Identification and Characterization of a Highly Conserved Cell Surface Receptor Utilized by Engineered BBB-Penetrant AAV Capsids with Enhanced Brain Tropism in Non-Human Primates and Mice

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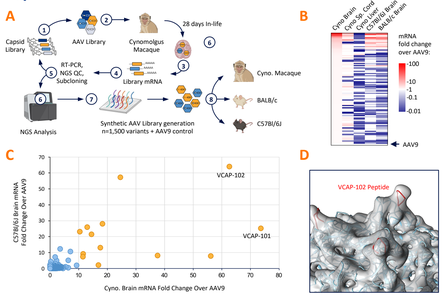
INTRODUCTION

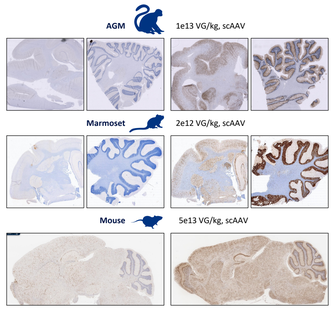
Novel engineered blood-brain barrier (BBB)-crossing AAV capsids have demonstrated significant improvements in CNS tropism and thus increased therapeutic efficiency. However, the unpredictability of cross-species activity by engineered capsids has hampered progress to the clinic. Therefore, the identification of the receptors utilized by such novel capsids is important to predicting their utility in treating human conditions. For instance, the recent identification of receptors for multiple CNS-tropic capsids have explained their lack of cross-species activity as either due to lack of a human receptor ortholog or poor conservation of critical residues. Here we report the identification of a highly conserved brain vascular receptor, ALPL (Alkaline Phosphatase), specifically bound by the cross-species VCAP-101/102 engineered capsid class. This capsid family was previously reported to exhibit a 50-fold increase in BBB penetrance in both macaques and mouse. Ectopic overexpression of the human ALPL isoform in cultured cells led to a significant increase in capsid binding and transduction while no difference was observed with the parental capsid, AAV9. Importantly, ALPL isoforms from macaque, mouse, and pig also facilitated transduction by VCAP-102, highlighting the cross-species functionality of this capsid class. Neutralization of capsid-receptor interaction with anti-ALPL antibodies or small molecule inhibitors completely reverted the gain of transduction by VCAP-102, further supporting a direct role in transduction. Additional studies in a transwell cell culture model demonstrated that ALPL dramatically increased the transcytosis of capsids across polarized cells, strongly supporting a mechanistic role in capsid translocation across the BBB in vivo. Previous studies have demonstrated that expression of ALPL significantly increases with age in both humans and rodents, and we observed a corresponding 2-fold increase of CNS transduction in aged mice relative to their young counterparts. Conversely, mice pre-treated with a small molecule inhibitor of ALPL showed significantly reduced brain transduction, while no measurable effect was observed on liver transduction. Importantly, the CNS transduction by other capsids with a different receptor usage was unaffected by the inhibitor, ruling out the possibility of non-specific effects on viral transduction. Employing *in silico* structural modeling, we have further characterized the molecular mechanism governing the interaction between the capsid and ALPL. In summary, our discovery of a conserved cross-species receptor facilitating BBB passage by a novel engineered AAV capsid class represents a significant step forward in the development of targeted CNS therapeutics. Understanding the molecular mechanisms underpinning this interaction provides a foundation for the rational design of next-generation AAV vectors.

Figure 1. TRACER-based *In vivo* Screen Design and Top Hits



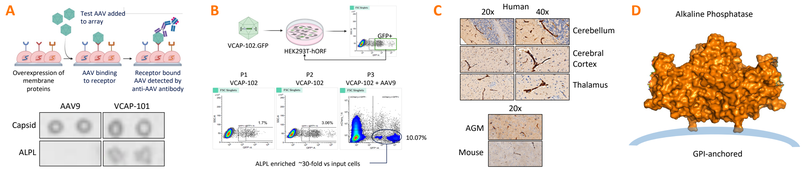
(**A**) Design of TRACER-NHP directed evolution pipeline. (**B**) Heat map of top 100 most enriched capsids in macaque brain from step (8). Color scale represents the mRNA enrichment score of the top 100 capsid versus AAV9 in indicated tissues. SC: Spinal Cord. (**C**) Comparative performance of 1500 capsids in cynomolgus macaque and C57Bl/6J mouse brain. Capsids with >10-fold enrichment relative to AAV9 in macaque brain are highlighted in orange. (**D**) 3D structure of the 3-fold protrusions of VCAP-102 obtained by cryo-EM. The inserted 6-AA peptide in the VR-IV loop is highlighted in red.

