 (Fig. 6A, B). Interestingly, MAAP9, which was associated earlier with slower secretion kinetics, showed a similar co-localization to both Rab7 and Rab11 subcellular compartments (Supplementary Fig. 6). These observations complement previous reports that AAV particles can associate with secreted EVs.15,17,27,28 Further, our results also highlight potential differences in the structural attributes of different MAAPs that may explain the ability to exploit distinct secretory mechanisms that enables cellular egress of different AAV serotypes. Although the exact components of the vesicular secretion pathway exploited in MAAP-aided AAV egress still need to be elucidated, we carried out biochemical assays to explore potential MAAP-AAV capsid interactions. Notably, we did not find any evidence of direct interaction between MAAP and AAV capsid proteins or MAAP and AAV as determined by immunoprecipitation analysis (Supplementary Fig. 7A, B). However, it is important to note that MAAP8 fused to the BirA biotin ligase BioID was able to successfully biotinylate the AAV capsid proteins in vitro indicating that AAV and MAAP do share a proximal interaction within the cell (Supplementary Fig. 7C–F).

We then characterized EVs further using the ExoView platform (NanoView Biosciences, USA) as outlined in the methods. Briefly, the ExoView tetraspanin kit features chips with immobilized antibodies against the tetraspanins CD81, CD63 and CD9, which were used to capture EVs from media of cells expressing HA tag alone or MAAP8-HA followed by interferometric analysis and detection of EVs with fluorophore-conjugated antibodies against these tetraspanins. After EV capture and analysis, we determined that overexpression of MAAP8 does not increase the number or size of EVs in the detectable range (50–200 nm) secreted into the media (Fig. 6C, D). However, it should be noted that this assay does not detect large vesicular bodies previously recovered by SEC. Moreover, using a fluorescently labeled anti-HA tag antibody, we confirmed that MAAP8 associates definitively with captured EVs (Fig. 6E, F). Furthermore, permeabilizing captured EVs to release luminal contents (represented by Syntelin as a marker) revealed that MAAP8 was in fact associated with the outer surface of EVs (Fig. 6G, H). Taken together, these data reveal that MAAP is a virally encoded egress factor that promotes cellular exit of AAV particles at least partially through association with EVs.

