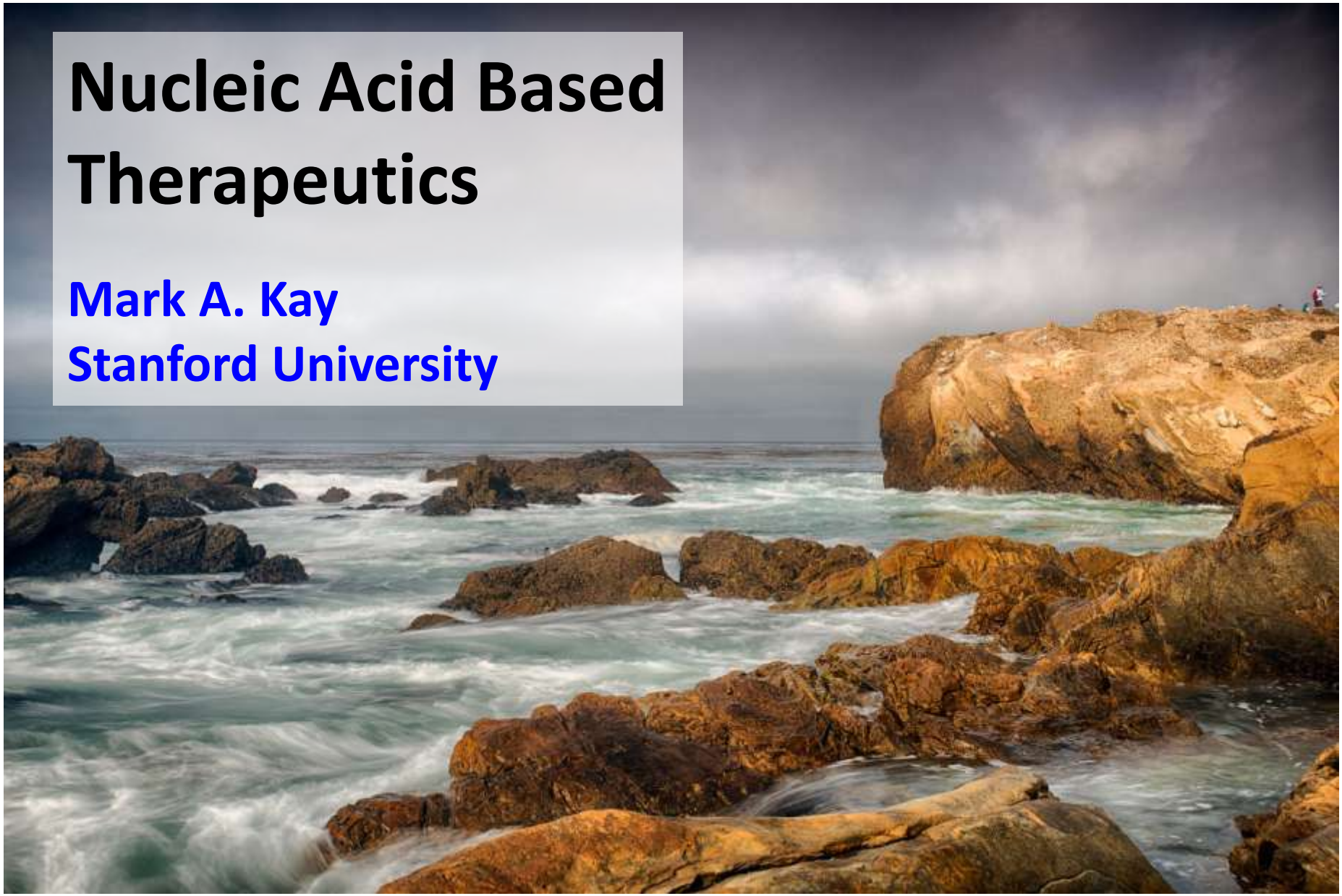
Nucleic Acid Based Therapeutics

Mark A. Kay
Stanford University



Lab Interests: ***Non-coding RNAs, Gene therapeutics-AAV and plasmid DNA vectors***

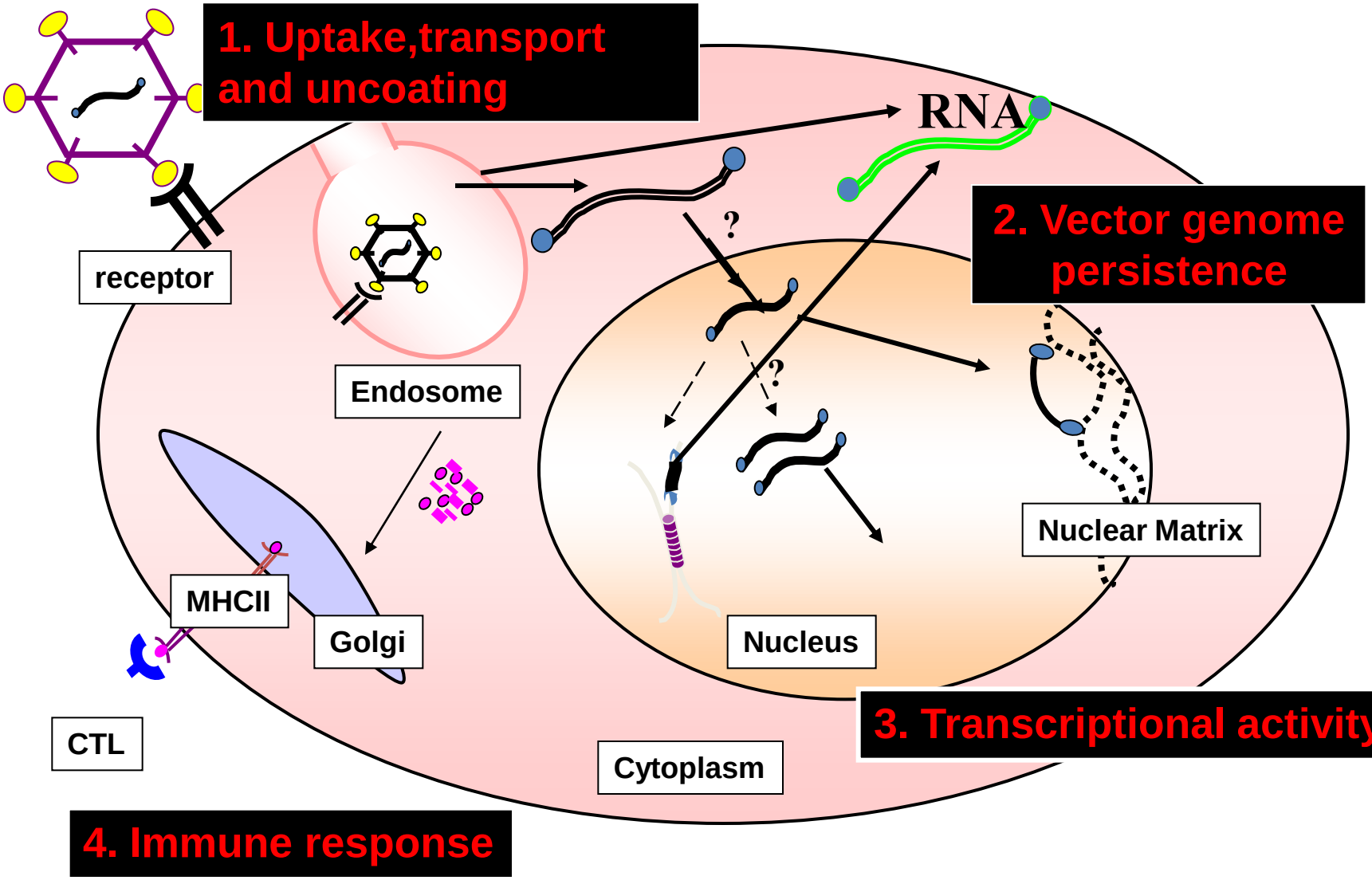
What Do We Want to Accomplish?

