Cellular, Tissue, and Gene Therapies Advisory Committee September 2-3, 2021 Meeting Presentation

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AAV INTEGRATION STUDIES IN LARGE ANIMAL MODELS: NON-HUMAN PRIMATES AND DOGS

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Cellular, Tissue, and Gene Therapies
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DISCLOSURES

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AAV INTEGRATION STUDIES IN LARGE ANIMAL MODELS

Species	Disease Model	AAV Vector; Route	AAV Dose (vg/kg)	Duration of F/U	Reference
NHP	WT n=18	AAV-RSV-LEA29Y AAV1 or AAV8 IV or IM	$5x10^{12}$	1.2-2.8 yrs	Nowrouzi et al. 2012 Mol Ther 20(6):1177
NHP	WT n=6	AAV5-hPBGD	$1x10^{13}$ $5x10^{13}$	1 month	Gil Farina et al. 2016 Mol Ther 24(6):1100
NHP	WT n=6	AAV-hFIX AAV8 or AAV5 In utero	$1.4\text{-}1.9x10^{13}$	11-63 months	Mattar et al. 2017 Mol Ther 25(8):1843
NHP	WT n=12	AAV5-hFIX IV	$5x10^{11}$, $5x10^{12}$ $2x10^{13}$, $9x10^{13}$	6 months	Spronck et al. 2020 Res Pract Thromb Haem 4, S1:562
NHP	WT n=12	AAV5-hFVIII IV	$2x10^{13}$ $6x10^{13}$	13 wks and 26 wks (6 mo)	Sullivan et al. 2021 Mol Ther 29(4S1):425
Dog	Hemophilia A n=9	AAV-TBG-cFVIII AAV-hAAT-cFVIII AAV8 or AAV9 PV or IV	$1x10^{13}$ $2x10^{13}$ $4x10^{13}$	2-10 yrs	Nguyen, Everett et al. 2021 Nat Biotechnol 39(1):47
Dog	Hemophilia A n=8	AAV-TTR-cFVIII AAV2, 6, 8 PV	$6x10^{12}$ to $2.7x10^{13}$	8-12 yrs	Batty et al. 2020 Res Pract Thromb Haem 4, S1:550

METHODS FOR AAV INTEGRATION STUDIES

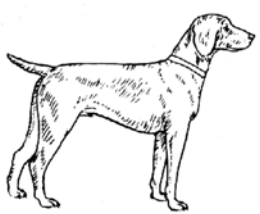
	LM-PCR Ligation-Mediated PCR	LAM-PCR Linear Amplification-Mediated PCR	TES Target enrichment sequencing
DNA SAMPLE PREPARATION	Sonication Adapter ligation	Linear PCR Magnetic capture Double strand synthesis Restriction digest Linker ligation	Mechanical shearing Adaptor ligation Pre-capture PCR Bait hybridization Magnetic capture
PCR	Primers in ITR (or other vector sequence) and adapter	Single primer or multiple primers across vector cassette and linker	Post-capture PCR
SEQUENCING	Illumina	Illumina	Illumina
ANALYSIS	Custom software, AAVenger Nguyen, Everett et al. Nat Biotech 2021	Customized bioinformatic analysis	Customized bioinformatic analysis
REFERENCES	Nguyen, Everett, et al. Nat Biotech 2021 Sherman et al. Mol Ther Meth Cl Dev 2017 Berry et al. Mol Ther Meth Cl Dev 2017 Berry et al. Bioinformatics 2012	Gil-Farina et al. Mol Therapy, 2016 Schmidt et al. Nature Methods, 2007	Batty et al. Res Pract Thromb Haem 4, S1:550, 2020 GeneWerk

COMPARISON OF AAV INTEGRATION STUDIES IN NHP

AAV Vector	AAV Dose (vg/kg)	Duration of F/U	Methods	Reference
AAV-RSV-LEA29Y AAV1 or AAV8	$5x10^{12}$	1.2-2.8 yrs	LAM-PCR 454 sequencing	Nowrouzi et al. 2012
AAV5-hPBGD	$1x10^{13}$ $5x10^{13}$	1 months	LAM-PCR Illumina	Gil Farina et al. 2016
AAV-hFIX AAV8 or AAV5	$1.4\text{-}1.9x10^{13}$	11-63 months	LAM-PCR Illumina	Mattar et al. 2017
AAV5-hFIX	$5x10^{11}$, $5x10^{12}$ $2x10^{13}$, $9x10^{13}$	6 months	LAM-PCR Illumina	Spronck et al. 2020
AAV5-hFVIII	$2x10^{13}$ $6x10^{13}$	13 wks and 26 wks (6 mo)	TES Illumina	Sullivan et al. 2021