Discussion
Dissecting new aspects of AAV biology is critical for continued improvement of recombinant vectors for gene therapy applications. For instance, our lab and others previously dissected the functional attributes of the Assembly-Activating Protein (AAP), which is encoded from an alternative ORF in the AAV cap gene.3,29,30 These studies provided mechanistic insight into AAV capsid assembly, in addition to highlighting capsid mutations that
can adversely impact assembly and vector yield. Here, we provide structural and functional insights into the recently identified membrane-associated accessory protein (MAAP), which is encoded from a different, alternative ORF within the AAV cap gene. We report that this highly conserved protein displays critical hydrophobic and cationic domains essential for membrane anchoring and promotion of extracellular secretion of AAV particles. Although attenuation of MAAP expression does not affect viral yield, we demonstrate that extracellular secretion of AAV particles is drastically delayed with increased cellular retention of AAV particles. Our findings have immediate implications for recombinant AAV vector production, which is currently undergoing a revolutionary manufacturing scale-up phase. Speciﬁcally, we demonstrate that MAAP transcomplementation can signiﬁcantly alter the kinetics of AAV extracellular secretion, potentially facilitating downstream purification only.
Fig. 4 Trans-complementation of MAAP8 rescues secretion defect across multiple MAAPΔ AAV serotypes. scC8h-GFP vectors produced with WT Cap or MAAPΔ Cap for AAV1 (B), AAV2 (D), AAV8 (E, F), and AAV9 (G, H) complemented in trans with a VP/AAP-null AAV8 plasmid to replicate endogenous levels of MAAP expression. Total vector genomes (A, C, E, G) and the proportion of vector found in the media and cells (B, D, F, H) 3 days post-transfection. Each bar is a representation of three experiments that are biological replicates. Data are presented as mean values ± SD. Significance was determined by two-way ANOVA, with Tukey’s post-test. (AAV1 Cells vs. AAV1 MAAPΔ Cells, ***p < 0.0001; AAV1 MAAPΔ Cells vs AAV1 MAAPΔ + MAAP8 Cells, ****p < 0.0001; AAV1 Total vs AAV1 MAAPΔ Total, **p = 0.0002; AAV1 MAAPΔ Total vs AAV1 MAAPΔ + MAAP8 Total, p = 0.0116; AAV8 Media vs AAV8 MAAP8 Media, ***p < 0.0001; AAV8 Media vs AAV8 MAAPΔ + MAAP8 Media, p = 0.0211; AAV8 MAAPΔ Media vs AAV8 MAAPΔ + MAAP8 Media, **p < 0.0001; AAV8 Cells vs AAV8 MAAPΔ Cells, **p < 0.0001; AAV8 MAAPΔ Cells vs AAV8 MAAPΔ + MAAP8 Cells, ***p < 0.0001; AAV8 Total vs AAV8 MAAPΔ Total, ***p < 0.0001; AAV8 Total vs AAV8 MAAPΔ + MAAP8 Total, ****p < 0.0001; AAV9 Media vs AAV9 MAAP8 Media, **p = 0.0071; AAV9 MAAPΔ Media vs AAV9 MAAPΔ + MAAP8 Media, p = 0.0223; AAV9 Cells vs AAV9 MAAPΔ Cells, ***p = 0.0003; AAV9 MAAPΔ Cells vs AAV9 MAAPΔ + MAAP8 Cells, ***p = 0.0003; AAV9 Total vs AAV9 MAAPΔ Total, ***p = 0.0001; AAV9 Total vs AAV9 MAAPΔ + MAAP8 Total, **p = 0.0075).

from cell culture media. Further, we note that mutation of the cap gene in engineered AAV capsids within the overlapping MAAP reading frame could impact vector yield and timing of harvest from cell lysate versus media fractions. Interestingly, we observed a significant titer increase on Day 3 with MAAP1A, MAAP8A, and MAAP9A vectors. One possible explanation for the increased titer is that in cells transfected with MAAP8 plasmids, empty rAAV capsids are not as readily sequestered into the media and are retained in the nucleus, thereby increasing their availability for REP mediated genome packaging. In addition, we also observed that MAAP contributes to rapid secretion of what appears to be a diverse population of EVs, independent of other AAV components. While this aspect needs to be explored further, it is exciting to note that MAAP might provide an orthogonal solution to loading therapeutic cargo onto EVs.

From a structural perspective, MAAP is rich in alpha helical domains, interspersed by T/S rich linker regions and basic residues. The most notable attribute of MAAP is a cationic amphipathic C-terminal domain, which is critical for membrane anchoring and extracellular secretion. Recent studies have revealed that PTGFRN and BASP1 can be utilized as scaffolds for loading various molecules onto the surface or into the lumen of EVs. BASP1 is an inner membrane leaflet localized protein that utilizes a polybasic effector domain (PED) to bind to the EV membrane. Interestingly, the PED, along with N-terminal myristoylation of BASP1, is essential for proper loading of carrier proteins to the inner membrane of EVs. Similarly, the matrix (MA) membrane proximal domain of HIV-1 Pr55GAG contains a highly basic region that is important for efficient membrane binding and proper targeting of MA to the plasma membrane. MAAP also contains multiple basic regions that are essential for proper molecular function; however, whether MAAP undergoes post-translational lipid modification remains to be determined. Nevertheless, our data suggests that similar to PTGFRN, MAAP can potentially be fused to protein cargo for loading onto EVs.

