AAVmod2, an AAV Capsid Engineered to Independently Detarget the Liver and Enhance Gene Delivery to Skeletal Muscle

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AAVmod2 demonstrates liver detargeting and enhanced muscle tropism in mice compared to AAV9

Current limitations with AAV

- Therapeutic application of AAV for neuromuscular diseases is limited by liver toxicity and high doses
- Genetic modification of AAV has the potential to improve AAV, but it is unknown how combinations of modifications will affect AAV function

AAVmod2 solution

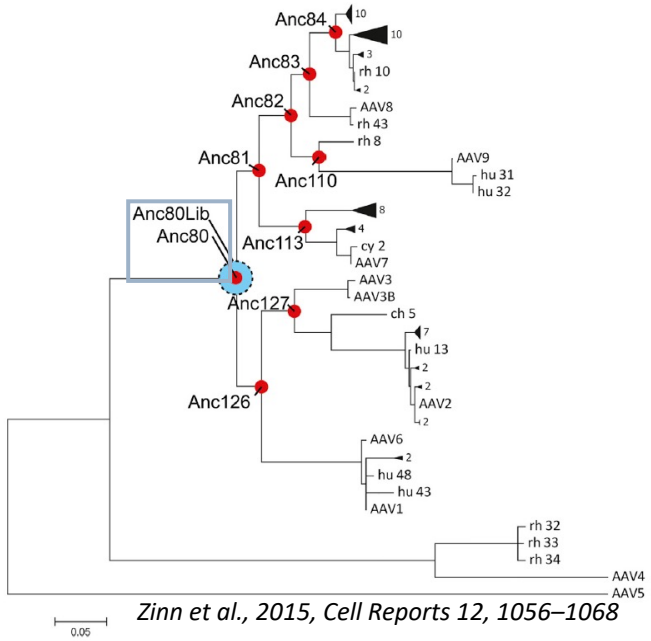
- We engineered a capsid with both a liver detargeting modification (Mod1) and a modification that enhances muscle expression (Mod1.5) into AAV9 creating (Mod2)
- Compared to AAV9, systemic dosing of Mod2 demonstrated an additive phenotype of both liver detargeting and enhanced muscle expression in two different strains of mice

Implications

- AAV Mod2 has an expression profile with potential utility for neuromuscular diseases that merits additional study in NHPs to further test its utility for human use

Discovery of liver detargeting modification (Mod1) from Ancestral Sequence Reconstruction (ASR)

AAV phylogeny

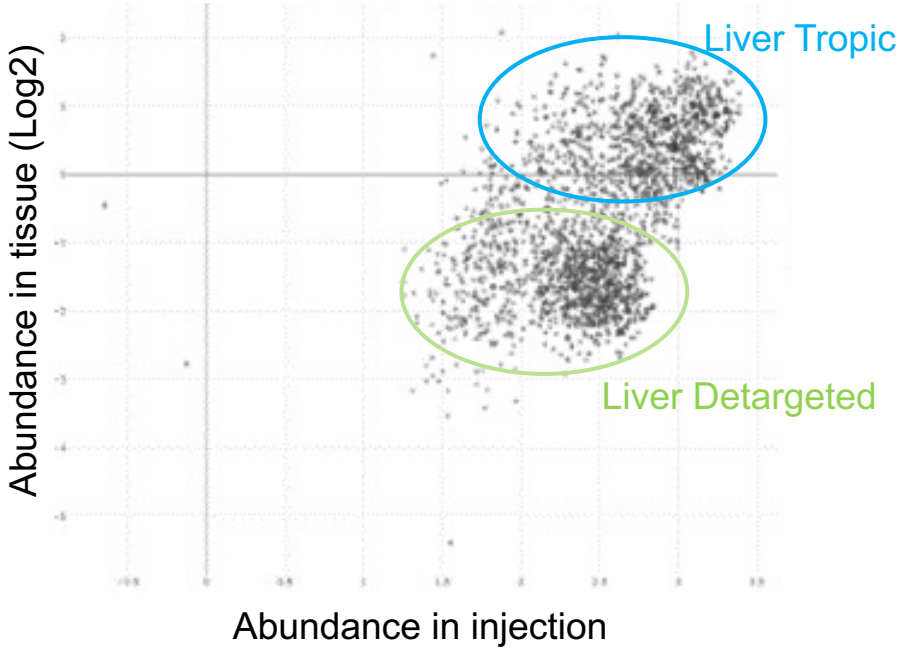


Zinn et al., 2015, Cell Reports 12, 1056–1068

NHP study

- ASR was used to produce pooled barcoded Anc80 library
- 2048 members with toggles at 11 ambiguous positions
- Injected into 2 NHP
- Tissue harvested at day 28
- Barcodes were quantified