- Vector-vector sequences observed (75%-99% of genomes)
- Complex rearranged vector sequences detected
- Integration distributions
  - No preference for gene coding regions and no clustering of integration sites reported (Nowrouzi 2012, Gil-Farina 2016, Mattar 2017)
  - Recurrent integrations observed around transcription units (Spronck 2020, Sullivan 2021)
- Integration frequency reported to be low; but methodological concerns are major
- Majority of integrations detected as single events (1-2 cells) but a few were detected in up to 7 cells (Sullivan et al. 2021)
- No evidence of nodules, tumors or malignancies in the NHP

AAV INTEGRATION STUDIES IN DOG MODELS

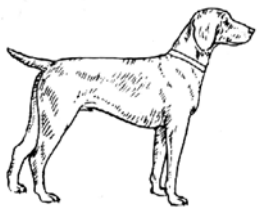


Hemophilia A dogs

- Hemophilia A dog colonies
  - UNC, Chapel Hill, North Carolina
  - Queen's University, Kingston, Ontario
- Inversion in intron 22 of the factor 8 gene that is analogous to the most common mutation found in humans
- Severe hemophilia A (<1% cFVIII activity)
- AAV studies initiated in early 2000's

AAV vector	AAV serotype	AAV Dose (vg/kg)	Duration of F/U	Methods	Reference
AAV-TBG-cFVIII AAV-hAAT-cFVIII	AAV8 AAV9	$1x10^{13}$ $2x10^{13}$ $4x10^{13}$	2-10 yrs	Ligation Mediated PCR Illumina	Nguyen, Everett et al. 2021 Nat Biotechnol 39(1):47
AAV-TTR-cFVIII	AAV2 AAV6, AAV8	$6x10^{12}$ to $2.7x10^{13}$	8-12 yrs	LAM-PCR and TES Illumina	Batty et al. 2020 Res Pract Thromb Haem 4, S1:550