Furthermore, our work has shed light on the interaction between MAAP, AAV, and EVs. Using established MISEV criteria, we showed that a sub-population of secreted AAV particles associate with multiple EV markers (CD81, CD63 and CD9), while a fraction of AAV particles appears to be free of any vesicular association. Notably, the EV fractions showed strong MAAP association further implicating this viral protein in vesicle mediated AAV cellular egress. It is plausible that the free particles were previously associated with EVs that lysed during processing steps or MAAP alters the cell membrane through other mechanisms, which remain the subject of investigation. From a cell biology perspective, our data supports a model wherein MAAP hijacks the EV/exosomal pathway. Exactly how MAAP and AAV capsids work in tandem to utilize this pathway prior to secretion warrants further investigation. It is plausible that MAAP may enable passive loading of AAV particles into EVs during secretion or alternatively, actively recruit AAV capsids into EVs through an unknown cellular bridging factor. Indeed, studies exploring cellular egress of autonomous parvoviruses, such as Minute Virus of Mice (MVM), have shown viral particles to be sequestered into COP II vesicles in the endoplasmic reticulum (ER) and transported via the Golgi compartment to the plasma membrane. Remodeling of the cytoskeletal filaments through gelsolin mediated actin fiber degradation in infected cells have been shown to be essential for egress. Further, several biochemical factors such as SAR1, SEC24, RAB1, the ERM family proteins, radixin, and moesin have been implicated in this process of cellular exocytosis. However, it should be noted that a virulence factor akin to MAAP in other paroviruses has not been identified to date. In summary, we conclude that while dependoviruses such as AAV rely on helper co-infection to trigger viral release through a lytic process, MAAP-mediated viral egress likely accelerates and regulates dissemination and spread. In summary, our studies unequivocally implicate MAAP as a novel AAV egress factor.

Methods

Plasmid constructs. MAAP DNA sequences from AAV serotypes 1, 2, 5, 8, and 9 were synthesized and cloned into pcDNA3.1(+) C-HA and pcDNA3.1(+) C-eGFP expression vectors using HindIII and XbaI sites for MAAP 1, 2, 8, and EcoRI sites for MAAP5 (Genscript). All MAAP expression constructs were synthesized and cloned with an ATG start codon. The AAV8-Rep/Cap-VP+ plasmid is a AAV2-Rep/AAV8-Cap plasmid with the start codons of VP1/2/3 and AAP mutated by site-directed mutagenesis to prevent expression. The AAV8-Rep/Cap-VP+ additionally has a mutated MAAP start codon to prevent MAAP expression. The AAV8-MAAP8 plasmid is a 2 AAV2-Rep/AAV8-Cap plasmid with the start codons of MAAP8 mutated by site-directed mutagenesis to prevent expression. The AAV8-MAAP8-3X-FLAG plasmid was generated by utilizing site-directed mutagenesis to incorporate a 3X-FLAG tag in frame onto the C-terminus of MAAP8 in the AAV8-Rep/Cap-VP+ plasmid. AAV8-MAAP8-3X-FLAG truncation mutants were generated through site-directed mutagenesis. MAAP8 was cloned into the MCS-13X-Linker-BioID2-HA expression vector (Addgene #80899) using Nhel and Age1 sites. All plasmid constructs were verified by DNA sequencing analysis.