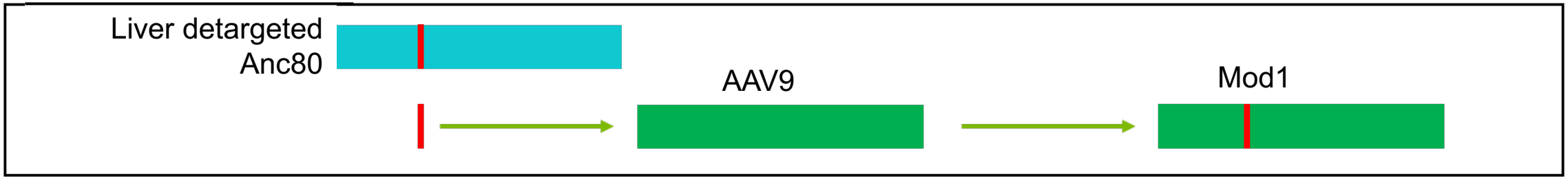
RNA Sequencing counts



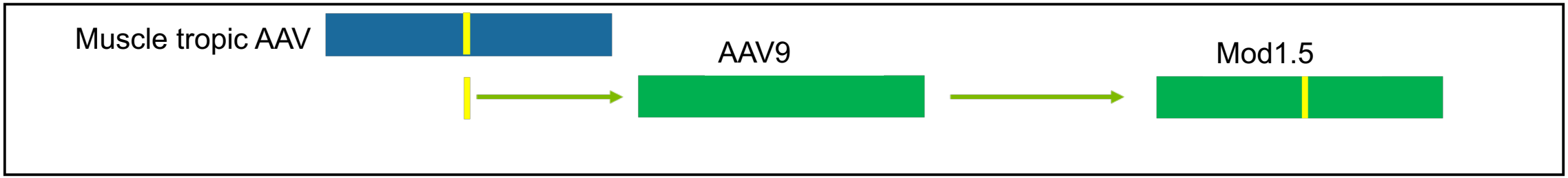
- Sequencing counts of variants segregated into liver tropic and liver detargeted clusters
- A single position divided liver tropic and liver detargeted variants