LONG TERM DOSE-DEPENDENT EXPRESSION OF cFVIII IN HEMOPHILIA A DOGS AFTER AAV-cFVIII DELIVERY

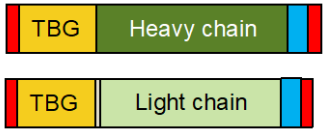


Hemophilia A dogs

<1% cFVIII activity

Two chain delivery
of canine FVIII

AAV8 or AAV9

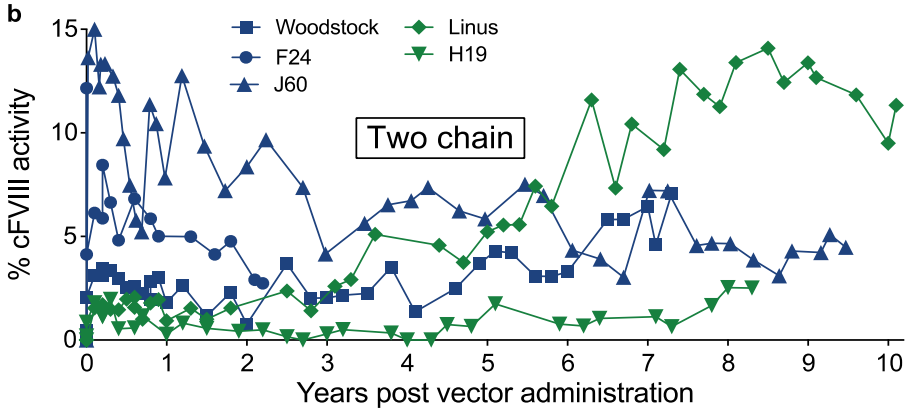


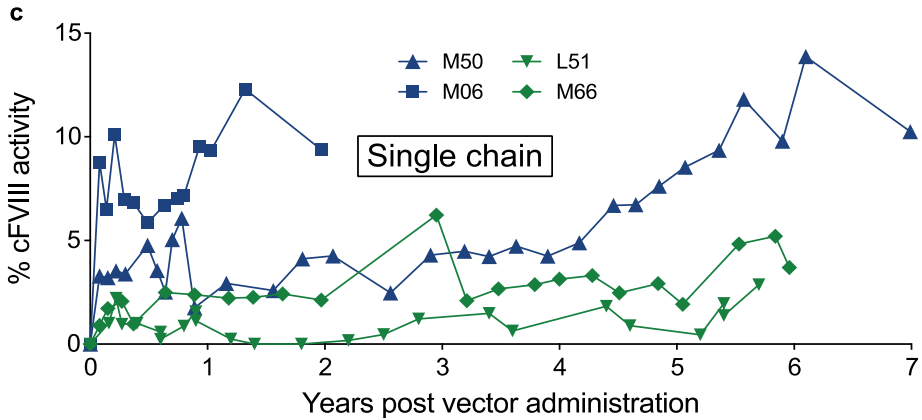
2.5 x 10^{13} vg/kg
1.2 x 10^{13} vg/kg

Single chain delivery
of B-domain deleted cFVIII

AAV8

4 x 10^{13} vg/kg
2 x 10^{13} vg/kg





Nguyen, Everett Nature Biotechnol 39(1):47, 2021

DNA ANALYSIS OF AAV GENOMES AFTER GENE THERAPY

- Liver samples were collected from the dogs at the end of the study for DNA analysis

Vector copy number analysis	Quantitative PCR	Estimate of vector DNA copy number Episomal and integrated forms
AAV integration site analysis	Ligation mediated PCR Illumina sequencing Custom software pipeline, AAVenger	DNA sequence data on the sites of integration and vector genomes

Penn
University of Pennsylvania
Frederic Bushman, Ph.D.

Nguyen, Everett Nature Biotechnol 39(1):47, 2021
Berry et al., Mol Ther Methods Clin Dev 4:17, 2017
Sherman et al., Mol Ther Methods Clin Dev 4:39, 2017

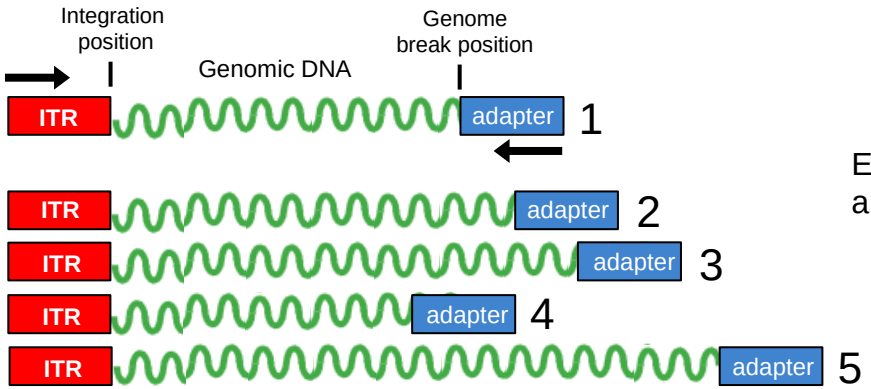
METHODS FOR DETERMINING THE NUMBER OF INTEGRATION EVENTS

Integration site analysis

Ligation mediated PCR
Illumina sequencing
Custom software pipeline AAVenger

- In order to identify integration sites and estimate clonal abundances, DNA samples were sheared using sonication after which adapter sequences were attached.
- Genomic fragments containing the interior edge of the ITR sequences were selectively amplified and sequenced.
- Counting the number of unique genome break positions associated with each integration provided an estimate of its clonal abundance.

Estimating clonal populations with SonicAbundance method



Example of a clone with an abundance of 5 cells.

Sherman, Nobles et al., 2017
Berry, Nobles et al., 2017

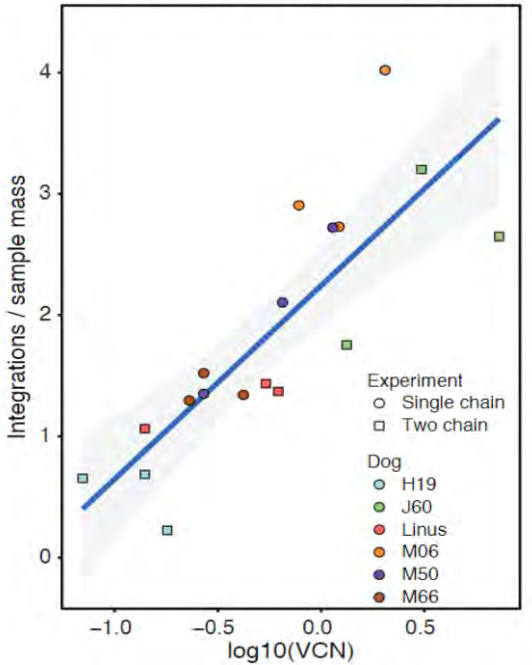
CORRELATION BETWEEN VECTOR COPY NUMBER AND INTEGRATION EVENTS AFTER AAV-FVIII DELIVERY