Bioinformatics analysis and structural models. The amino acid sequences of 15 AAV serotypes were retrieved from GenBank. MAAP start and stop sites were defined as previously described. Protein sequences were aligned using the ClustalW multiple-alignment tool and generated using Unipro UGENE software. MAAP amino acid sequences from multiple AAV isolates were aligned using ClustalW, and phylogenetic trees were generated using the MEGAv7.21 software package. The phylogeny was produced using the neighbor-joining algorithm, and amino acid distances were calculated using a Poisson correction. Statistical testing was done by bootstrapping with 1000 replicates to test the confidence of the phylogenetic analysis and to generate the original tree. The percentage of replicate trees in which associated taxa clustered together in the bootstrap test is displayed next to the branches. Secondary structural elements were predicted using the Jpred tool. To predict membrane-binding, amphipathic α-helices, we used Amphipred (parameters: high specificity/low sensitivity). MAAP structural models were generated using the TrRosetta deep-learning-based modeling method. Secondary structural depictions of these models were visualized using the PyMOL Molecular Graphics System (Schrödinger; https://www.pymol.org/2/).
Fig. 5 MAAP promotes association of AAV with EVs. A Schematic of EV isolation by iodixanol density gradient from HEK293 suspension culture. B Immunoblots of iodixanol fractions from suspension cells producing recombinant MAAP8Δ vector complemented in trans with CMV-HA and C CMV-MAAP8-HA. EVs, capsid and MAAP were analyzed from the media of HEK293 producing cells at day 3 post transfection. Capsid and MAAP proteins were analyzed by SDS-PAGE under reducing conditions while EV markers (CD81, CD63, CD9) were analyzed by SDS-PAGE under non-reducing conditions (n = 2). D Graph displaying percent vector genome titer relative to total viral genomes for each fraction from iodixanol gradient purified MAAP8Δ vector complemented in trans with CMV-HA and CMV-MAAP8-HA. E Schematic of EV isolation by size exclusion chromatography (SEC) from HEK293 suspension culture. F Immunoblots of SEC fractions from suspension cells producing recombinant MAAP8Δ vector complemented in trans with CMV-HA and G CMV-MAAP8-HA. EVs and MAAP were analyzed from the media of HEK293 producing cells at day 3 post transfection. MAAP protein was analyzed by SDS-PAGE under reducing conditions while the EV marker CD63 was analyzed by SDS-PAGE under non-reducing conditions (n = 2). H Graph displaying percent vector genome titer relative to total viral genomes for each fraction from SEC purified MAAP8Δ vector complemented in trans with CMV-HA and CMV-MAAP8-HA.
Fig. 6 MAAP interacts with the surface of extracellular vesicles. A HEK293 cells were transfected with expression vectors encoding Rab7-GFP, Rab11-GFP, MAAP8-HA. MAAP-HA was detected by immunofluorescence with an AlexaFlour647 secondary antibody (MAAP8-HA-A647). A Z-stack of confocal optical sections at 1-μm steps was acquired. A 3-μm-thick medial stack is shown. Images are representative of three experiments. Scale bars, 10 μm. B Co-localization between MAAP8-HA and Rab7-GFP or Rab11-GFP in the whole cell as assessed by Pearson’s correlation coefficient (R) as described in Materials and Methods. Each dot represents one cell. Horizontal bars represent the mean ± SEM. A two-sided Mann–Whitney rank test was used to determine significance. (Rab7+ vs Rab11+, ****p < 0.0001). C Chips coated with separate capture spots for anti-CD81, anti-CD63, and anti-CD9 were used to capture EVs from media of cells expressing CMV-HA and CMV-MAAP8-HA. Total captured EVs on each chip were quantified (n = 3). Data are presented as mean values ± SD. D Representative size distribution profile of tetraspanin-positive EVs determined by label-free interferometry (n = 3). E CD81 capture probe images of EVs captured from media of cells expressing CMV-HA and CMV-MAAP8-HA. EVs were probed with anti-CD81-CF488a (blue), anti-HA-Alexa Fluor 555 (green), anti-CD9-CF647 (red), and anti-CD63-CF647 (red) (n = 3). F Quantification of percent HA labeled EVs captured from media of cells expressing CMV-HA and CMV-MAAP8-HA (n = 3). Data are presented as mean values ± SD. G EVs from media of cells expressing CMV-MAAP8-HA were captured with anti-CD81, anti-CD63, and anti-CD9 coated chips. EVs were fixed, permeabilized and probed with anti-HA-Alexa Fluor 555 to determine the EV loading orientation of MAAP8-HA (n = 3). Data are presented as mean values ± SD. H Cartoon schematic depicting EV surface loading of MAAP.
Cellular assays, immunoprecipitations, and western blotting. For protein expression analysis, HEK293 cells seeded overnight in 6-well plates at a density of 3 × 10^5 cells per plate were transfected with 1x Halt Protease Inhibitor (ThermoFisher) for 45 min at 4 °C. Lysates were spun at max speed for 10 min at 4 °C to remove cellular debris. 1x LDS sample buffer with 10 mM DTT were added to cleared lysate and boiled for 2 min. Samples of cleared lysate were run on Mini-Protean TGX 4–15% gels (Biorad), transferred onto PVDF with the Trans-Blot Turbo system (BioRad) and blocked in 5% milk/1x TBS/T. Blots were probed with mouse monoclonal anti-GFP (Abcam, 1:10,000 dilution, GC0996; Santa Cruz Biotechnology, anti-Fluorescent) antibody. mouse monoclonal B1 hybridoma supernatant (1:50, 03-65158; ARP), mouse monoclonal anti-FLAG M2 antibody (1:1000 dilution, F18045UG; Sigma), mouse monoclonal anti-beta-actin (1:1000 dilution, 8226; Abbam) as the primary antibody. Following three 1x TBS washes, samples were incubated with secondary antibodies conjugated to HRP (goat anti-rabbit-HRP, 111-035-003; Jackson ImmunoResearch). Blots were developed using SuperSignal West Fermo substrate (ThermoFisherScientific/Life Technologies) according to manufacturer instructions. For immunoprecipitation studies, HEK293 cells were transfected with pXPR9 and MAAP9-pcDNA3.1(+)-C-HA for 72 h, then washed with 1x PBS and harvested in NP-40 with 1x Halt Protease Inhibitor (ThermoFisher) for 1 h at 4 °C. Lysates were spun at max speed for 20 min at 4 °C to remove cellular debris. In all, 10 μL (2.5 μg) of anti-HA SG77 antibody were added to 500 μL cleared lysate and incubated at 4 °C for 3 h with rotation. Added 40 μL of pre-washed Protein G magnetic beads to each well and incubated with antibodies overnight on a rotator at 4 °C. Bound protein was eluted in 10 mM DTT and 1x LDS for 5 min at 95 °C. Samples were then analyzed via SDS-PAGE (NuPAGE 4–12% Bis-Tris Gel) and transferred onto nitrocellulose membrane (ThermoScientific). Following blocking in 5% milk/1x TBS, samples were incubated with primary antibodies to either capsid (mouse monoclonal anti-beta-actin, 1:1000 dilution, GC0996; Santa Cruz Biotechnology, anti-Fluorescent), mouse monoclonal B1 hybridoma supernatant (1:50, 03-65158; ARP), actin (mouse monoclonal anti-beta-actin, 1:1000 dilution, 8226; Abbam), CD81 (mouse monoclonal anti-CD81 M3B, 1:1000, 10630D), C63 (mouse monoclonal anti-CD63 T63, 1:1000, 10628D), CD9 (mouse monoclonal anti-CD9 T9, 1:1000, 10626D) or MAAP (mouse monoclonal anti-HA HS antibody, 1:1000 dilution, MA5-27543; ThermoFisher Scientific) overnight in 5% milk/1x TBS. Following three 1x TBS washes, samples were incubated with secondary anti-mouse antibody conjugated to HRP (goat anti-mouse-HRP, 32430; ThermoFisher Scientific) at 1:2000 in 5% milk/1x TBS for 1 h. The signal was visualized via SuperSignal West Fermo Maximal Sensitivity substrate (ThermoScientific) according to manufacturer instructions.