Engineering of three variant capsids used in study

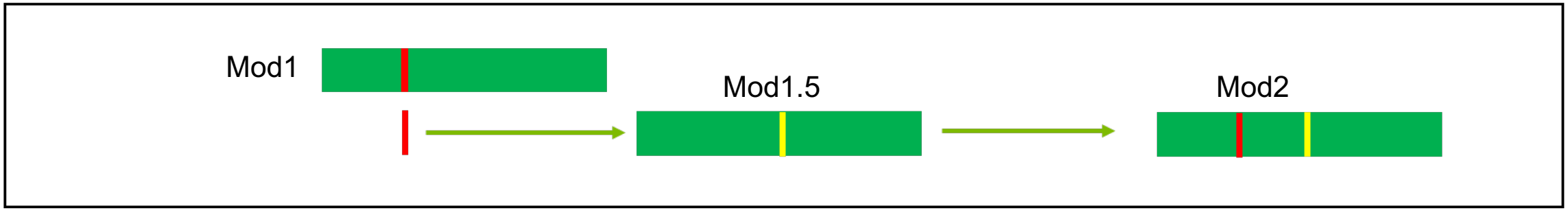
Screening informed AAV9 modification



Peptide insertion



Combination of peptide insertion and AAV9 modification



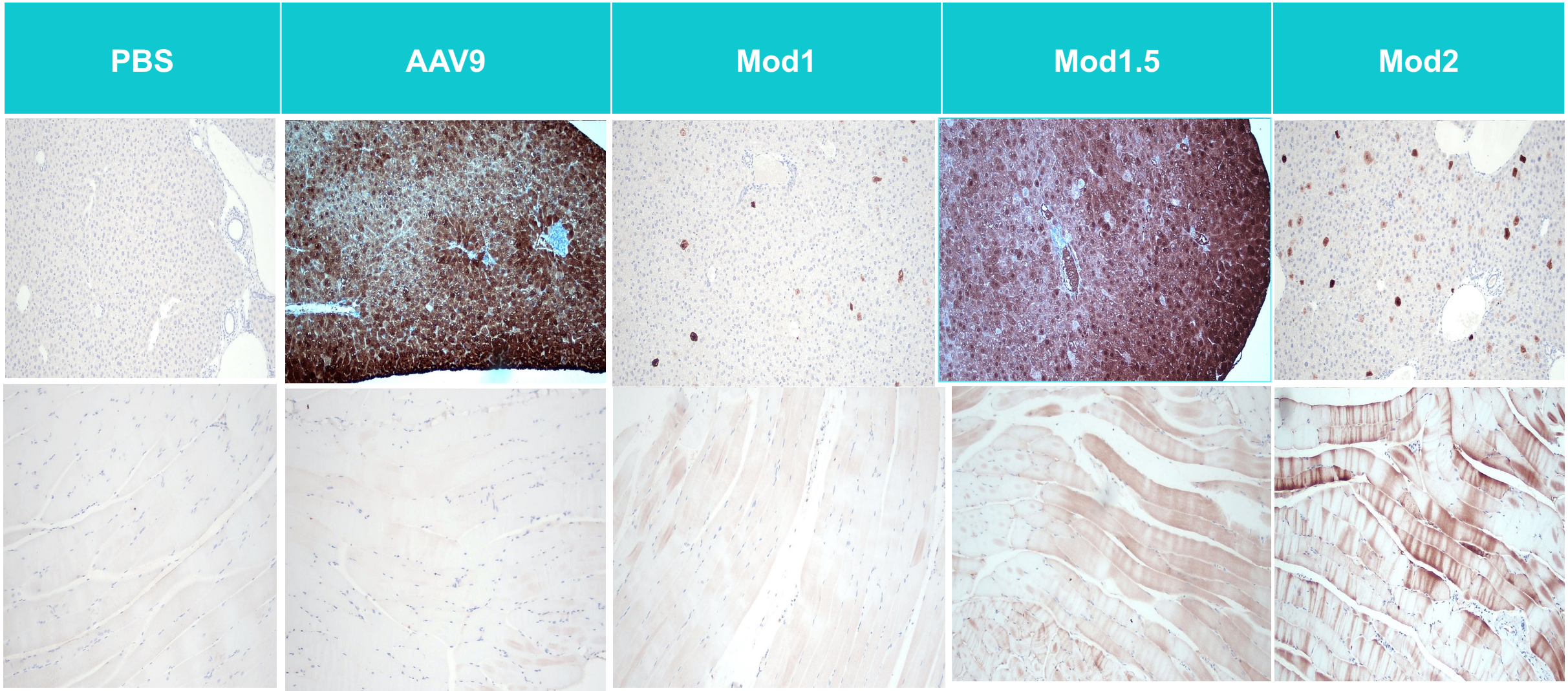
Murine evaluation of Mod1, 1.5 and 2 compared to AAV9

- 2 to 3 male C57Bl/6 mice were IV injected with one of two doses of vector or vehicle control
- At 28 days post-injection, tissues were harvested and fixed for IHC or treated with an RNA preserving agent and stored at -80°C for ddPCR analysis