Figure 2. Cross-species BBB-penetrance of VCAP-102 in AGM, Marmoset and Mouse



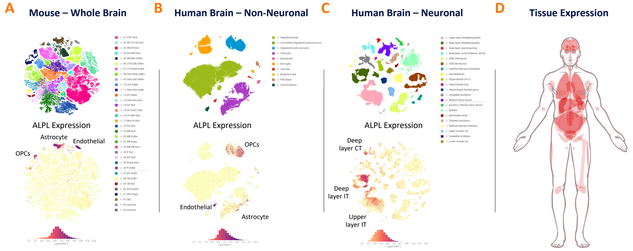
HA-tagged transgene detected by IHC from FFPE sections of African green monkey (AGM), marmoset or mouse 28 days after IV injection of indicated dose of AAV9 or VCAP-102 (containing a self-complementary transgene under the CAG promoter).

Figure 3. Identification of ALPL as a Cell Surface Attachment Receptor for VCAP-102



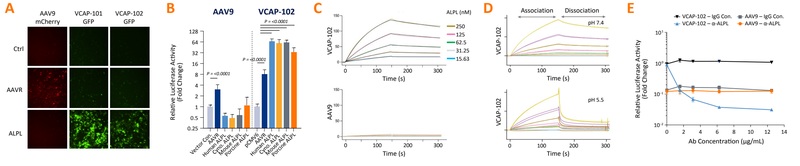
(**A**) Retrogenix microarray screen. Expression vectors encoding >6000 human membrane proteins were arrayed on slides and overlaid with HEK293 cells for reverse transfection. AAV was added and cell-bound capsids were detected with an anti-AAV9 antibody. Bottom panel: Image from microarray screen demonstrating detection of virus spotted in gelatin (positive control) and VCAP-101 bound to cells expressing ALPL. (**B**) Human ORFeome screen protocol. A lentiviral library containing 17,000 human ORFs was used to generate a stable HEK293T pool that was subjected to iterative rounds of transduction with VCAP-102-EGFP followed by sorting of GFP(+) cells. AAV9-mCherry was added to the third round of screening to identify EGFP(+)/mCherry(-) cells expressing a VCAP-102-specific receptor. Bottom panel: FACS gating strategy showing stepwise enrichment of permissive GFP(+) cells. (**C**) Detection of ALPL by immunohistochemistry (IHC) in brain sections from human, African green monkey and mouse. (**D**) Structure of ALPL dimer generated with PyMol (PBD: 7YIV).

Figure 4. ALPL is Widely Expressed and Enriched in Brain Endothelial Cells in Mouse and Human



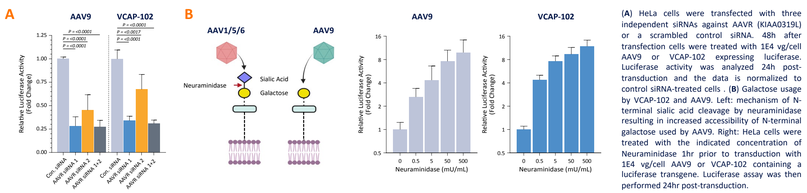
(**A-C**) UMAP (a) and t-SNE (b,c) plots generated using Allen Brain Cell Atlas (RRID:SCR_024440) brain map tool (https://knowledge.brain-map.org/). (**A**) Primary publication: Yao et al. (2023) A high-resolution transcriptomic and spatial atlas of cell types in the whole mouse brain. DOI 10.1038/s41586-023-06812-z. b,c , Primary publication: Siletti et al. (2023) Transcriptomic diversity of cell types across the adult human brain. DOI: 10.1126/science. add7046. (**D**) Image of ALPL expression in human tissues generated by Human Protein Atlas (proteinatlas.org)