BioID2 expression and pulldown. For protein expression analysis, HEK293 cells seeded overnight in 6-well plates at a density of 3 × 10^5 cells per plate were transfected with a total of 2 μg of 1X-BioID2-HA or MAAPA8-13X-BioID2-HA DNA. Cells were then supplemented with 50 μM biotin 24 h post transfection and allowed to label for 2 h. Cell pellets were recovered 24 h biotin supplementation and were washed in ice with 1x Halt Protease Inhibitor (ThermoFisher) for 45 min at 4 °C. Lysates were spun at max speed for 10 min at 4 °C to remove cellular debris. 1X LDS sample buffer with 10 mM DTT were added to cleared lysate and boiled for 2 min. Samples of cleared lysate were run on Mini-Protean TGX 4–15% gels (Biorad), transferred onto PVDF with the Trans-Blot Turbo system (BioRad) and blocked in 5% milk/1x TBS/T. Blots were probed with rabbit polyclonal anti-HA SG77 antibody (1:1000 dilution, 71-5500; ThermoFisher Scientific), mouse monoclonal anti-beta-actin (1:1000 dilution, 8226; Abbam), and goat polyclonal anti-biotin (1:1000 dilution, 31852; Thermosther) as the primary antibody. Following three 1x TBS washes, samples were incubated with secondary antibodies conjugated to HRP (goat anti-mouse-HRP, 32430; ThermoFisher Scientific) at 1:2000 in 5% milk/1x TBS for 1 h. The signal was visualized via SuperSignal West Fermo Maximal Sensitivity substrate (ThermoScientific) according to manufacturer instructions.