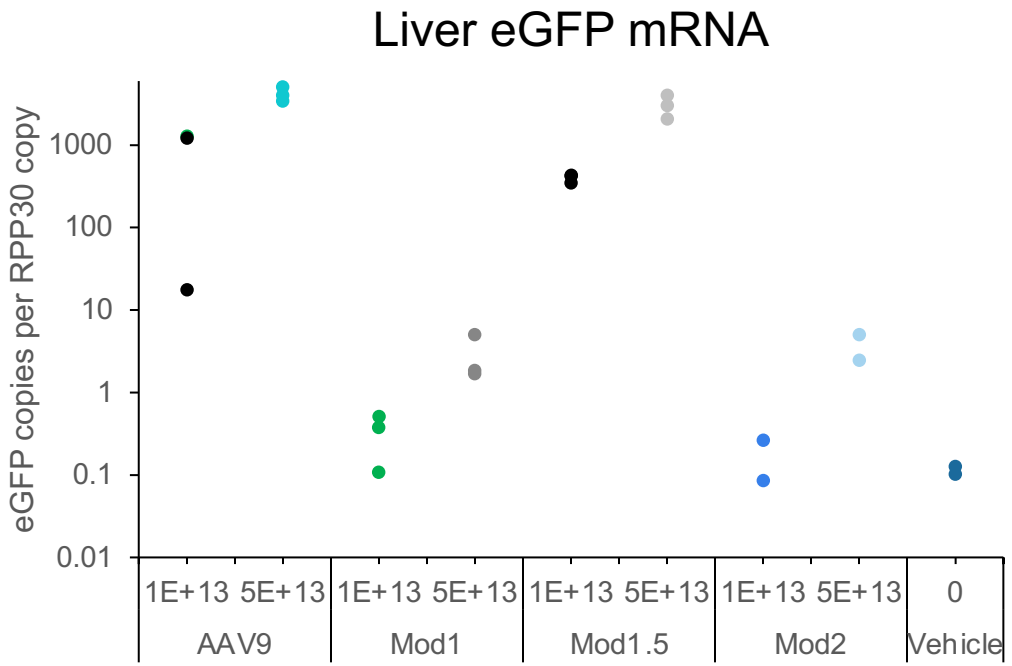
Treatment	Route	Dose (vgs)	No. of animals	Study duration	Necropsy
Vehicle control	IV	0.00E+00	2	28 days	Organ collection
AAV9	IV	2e11	3		
Mod1	IV	2e11	3		
Mod1.5	IV	2e11	3		
Mod2	IV	2e11	3		
AAV9	IV	1e12	3		
Mod1	IV	1e12	3		
Mod1.5	IV	1e12	3		
Mod2	IV	1e12	2		

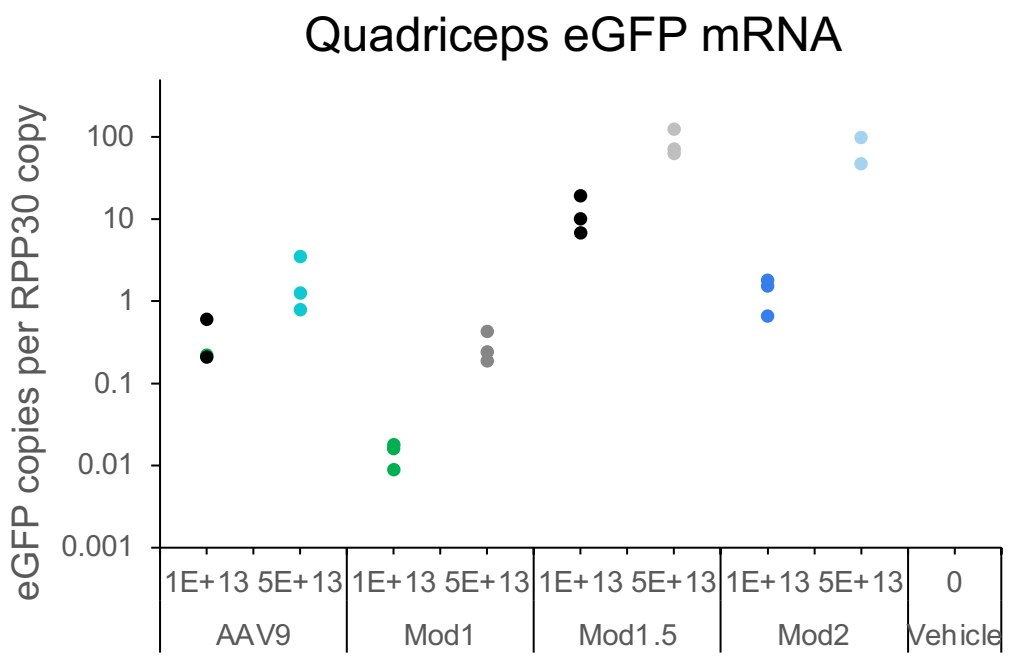
	IHC	Vector Genome ddPCR	GFP mRNA ddPCR
Quadriceps	X	X	X
Triceps Surae (Calf)		X	X
Liver	X	X	X
Heart		X	X
Diaphragm		X	X