Summary of vector copy number and integration events

Delivery Approach	Dog	Years Post AAV Delivery	# of liver samples	Vector copy number per diploid genome	# of integration sites recovered (n=3 per dog)
Two Chain	J60	9.5	29	3.4 ± 2.4	271
Two Chain	Wood-stock	8	5	0.3 ± 0.1	--
Two Chain	Linus	10	15	0.3 ± 0.2	131
Two Chain	H19	8	11	0.2 ± 0.1	160
Single Chain	M50	7	29	0.4 ± 0.3	258
Single Chain	M06	2	8	1.4 ± 0.7	764
Single Chain	M66	6	13	0.3 ± 0.2	161
Single Chain	L51	6	5	0.01 ± 0.02	--

Total 1741

Integrations events vs. vector copy number



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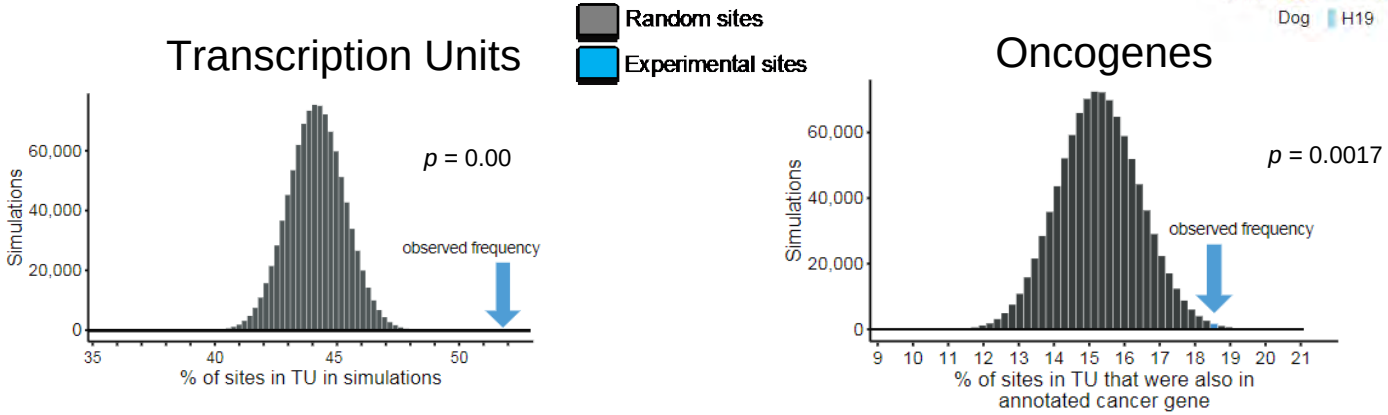
DISTRIBUTION OF AAV INTEGRATION SITES

- Distributed throughout the canine genome
- Integration favored in transcription units and oncogenes
- Integration sites in genomic features associated with active transcription