Recombinant and wild type AAV production, purification, and quantification. HEK293 (human embryonic kidney cells obtained from the University of North Carolina Vector Core) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 1% DNase in RPMI supplemented with 5% CO₂ at 37 °C. Recombinant AAV vectors were produced by transfecting HEK293 cells at ~75% confluence with polyethylenimine (PEI Max: Polysciences, 24765) using a triple plasmid transfection protocol with the AAV Rep-Cap plasmid, Adenoviral helper plasmid (pXx680), and single-stranded genomes encoding firefly luciferase driven by the chicken beta-actin promoter (scBRA-Luc) and self-complementing AuxFla (scBHF-GFP). Lysates were spun at max speed for 10 min at 4 °C in a Sure Spin 300 (Roche Applied Sciences, Pleasanton, CA) at 5400 rpm for 30 min. Following centrifugation at 21,000 rcf for 2 min, NaCl to 300 mM was added to AAV2 lysates prior to centrifugation to prevent virus binding of the cell debris. Collected media and lysates were assayed with qPCR for DNA-resistant viral genomes as described above. For Day 3 and 5 virus preparations, media was collected after 3 days in culture and cells and media were harvested as described on the last day. For WT-like AAV transfections, cells were transfected with Adenovirus helper plasmid (1.9 μg) and WT or MAAPA ITR- AA8-VegfA/AAV9 plasmid (0.9 μg), with media and cells collected on days 3 and 5 post transfection as described above.

Confocal fluorescence microscopy. HEK293 cells were seeded on slide covers in 24-well plates at a density of 3x10⁵ cells/well and allowed to adhere overnight. Cells were then grown for 3 days with viability measured daily. Cell density and viability were measured daily, and media and cells were collected on days 3 and 5 post transfection as described above.

Quantitative PCR analysis of AAV vector yield. HEK293 cells in six-well plates were transfected using PEI at ~75% confluence with Adenovirus helper plasmid (1 μg), WT or MAAPA AAV-Rep/Cap plasmid (1 μg), ITR-transgene plasmid (500 ng), and AAV8-Rep/Cap-VP or -MVP* (500 ng). The AAV8-Rep/Cap-VP plasmid is a AAV2/2-Rep/AAV8 plasmid with the start codons of VFP23 and AAV2/AAP in mutual antisense orientation and quantified by quantitative PCR using a Roche Lightcycler 480 (Roche Applied Sciences, Pleasanta, CA) with primers amplifying the AAV2/AAV8 ITR regions (Supplementary Table 1).