- **Add a gene**-*restore a missing gene function or supply an RNA or protein that has pharmacological effect*

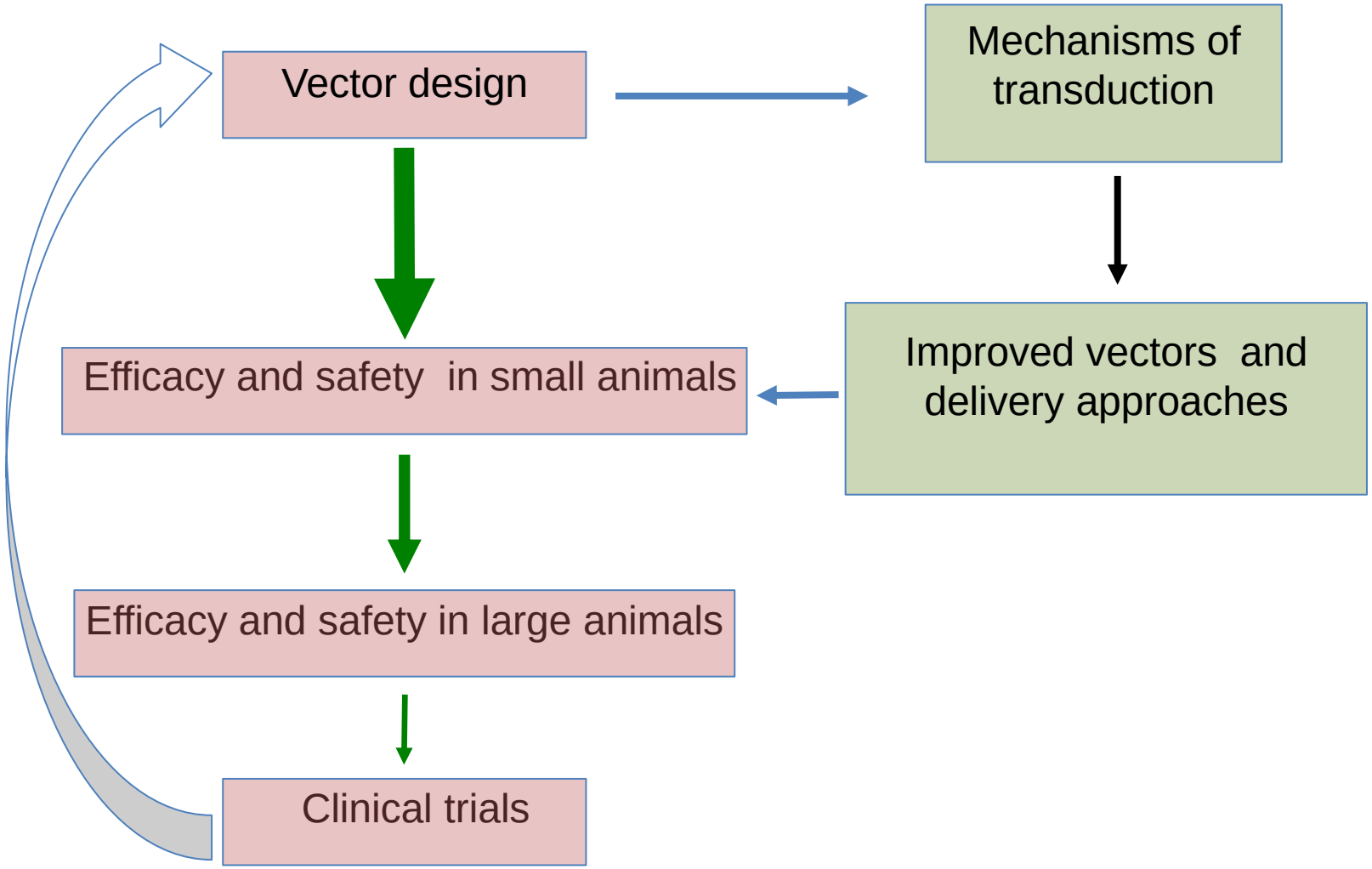
- **Fix a gene** change the DNA-*mutation repair*