Figure 5. Direct ALPL: VCAP-102 Interaction Mediates Viral Transduction and Transcytosis



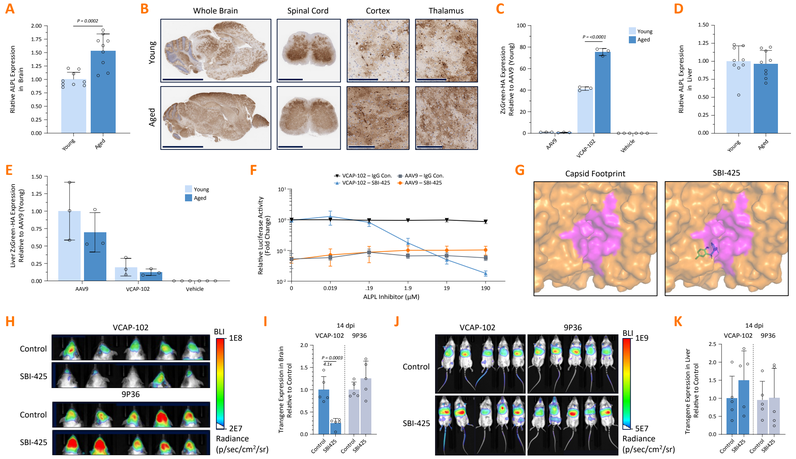
(**A**) Ectopic expression of ALPL increases VCAP-101 and VCAP-102 transduction. HEK293T cells transfected with indicated plasmid were treated with AAV9-mCherry or VCAP-102-GFP. Fluorescence was visualized after 24h. (**B**) Functional interaction of VCAP-102 with ALPL across species. HEK293T cells transfected with indicated AAVR or ALPL plasmids were transduced with AAV9- or VCAP-102-luciferase. (**C**) Direct binding of VCAP-102 to human ALPL. Binding kinetics between VCAP-102 and human ALPL were analyzed by surface plasmon resonance (SPR). VCAP-102 or AAV9 capsids were immobilized and ALPL was used as an analyte. (**D**) pH-dependent dissociation of ALPL:VCAP-102 complex, measured by SPR. (**E**) Blocking of VCAP-102 transduction by ALPL antibody. HeLa cells were incubated with anti-ALPL antibody or an isotype control before adding AAV9 or VCAP-102 expressing luciferase. (**F**) Impact of ALPL depletion on VCAP-102 transduction. HeLa cells were transfected with siRNAs against ALPL and transduced with AAV9 or VCAP-102 containing a luciferase transgene.. (**G**) Removal of cell surface GPI-AP proteins reduces VCAP-102 transduction. HeLa cells were treated with PI-PLC and transduced with AAV9 or VCAP-102 containing a luciferase transgene. (**H**) Plasma membrane localization of ALPL is necessary for VCAP-102 transduction. HEK293T cells were transfected with plasmid encoding an ALPL isoform defective for plasma membrane trafficking (ALPL-V2) and transduced with AAV9 or VCAP-102 encoding luciferase. (**I**) Tissue specific isoforms of ALPL do not mediate VAP-102 transduction. HEK293T cells transfected with the indicated plasmids were transduced with AAV9- or VCAP-102-luciferase. (**J**) ALPL mediates capsid transcytosis. MCDK or MDCK-ALPL cells grown on a transwell insert and AAV9 or VCAP-102 were added to the top chamber. AAV genomes in the bottom chamber was quantified 24h later by qPCR. Data normalized to AAV9. Insert shows detection of VCAP-102 capsid in the bottom chamber by dot blot with an anti-AAV9 antibody