Compared to AAV9, expression is enhanced in quadriceps and reduced in liver with Mod2



RT ddPCR data demonstrates expression is enhanced in quadriceps and reduced in liver with Mod2 compared to AAV9





Compared to AAV9:

- Mod1 decreases liver expression but does not enhance muscle expression
- Mod1.5 has similar liver expression and increased muscle expression
- Mod2 has decreased liver expression and increased muscle expression

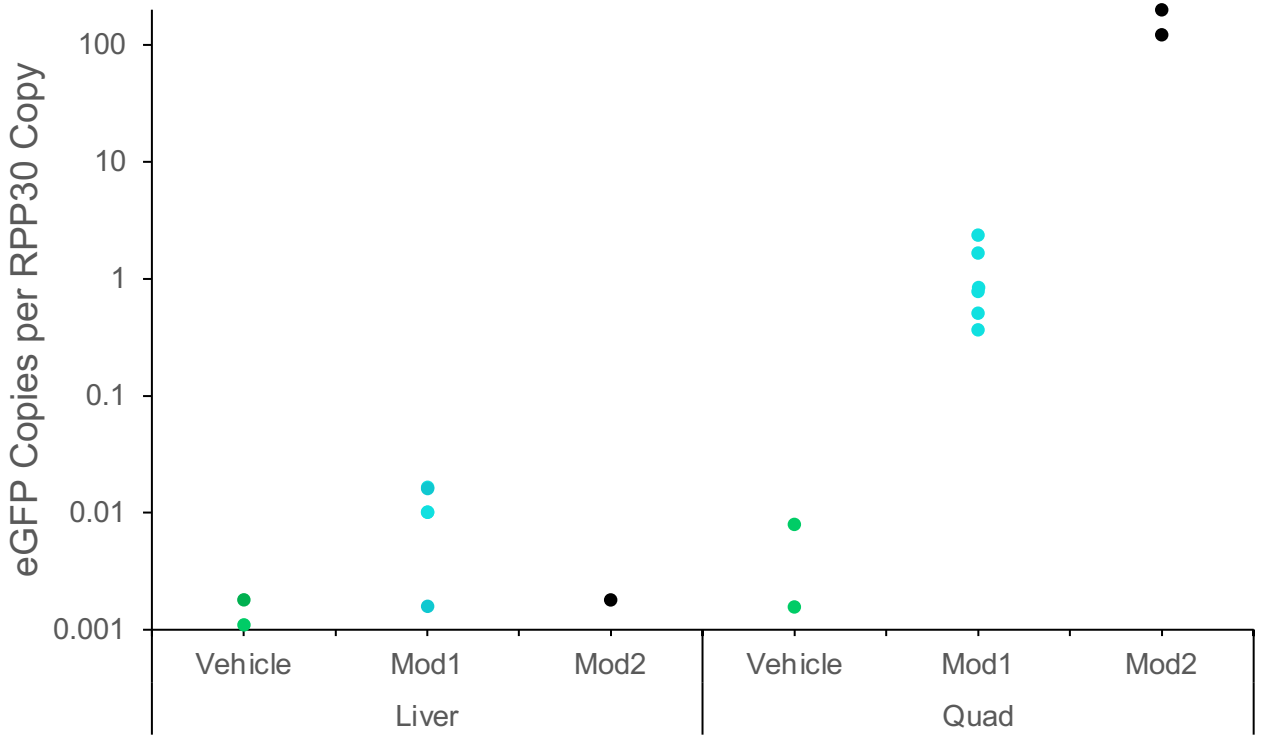
Mod2 liver detargeting and muscle enhancement in BALB/c