- **Silence a gene-***from a pathogen, gain of function mutation*

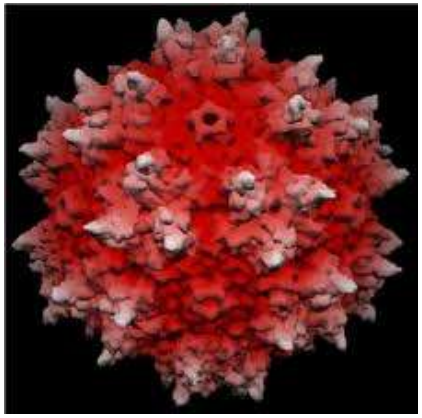
Potential factors limiting vector efficacy



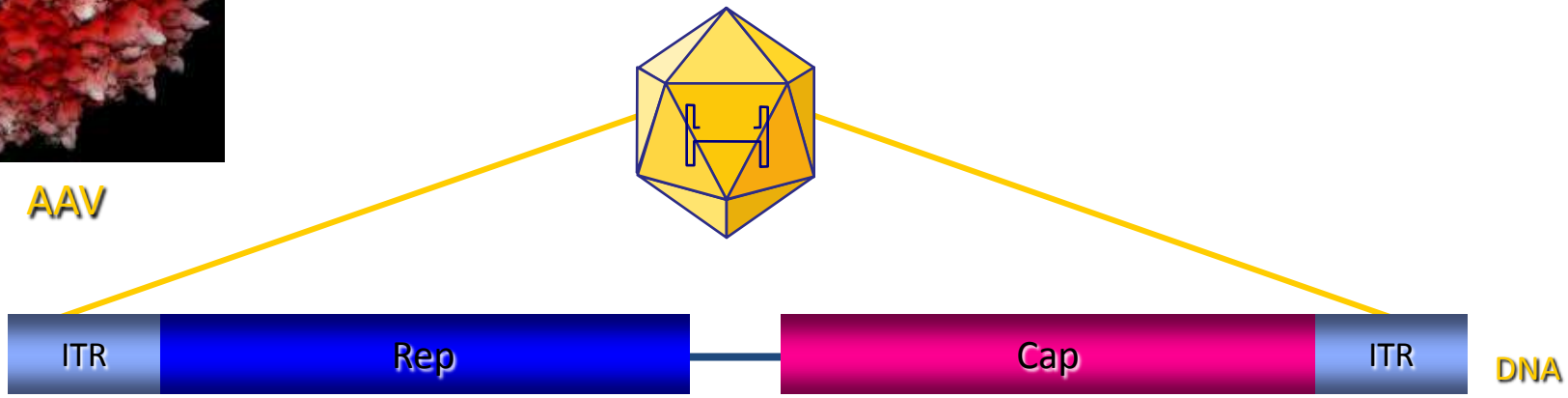
Bench to bedside approach



AAV Vectors

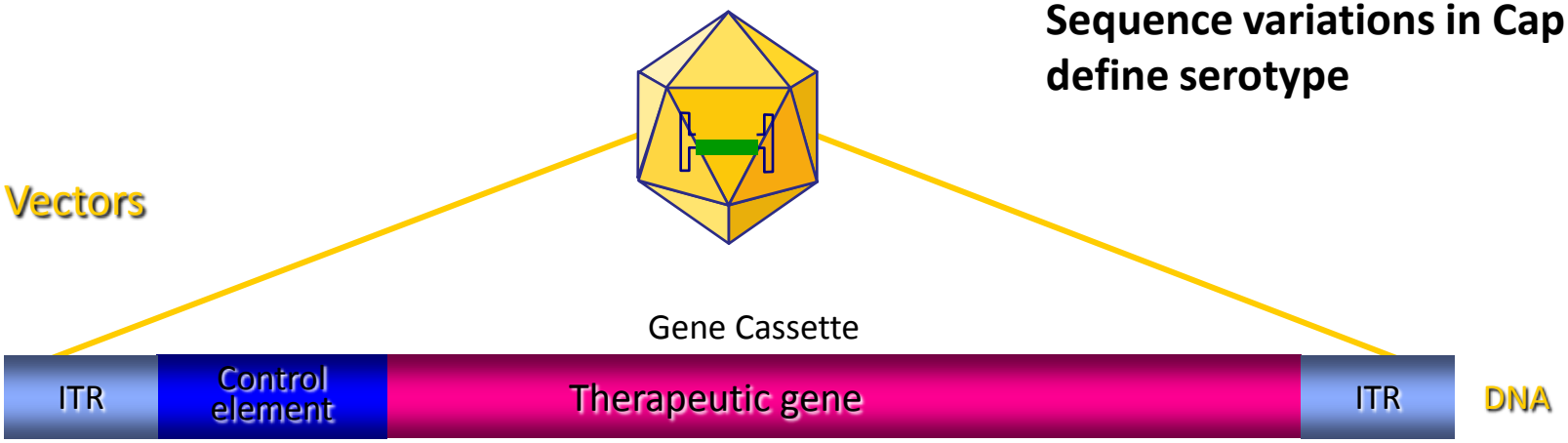


AAV

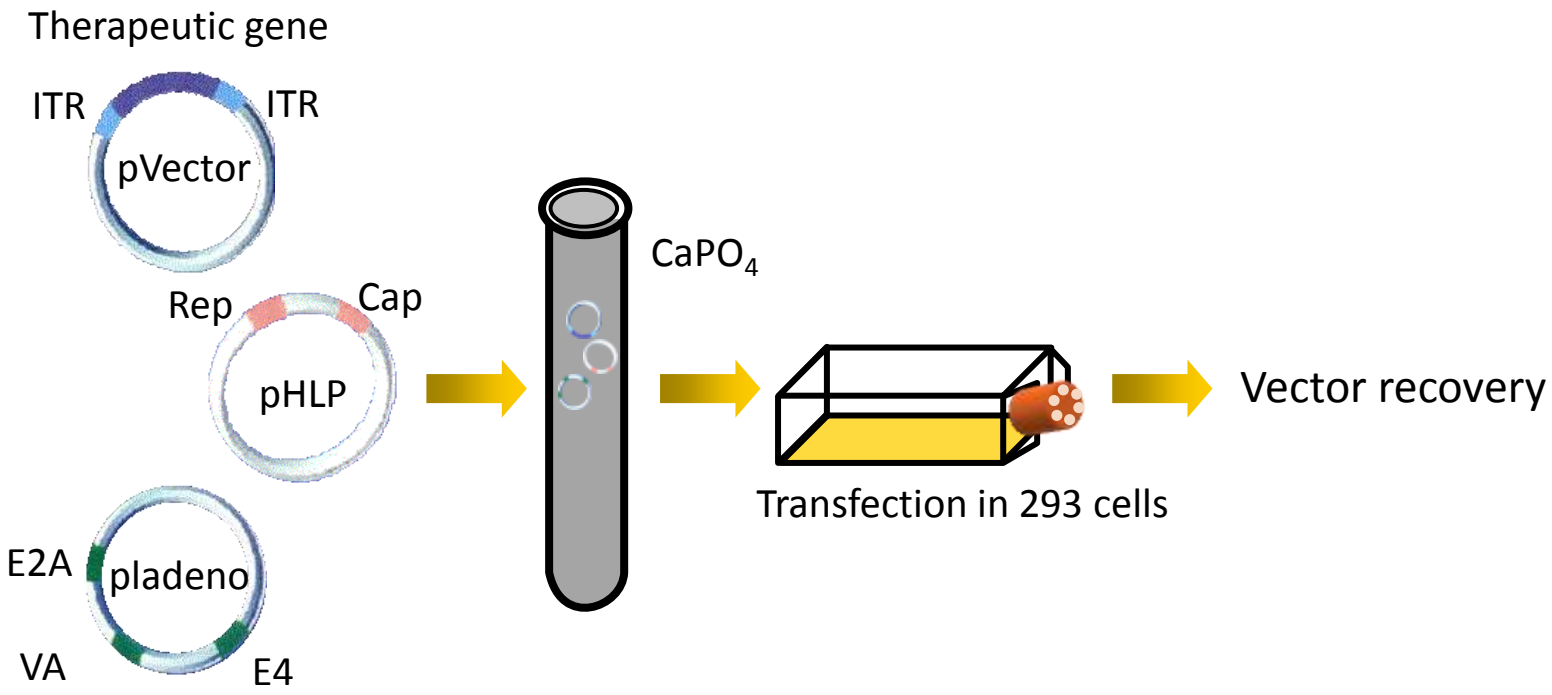


Sequence variations in Cap define serotype

AAV Vectors

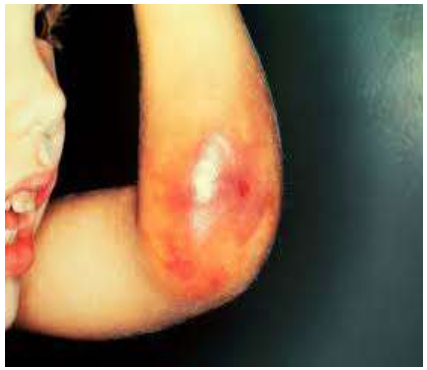


AAV vector production strategies: Helper virus-free system