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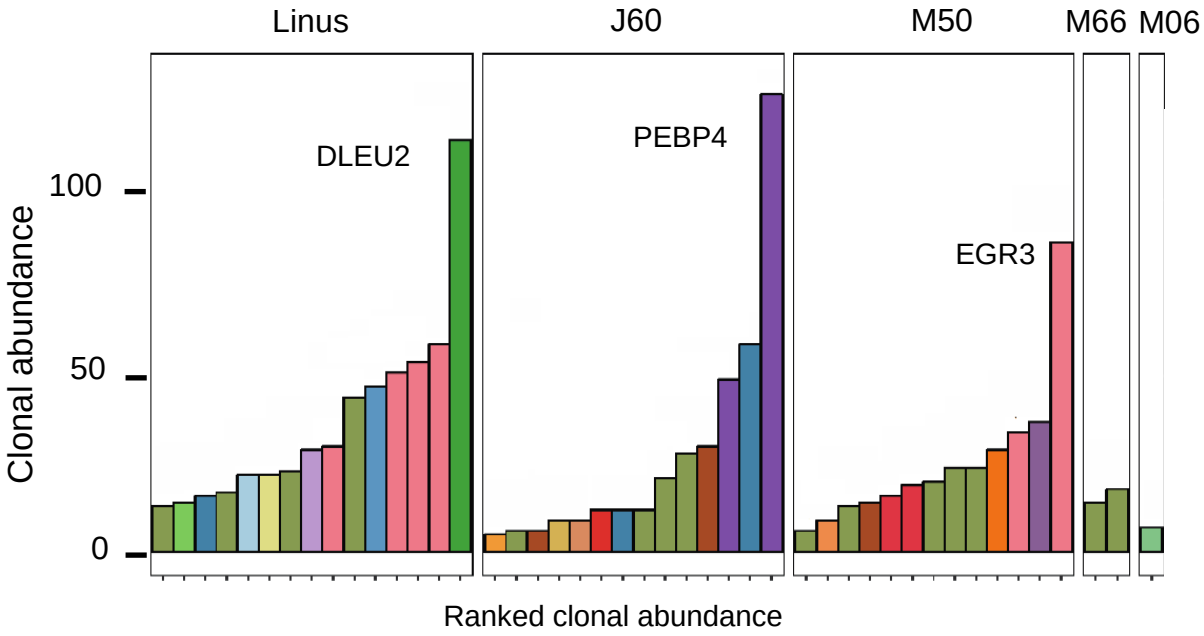
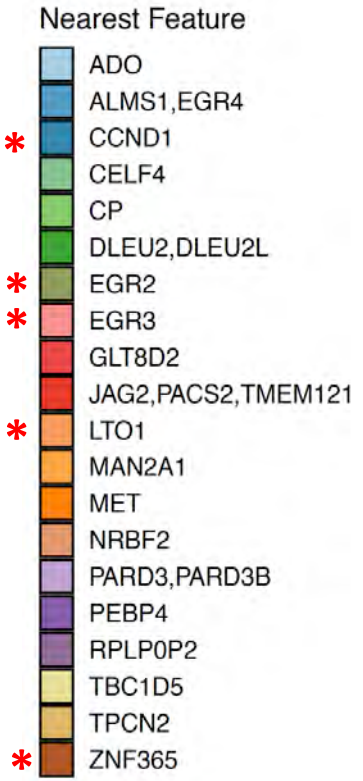
EVIDENCE FOR CLONAL EXPANSION AT SITES OF AAV INTEGRATION

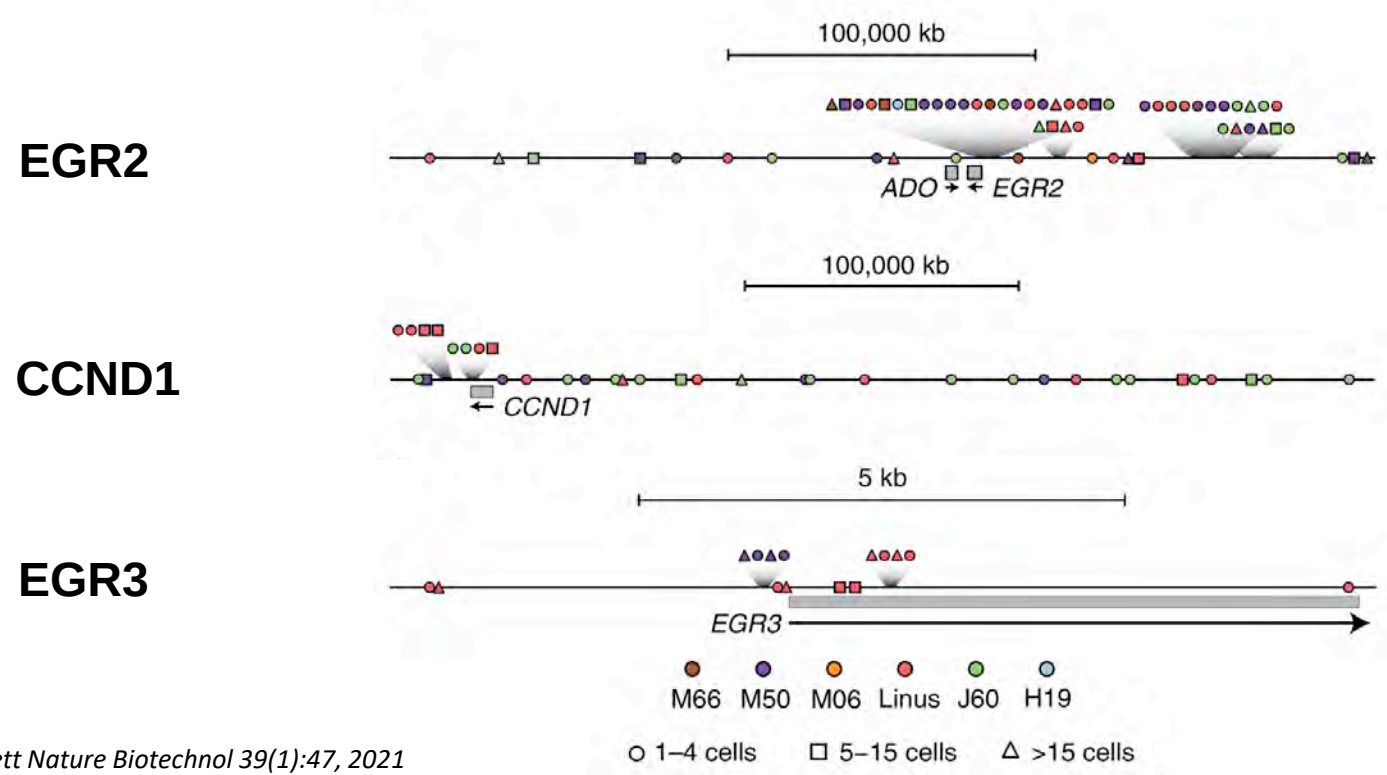
- Identified 54 abundant clonal populations (≥5 cells).
- Several clonal expansions had integrations near genes associated with growth control and cancers in humans.