- 3 to 6 male BALB/c mice were IV injected with Mod1, Mod2 or vehicle control
- Study and analysis was performed as before with the exception of additional tissue collection
  - Brain, spleen, spinal cord

Treatment	Route	Dose (vgs)	No. of animals	Study duration	Necropsy
Vehicle control	IV	0.00E+00	3	28 days	Organ collection
Mod1	IV	1e12	6		
Mod2	IV	1e12	3		

	Vector Genome ddPCR	GFP mRNA ddPCR
Quadriceps	X	X
Triceps Surae (Calf)	X	X
Liver	X	X
Heart	X	X
Diaphragm	X	X
Brain	X	X
Spinal Cord	X	X
Spleen	X	X

Mod2 liver detargeting and muscle expression is also observed in BALB/c mice



- No increase in liver tropism was observed with Mod2
- Mod2 expression was higher than Mod1 in quadriceps
  - Heart, triceps surae, and diaphragm showed a similar Mod2 enhancement by ddPCR
  - No significant differences were found in the spleen, spinal cord, or liver

Summary

- The use of AAV for gene therapy for neuromuscular diseases is limited by liver toxicity and high doses
- To overcome these issues, we identified two modifications that together increased muscle expression and reduced liver expression in mice compared to AAV9
- The first modification – termed Mod1 – was a single amino acid change that dramatically decreased liver expression while preserving muscle expression. This modification was identified by screening the Anc80 ancestral-sequence derived library for AAV variants that detargeted the liver.
- The second modification – termed Mod1.5 – was a peptide insertion that increased muscle expression while not affecting liver expression relative to wild-type AAV.
- The combination of Mod1 and Mod1.5 – termed Mod2 – led to increased muscle expression with decreased liver expression.
- Therefore, AAV Mod2 has an expression profile with potential utility for neuromuscular diseases that merits additional study in NHPs to further test its utility for human use

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Abstract

Engineering novel adeno-associated virus (AAV) vectors is an exciting emerging area in gene therapy to broaden its reach by addressing the limiting clinical characteristics of conventional serotypes. As has been previously reported, AAV alteration leading to a liver-detargeting phenotype was discovered within the Anc80library, one of nine libraries derived by ancestral sequence reconstruction. The residues necessary and sufficient for this phenotype were identified and confirmed in vivo. Importantly, the ability to detarget the liver has been successfully transferred to naturally occurring serotypes, and AAVmod1 has emerged as a potential lead vector with favorable transduction profile for muscle disorders while detargeting the liver. Demonstration of specific, possibly synergistic, overlay of independent structure/function phenotypes is critical to advance our understanding of vector modification and resulting alteration in tissue tropism.

The use of peptide insertion to enhance or alter AAV targeting is well known. Here, we describe a peptide insertion that improved muscle tropism in mice. Furthermore, the peptide insertion combined with AAVmod1 further enhanced the overall muscle transduction profile. C57BL/6 mice were injected with four CAG.GFP variants: AAV9, AAVmod1, AAVmod1.5 (peptide insertion), and AAVmod2 (mod1+peptide insertion), at doses of 1e13 and 5e13 vg/kg. Upon sacrifice 28 days later, tissues were flash frozen for biodistribution and fixed for immunohistochemistry (IHC). IHC showed characteristic staining of the liver and muscle with AAV9. As suggested by earlier work, AAVmod1 showed reduced liver staining and AAVmod1.5 enhanced muscle staining. Interestingly, the combination of modifications in AAVmod2 showed uncompromised features of both engineered vectors, and perhaps qualitatively enhanced muscle delivery. Biodistribution by ddPCR confirmed that the peptide insertion alone had improved targeting of skeletal muscle, 5x better by gc/diploid genome versus AAV9, with a 50x improved gene expression by eGFP mRNA versus the housekeeping gene RPP30. AAVmod1.5 is otherwise akin to its parental serotype in terms of robust hepatocyte transduction. AAVmod1 had a >10x reduction in liver gc/diploid genome and a>1000x reduction in liver expression. As observed by IHC, AAVmod2 liver-detargeting was unaffected by the muscle enhancement. Expression in the muscle by ddRT-PCR was equivalent between AAVmod1.5 and AAVmod2. Quantitative measure of GFP signal by IHC is ongoing.