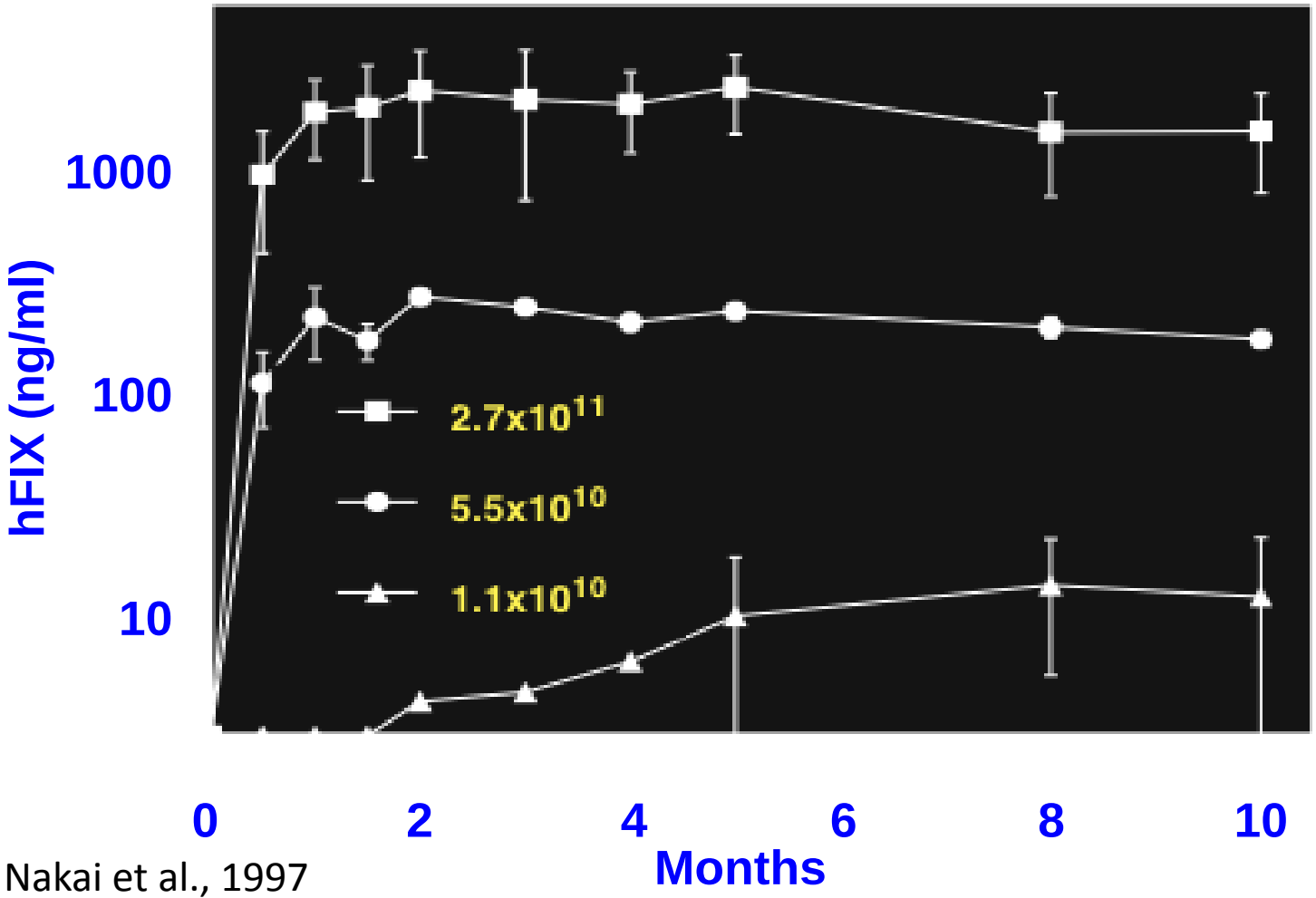
Hemophilia B Gene Therapy

- Blood coagulation deficiency occurring in ~1/30,000 male births
- Caused by a mutation in the *FIX* gene resulting in lack of protein production from the liver.
- Phenotype is dictated by amount of factor
- Large and small animal models
- Currently treated by life-long frequent FIX protein infusions.
- A model for gene therapy of monogenic diseases.
- Restoring a fraction of the normal level can ameliorate the bleeding disorder.