CLUSTERING OF INTEGRATION SITES IN THE CANINE GENOME

- Clusters identified at EGR2, EGR3, CCND1, ALB and DUSP1



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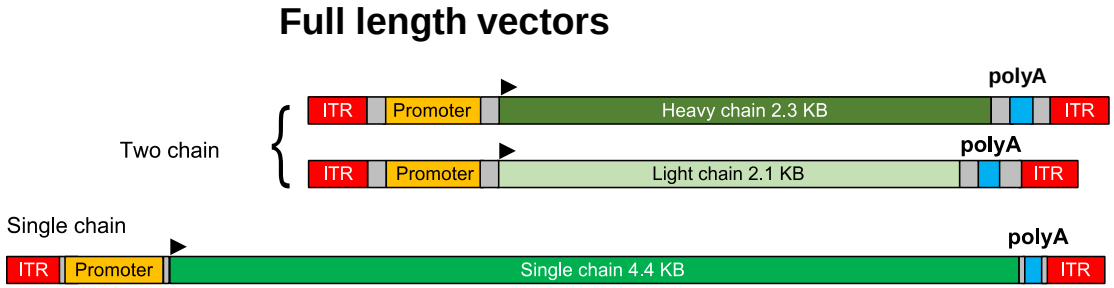
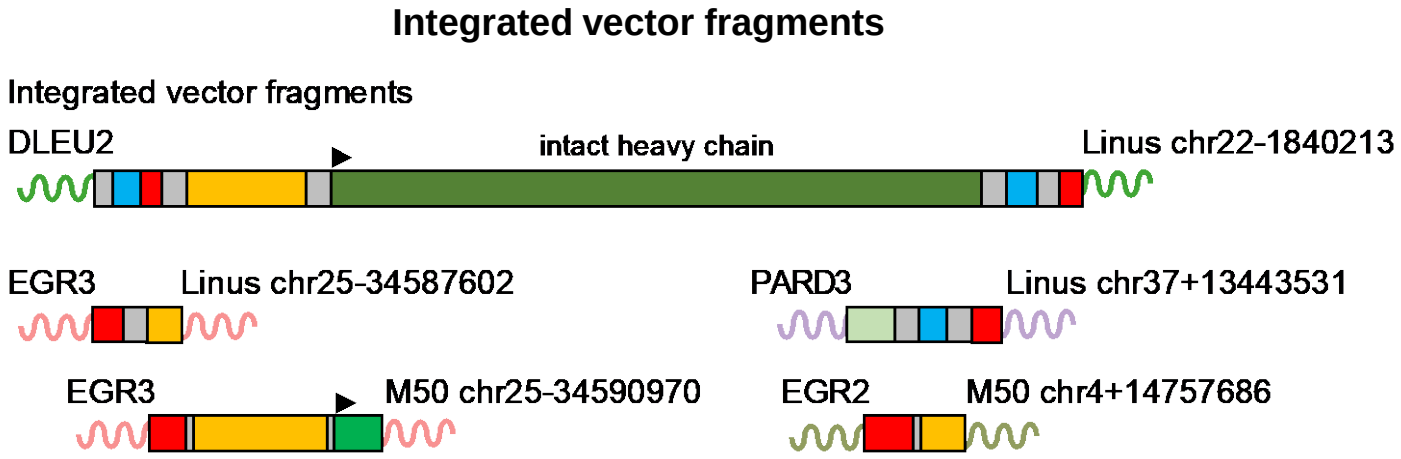
GENETIC MAP OF INTEGRATED VECTOR SEQUENCES