Extracellular vesicle isolation (iodixanol and size exclusion chromatography). For Fig. 3A–D and Supplementary Fig. 4, a previously established EV isolation protocol was utilized. Suspension adapted HEK293 (human embryonic kidney cells obtained from the University of North Carolina Vector Core) were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin and 1% DNase in RPMI supplemented with 5% CO₂ at 37 °C. Recombinant virus preparations were determined by quantitative PCR using a Roche Lightcycler 480 (Roche Applied Sciences, Pleasanta, CA) with primers amplifying the AAV2 AA8 ITR regions (Supplementary Table 1).
iodixanol solutions were carefully pipetted on top. Resuspended pellet in PBS was added on top of the gradient. The gradient was ultracentrifuged in a Sure Spin 630 36 mL rotor (Thermo Scientific) for 16 h at 150,000 rpm at 4°C to separate EVs from other cell culture supernatant contaminants. The gradient was then fractionated in 2 mL fractions and analyzed by biochemical analysis.

To purify EVs by size exclusion chromatography, CL-2B Sepharose resin (CL2B300, Sigma Aldrich) was washed with an equal volume of PBS in a glass container and placed at 4°C to let the resin settle completely. PBS washes were repeated two more times for a total of three washes. Columns were prepared fresh on the day of use. Washed resin was poured into an Econo-Pac Chromatography column (Bio-Rad, 7321010) and the bed volume brought to 10 mL. The top frit was immediately placed at the top of the resin and the column was subsequently washed with 20 mL PBS. Immediately before sample addition, the column was allowed to fully drip out and 1 mL of sample was added to the column. As soon as sample was added to the top of the column, PBS was added to the top of the column 1 mL at a time. Fraction numbers corresponded to 0.5 mL increments collected as soon as sample was added. Thirty total fractions were collected and analyzed by SDS-PAGE and western blot.

**Single-particle interferometric reflectance imaging sensing (NanoView) analysis.** Samples were processed as described previously44-47. Samples were diluted according to the manufacturer’s protocol for the ExoView tetradsen kit (NanoView Biosciences; EV-TETRA) and incubated for 16 h on ExoView tetradsen chips spotted with antibodies against CD91, CD63, CD9 and the mouse IgGlκ isotype control in triplicate. The chips were then washed in an automated chip washer and incubated with conjugated antibodies for fluorescent labeling of the captured EVs (anti-CD81-CF488a, anti-HA-Alexa Fluor 555 [AF555] (ThermoFisher, 26183-A555), anti-CD9-CF647, and anti-CD63-CF647 for 1 h. After labeling, the chips were washed and dried in the automated chip washer and placed in the reader for analysis. All data were gathered using an ExoView R100 reader equipped with ExoView Scanner 3.0 software and analyzed using ExoView Analyzer 3.0.

**Transmission electron microscopy.** Isolated vesicular fractions or AAV viral particles (1 × 1011 vg) in 1x PBS samples were adsorbed onto 400 mesh, carbon coated grids (Electron Microscopy Sciences) for 2 min and briefly stained with 1% uranyl acetate (Electron Microscopy Sciences) diluted in 50% ethanol. After drying, grids were imaged with a Philips CM12 electron microscope operated at 80 kV. Images were collected on an AMT camera.

**Luciferase expression assays.** Images were collected on an AMT camera.

**Data availability**

The data that support the findings of this study are available from the corresponding author upon request. Source data are provided with this paper.

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Author contributions

Z.E., P.H. and A.A. designed all experiments and interpreted the data. Z.E., P.H. and D.O. carried out all molecular biology, virus production, and imaging studies. L.A. and G.D. performed single-particle interferometric reflection imaging sensing (NanoView) analysis of exosomes. Z.E., P.H., H.V. and A.A. wrote the manuscript.

Competing interests

A.A. and Z.E. have filed patent applications on the subject matter of this manuscript. A.A. is a co-founder at StrideBio and TorqueBio and an advisor to Sarepta Therapeutics, Mammoth Biosciences, Atsena Therapeutics, Ring Therapeutics, AstraZeneca Pharmaceuticals. A.A. and Z.E. are advisors to Isolere Bio.

Additional information

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