rAAV-mediated transgene expression *in vivo*

AAV-EF1α-hFIX to C57BL/6 mice



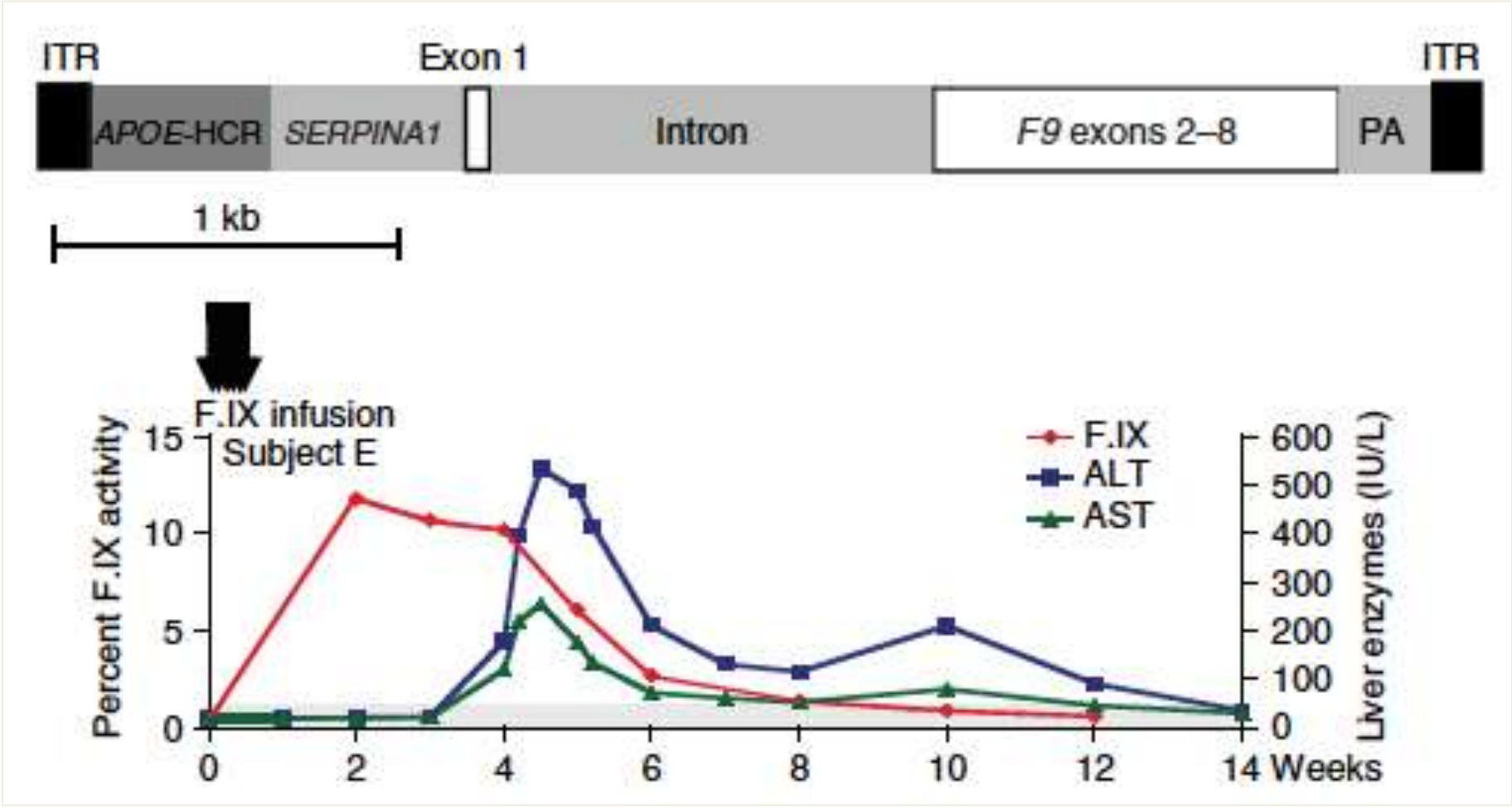
Nakai et al., 1997

Dogs Are Treated

- Dog colonies at University of North Carolina-Chapel Hill
- Dogs treated with AAV-FIX vectors
- Expression for >8 years *(Snyder et al., Nat. Medicine 1999, unpublished, others etc)*

Well recovered. This hemophilia B dog, shown with technician Pamela McElveen, had factor IX gene therapy almost 9 months ago.