- A full length coding sequence of a vector was identified in an expanded clone
- Numerous rearranged vectors were also detected
- Candidate explanation for the increase in FVIII expression is the clonal expansion of cells harboring integrated vectors





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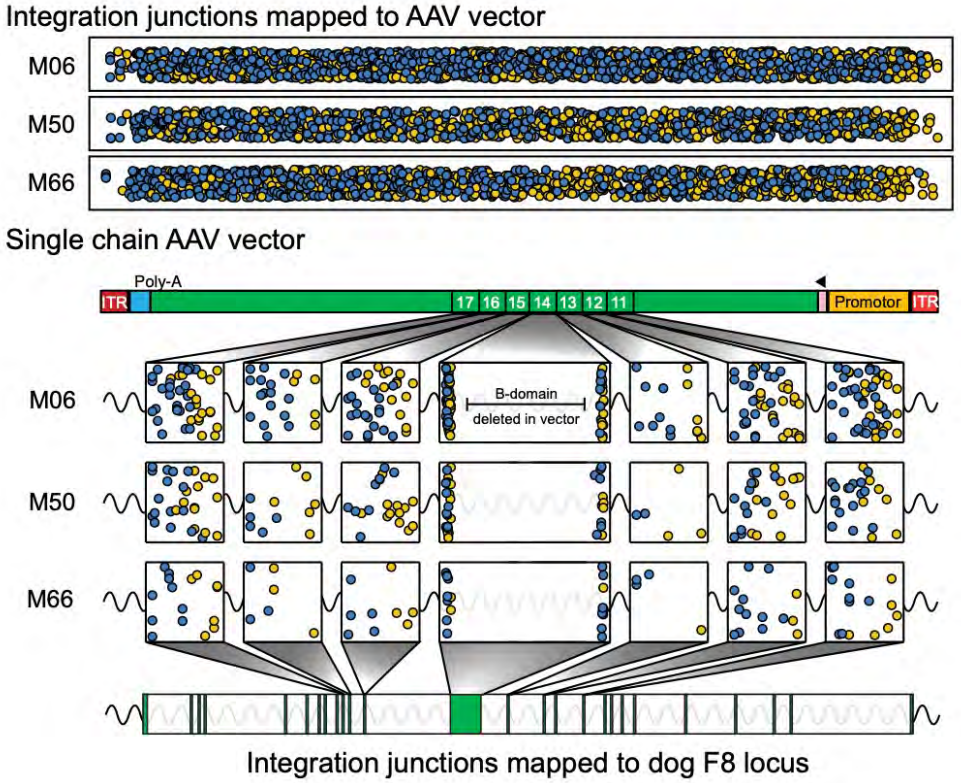
REARRANGEMENTS OF THE AAV VECTOR

- 82% of integration sites showed apparent integration of AAV into F8 itself
- No integration into genomic F8
  - No integration in introns
  - No integration in B-domain
  - Sequence reads cross exon-exon boundaries
- Evidence of extensive vector rearrangement
- Unknown if integrated or episomal forms
- Unknown if rearrangements occurred during vector production or in target cells



Nguyen, Everett Nature Biotechnol 39(1):47, 2021

SUMMARY OF AAV INTEGRATION IN HEMOPHILIA A DOGS

- Stable and sustained FVIII expression up to 10 years in a large animal model of hemophilia A.
  - Two of 9 dogs experienced a gradual rise in FVIII activity that was 4 times the steady state levels.
  - These studies support the hypothesis that this observation may be due to clonal expansion of cells with integrated vectors.
- AAV integration was favored in transcription units and oncogenes.
- The structures of AAV integrated forms often contained rearranged or truncated vector genomes. Vector-vector forms were the most common form detected.
- Clustering of integration sites was observed at five loci.
- Clonal expansion of integrated AAV genomes was observed in 5 of 6 dogs analyzed.
- Mild asymptomatic elevations of liver enzymes were not consistent with any specific liver pathology. Liver pathology was consistent with age-related findings also observed in naïve dogs.
- While AAV integration and clonal expansion were observed, the dogs had no evidence for tumorigenesis.