Figure 6. Impact of AAVR and Galactose on VCAP-102 Transduction



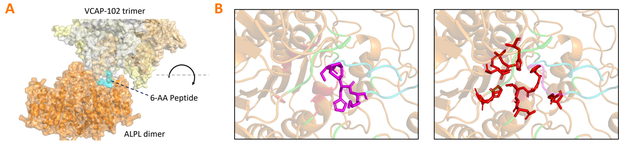
(**A**) HeLa cells were transfected with three independent siRNAs against AAVR (KIAA0319L) or a scrambled control siRNA. 48h after transfection cells were treated with 1E4 vg/cell AAV9 or VCAP-102 expressing luciferase. Luciferase activity was analyzed 24h post-transduction and the data is normalized to control siRNA-treated cells . (**B**) Galactose usage by VCAP-102 and AAV9. Left: mechanism of N-terminal sialic acid cleavage by neuraminidase resulting in increased accessibility of N-terminal galactose used by AAV9. Right: HeLa cells were treated with the indicated concentration of Neuraminidase 1hr prior to transduction with 1E4 vg/cell AAV9 or VCAP-102 containing a luciferase transgene. Luciferase assay was then performed 24hr post-transduction.

Figure 7. ALPL Impacts VCAP-102 Transduction *in vivo*



(**A**) ALPL expression in young (6 week) and aged (18 months) mice relative to mTBP housekeeping gene. Data normalized to young mouse data. (**B**, **C**) CNS transduction by VCAP-102 in young and aged mice. (**B**) Anti-HA staining 28d after transduction with 1E13 VG/kg VCAP-102 containing scAAV-CAG-zsGreen-HA. Scale bars: 5mm (whole brain), 1 mm (spinal cord), 200 µm (cortex, thalamus). (**C**) Transgene mRNA expression in the brain of young and aged mice. (**D**) Liver ALPL expression from young and aged mice in (**A**). (**E**) Transgene mRNA expression in the liver of young and aged mice. (**F**) HeLa cells were treated with ALPL inhibitor SBI-425 and exposed to 1E4 VG/cell AAV9 or VCAP-102-luciferase. Data is normalized to untreated cells. (**G**) Top-down view of ALPL (orange) showing *in silico* prediction of VCAP-102 capsid footprint (magenta) and SBI-425 binding site (right panel), modeled with Diffdock. (**H**) Brain transduction in mice treated with 35 mg/kg ALPL inhibitor SBI-425. VCAP-102 or 9P36 capsids with a luciferase transgene were administered IV at 2E13 VG/kg. *In vivo* luminescence shows luciferase expression in the brain 7 days after AAV administration. (**I**) Brain transgene mRNA in mice from (H) at day 14. Values normalized to vehicle control. (**J**) Ventral view of mice from (H) 7 days after AAV administration. (**K**) Liver transgene mRNA in mice from (H) at day 14.

Figure 8. *In silico* Prediction and Validation of ALPL-VCAP-102 Interaction



(**A**) AlphaFold2-multimer predicted structure of ALPL dimer (orange) and VCAP-102 VP3 trimer (white, yellow, wheat) with VCAP-102 6-AA peptide shown in turquoise. (**B**) Top-down view of the ALPL active pocket bound to the VCAP-102 peptide (turquoise). Left panel: conserved residues on the peptide are shown in magenta, ALPL residues predicted to interact with VCAP-102 are shown in green. Right panel: ALPL residues in contact with the conserved motif of the peptide are highlighted in red.

CONCLUSIONS

- We have identified ALPL as the vascular receptor mediating BBB-penetrance of a cross-species capsid family.
- ALPL is highly conserved across species, and we confirmed functional interaction of VCAP-102 with human, macaque, mouse and porcine ALPL *in vitro*.
- Direct interaction between of VCAP-102 and ALPL was demonstrated by SPR and displays pH dependent dissociation.
- ALPL ectopic expression increased transcytosis of VCAP-102 >100-fold in a transwell model.
- *In vivo* data supports the central role of ALPL in the BBB transport and CNS transduction of VCAP-102.
  - Aged dependent increase in ALPL expression results in a corresponding increase in VCAP-102 CNS transduction in mice.
  - Pre-treatment with a small molecule inhibitor of ALPL results in decreased CNS transduction of VCAP-102 in mice.
- *In silico* modelling predicts the VCAP-102 peptide binds within the catalytic pocket of ALPL, interacting with highly conserved residues.
  - Point mutation of these residues results in significant loss of transduction *in vitro*.
- This work provides strong *in vivo* and mechanistic evidence that ALPL can be harnessed to transport AAV across the BBB with high efficiency and broad distribution, and that these properties can be faithfully recapitulated in rodents, primates and potentially humans.

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