AAV-2 hFIX Delivery in Humans



Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the immune host response hemophilia

Manno et al., Nature Medicine 2006

- Therapeutic hFIX was demonstrated in a human
- Unlike all animal models expression in humans was temporary
- This limitation was likely related to a cell-mediated immune response directed against hepatocytes containing capsid peptides during their degradation
- No matter how good the animal models one cannot predict the outcome in humans until you try it in people

Pseudotyping Recombinant Vector Genomes

AAV2

AAV-2 is prototype vector-isolated from humans
Most of the population exposed (e.g. immunity)
Cell-mediated immune responses

AAV1

AAV3

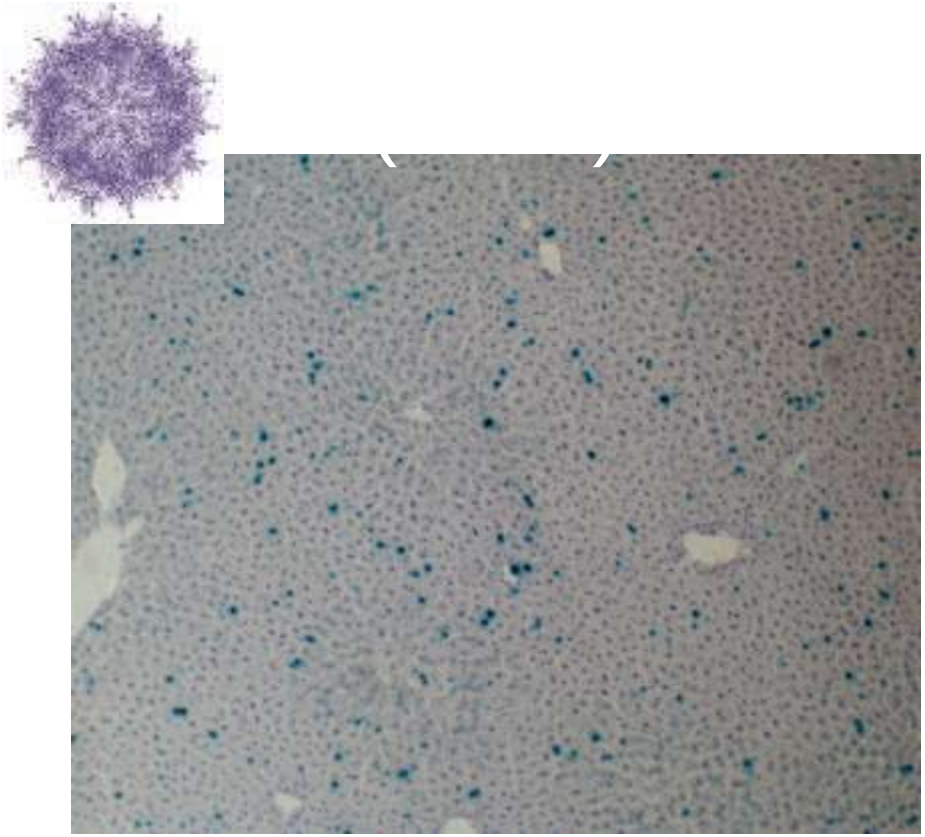
AAV4

AAV5

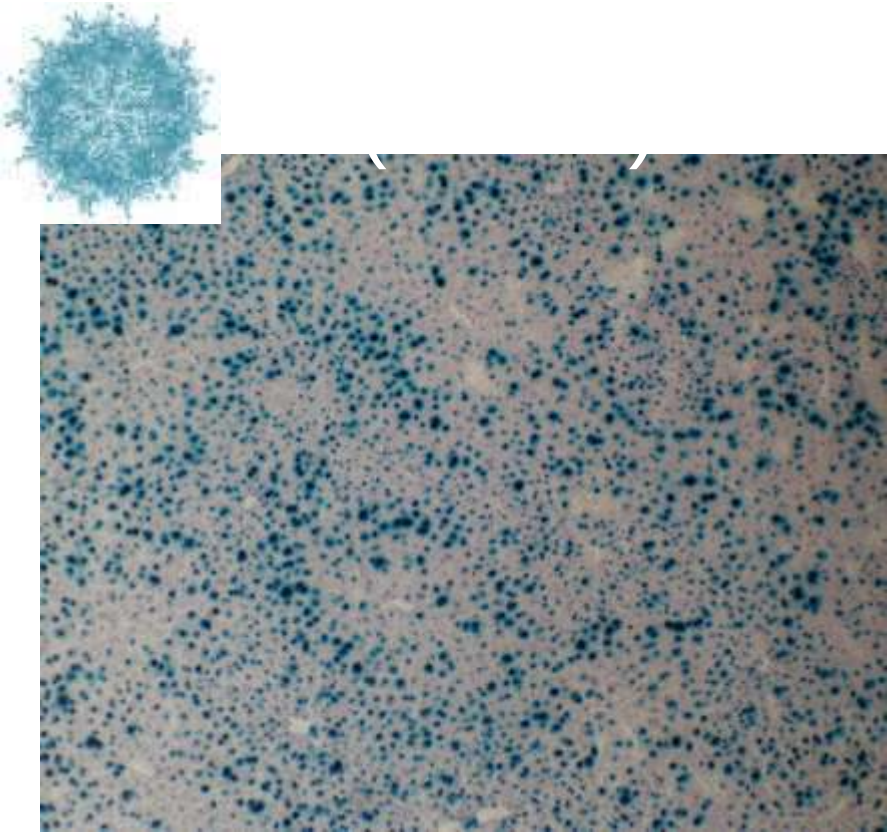
AAV6

Small number of amino acid changes can have profound effects
on the transduction parameters (immunity, efficiency, cell type)