Future studies will test the transduction profile ofAAVmod2 in BALB/c mice and NHPs.

Experimental design and methods: Study 1 C57Bl/6

Animals: 25 male C57Bl/6 mice. Survival time was 28 days after AAV delivery for all the animals.

Production of AAV vectors: Recombinant AAV vectors AAV9-eGFP WT, Mod1, Mod1.5, and Mod2 were produced by triple transfection of human embryonic kidney carcinoma 293 cells (HEK- 293)

Vector Infusion: All animals received AAV vector via intravenous tail vein injection at either 1e13 or 5e13 gc/kg; (Table 1).

Analysis of transgene expression: To assess transgene expression, quadriceps, triceps surae, heart, diaphragm, and liver sections were processed for ddPCR analysis. Livers and quadriceps sections were also processed for immunohistochemical analysis (IHC).

Immunohistochemistry: *GFP staining by 3,3'-diaminobenzidine (DAB)*: Sections (3 per each 6-mm block: separation of 2 mm) were washed 3 times in PBST followed by treatment with 1% H_2O_2. Sections were stained with the primary anti-GFP antibody diluted 1:1000 in Da Vinci Green Diluent as previously described (San Sebastian et al., 2013).

ddPCR: Fresh tissue pieces were collected in RNA later, treated over night at 4C, and then stored at -80C. DNA and RNA was extracted from 30 mg sections. DNA and RNA samples were assayed for eGFP vector genome or mRNA and normalized to murine RPP30 genomic copies or RPP30 mRNA copies. Triplicate technical replicates were performed.

Table 1

Treatment	Route	Dose (vgs)	No of animals	Study duration	Necropsy
Vehicle control	IV	0.00E+00	2	28 days	Organ collection
WT	IV	2e11	3		
Mod1	IV	2e11	3		
Mod1.5	IV	2e11	3		
Mod2	IV	2e11	3		
WT	IV	1e12	3		
Mod1	IV	1e12	3		
Mod1.5	IV	1e12	3		
Mod2	IV	1e12	2		

Table 2

	IHC	Vector Genome ddPCR	GFP mRNA ddPCR
Quadriceps	X	X	X
Triceps Surae (Calf)		X	X
Liver	X	X	X
Heart		X	X
Diaphragm		X	X

Experimental design and methods: Study 2 BalbC

Animals: 12 male BalbC mice. Survival time was 28 days after AAV delivery for all the animals.

Production of AAV vectors: Recombinant AAV vectors AAV9-eGFP WT, Mod1, Mod1.5, and Mod2 were produced by triple transfection of human embryonic kidney carcinoma 293 cells (HEK- 293)

Vector Infusion: All animals received AAV vector via intravenous tail vein injection at either 1e13 or 5e13 gc/kg; (Table 1).

Analysis of transgene expression: To assess transgene expression, quadriceps, triceps surae, heart, diaphragm, brain, spinal cord, spleen, and liver sections were processed for ddPCR analysis. Livers and quadriceps sections were also processed for immunohistochemical analysis (IHC).

Immunohistochemistry: *GFP staining by 3,3'-diaminobenzidine (DAB)*: Sections (3 per each 6-mm block: separation of 2 mm) were washed 3 times in PBST followed by treatment with 1% H_2O_2. Sections were stained with the primary anti-GFP antibody diluted 1:1000 in Da Vinci Green Diluent as previously described (San Sebastian et al., 2013).

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Treatment	Route	Dose (vgs)	No of animals	Study duration	Necropsy
Vehicle control	IV	0.00E+00	3	28 days	Organ collection
Mod1	IV	1e12	6		
Mod2	IV	1e12	3		

	Vector Genome ddPCR	GFP mRNA ddPCR
Quadriceps	X	X
Triceps Surae (Calf)	X	X
Liver	X	X
Heart	X	X
Diaphragm	X	X
Brain	X	X
Spinal Cord	X	X
Spleen	X	X