Nguyen, Everett Nature Biotechnol 39(1):47, 2021

COMPARISON OF AAV INTEGRATION STUDIES IN HEMOPHILIA A DOGS

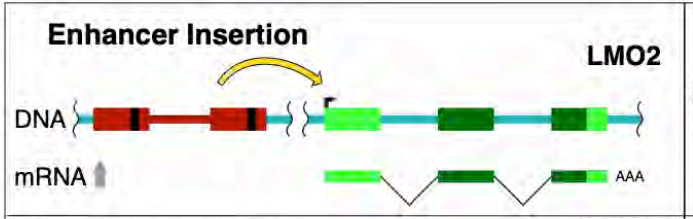
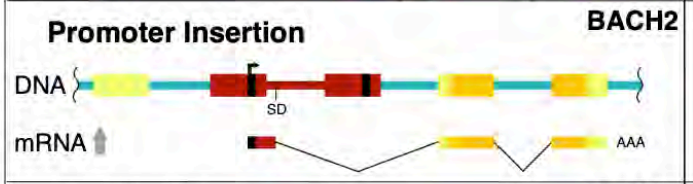
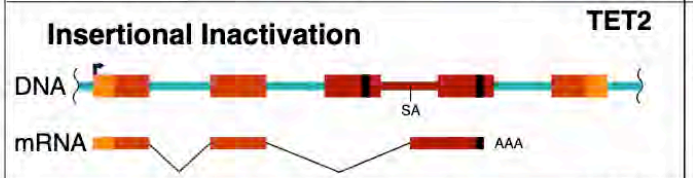
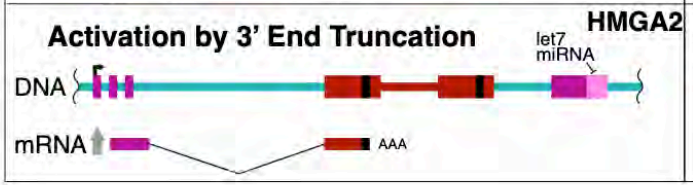
AAV vector	AAV serotype	AAV Dose (vg/kg)	Duration of F/U	Methods	Reference
AAV-TBG-cFVIII AAV-hAAT-cFVIII	AAV8 AAV9	$1x10^{13}$, $2x10^{13}$, $4x10^{13}$	2-10 yrs	Ligation-Mediated PCR Illumina	Nguyen, Everett et al. 2021 Nature Biotechnol 39(1):47
AAV-TTR-cFVIII	AAV2 AAV6, AAV8	$6x10^{12}$ to $2.7x10^{13}$	8-12 yrs	LAM-PCR and TES Illumina	Batty et al. 2020 Res Pract Thromb Haemost. 4S1:550

- Vector genome forms
  - Vector-vector forms are the most abundant forms detected (>80%)
    - not known if episomal or integrated forms
  - Integrated forms (vector-genome forms) represented 5% -18%
- Location of integration events
  - Transcription units favored when annotation is transferred from human onto canine genome
  - Not observed with canine annotation only
- Clustering at common integration sites detected
- Majority of integration events were detected at single sites; clonal expansions were detected in some dogs (Nguyen, Everett et al. 2021)
- While integration events were observed in all animals, no tumors were detected.

CHALLENGES OF AAV INTEGRATION STUDIES

- AAV primarily remains episomal
  - Assays yield mixtures of episomal and integrated forms
  - Vector-vector sequences suggest concatemeric forms but not known if integrated or episomal forms
- Secondary structures in ITR can impact recovery of integration events
- AAV vector genomes are often rearranged and/or truncated
  - May impact recovery of integrated vector genomes
  - Nonfunctional vector genomes detected
- Tissue sampling represents a small population of the cells in the tissue
  - Serial sampling of the same cell population is not possible in solid tissues
- Population size of the integrated vectors can be challenging to estimate
  - Analysis yields subsample of the full population
- Comparative genomics with different model organisms and the quality of the annotation can lead to apparent differences
- Vector design may influence the effects of AAV integration (promoter, etc.)
- Duration of follow up may impact findings
  - Not known when AAV integration occurs and what occurs longitudinally

POTENTIAL MECHANISMS FOR CLONAL EXPANSION

	Virus or Vector	Clinical Setting
	*Gammaretroviral vectors*	*SCID-X1, WAS, CGD, gene therapy*
	*HIV*	*Clonal expansion in HIV latency*
	*Lentiviral vector*	*CART cellular immunotherapy*
	*Lentiviral vector*	*Gene therapy for Beta thalassemia*

Bushman Mol Therapy 28(2): 353, 2020

CONCLUSIONS

- The AAV genomes are a mixture of episomal and integrated forms.
- AAV genomes are complex and highly rearranged leading to complexity of molecular structures.
- Clonal expansions have been detected but mechanisms and biological significance are not known.
- Technology and analysis tools are inherently limited; alternative technologies are under evaluation.

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Penn Vector Core

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