AAV2 vs AAV8 in the liver



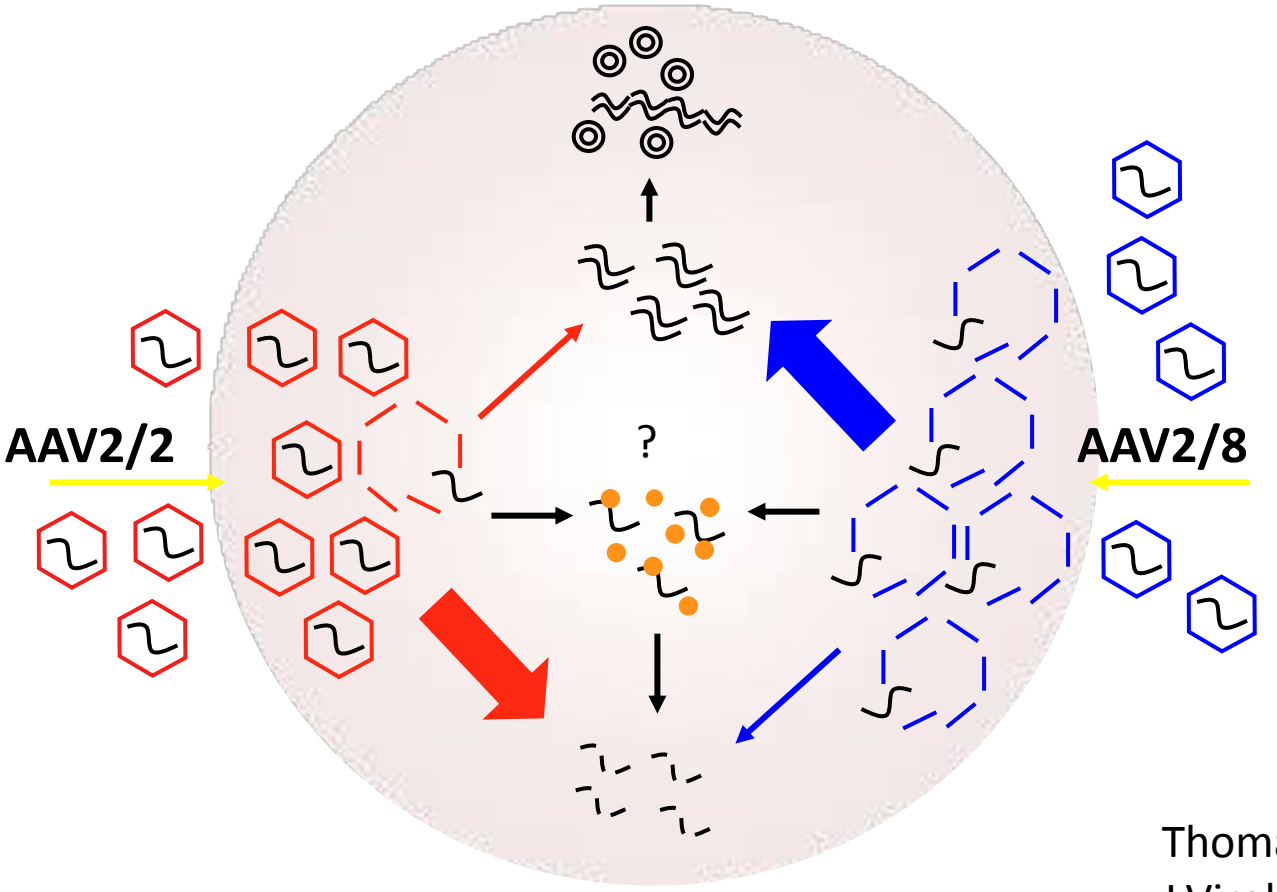
AAV2-EF1α-nlslacZ
(3.9x10^{12} vg/mouse)



AAV8-EF1α-nlslacZ
(7.2x10^{12} vg/mouse)

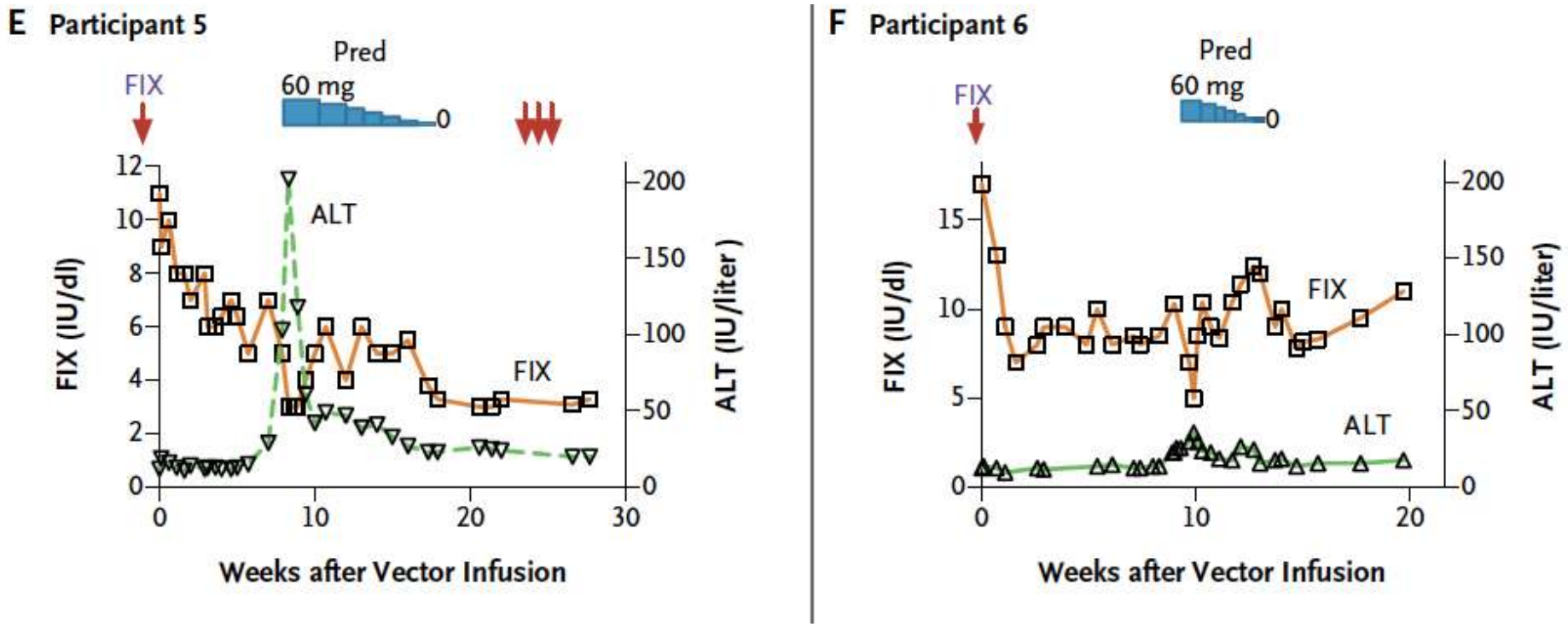
We have measured transduction 3 different ways
We can safely/reproducibly transduce ~100% of hepatocytes in vivo
Mechanism of transduction differences complex

Kinetics of capsid uncoating and transgene expression



Thomas et al.
J.Virology 2004

AAV-8 Gene Therapy for Hemophilia B Human Data



New England Journal of Medicine 12/2011

What is still needed?

- **Some humans pre-existing immunity inhibiting any gene transfer**

- **Dose response in human is >10x less than expected based on animal studies**

How Does One Predict Clinical Outcomes from Animal Studies?

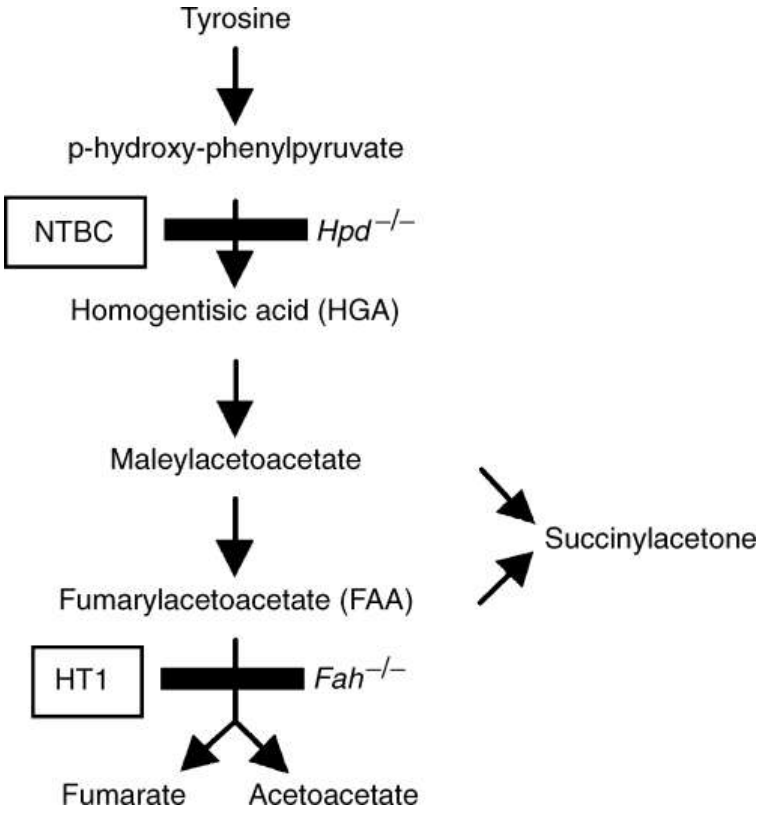
Which Animal Models are the most predictive?

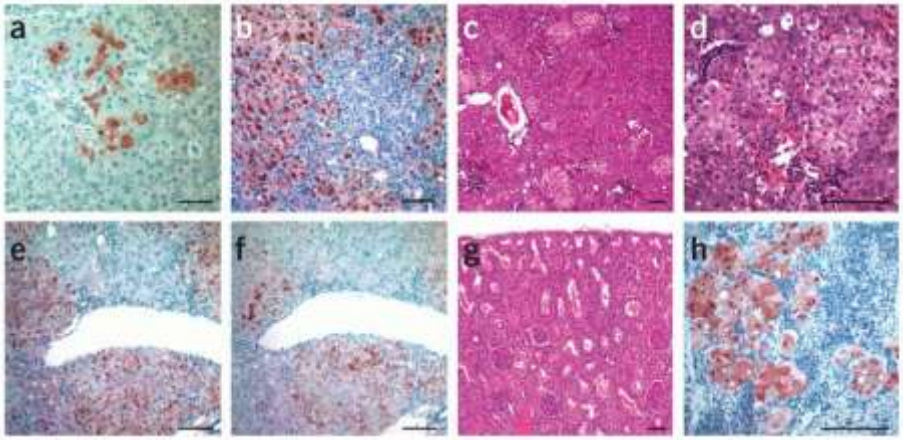
Reconstitution of mouse liver with human hepatocytes

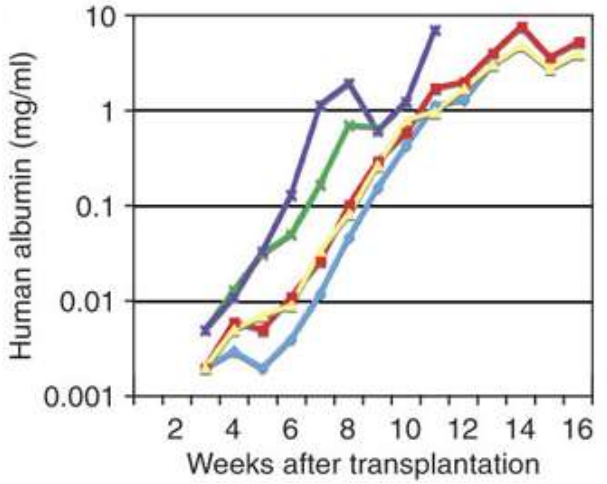
Robust expansion of human hepatocytes in *Fah*$^{-/-}$/*Rag2*$^{-/-}$/*Il2rg*$^{-/-}$ mice

Hisaya Azuma[1], Nicole Paulk[1], Aarati Ranade[2], Craig Dorrell[1], Muhsen Al-Dhalimy[1], Ewa Ellis[2], Stephen Strom[2], Mark A Kay[3], Milton Finegold[4] & Markus Grompe[1]

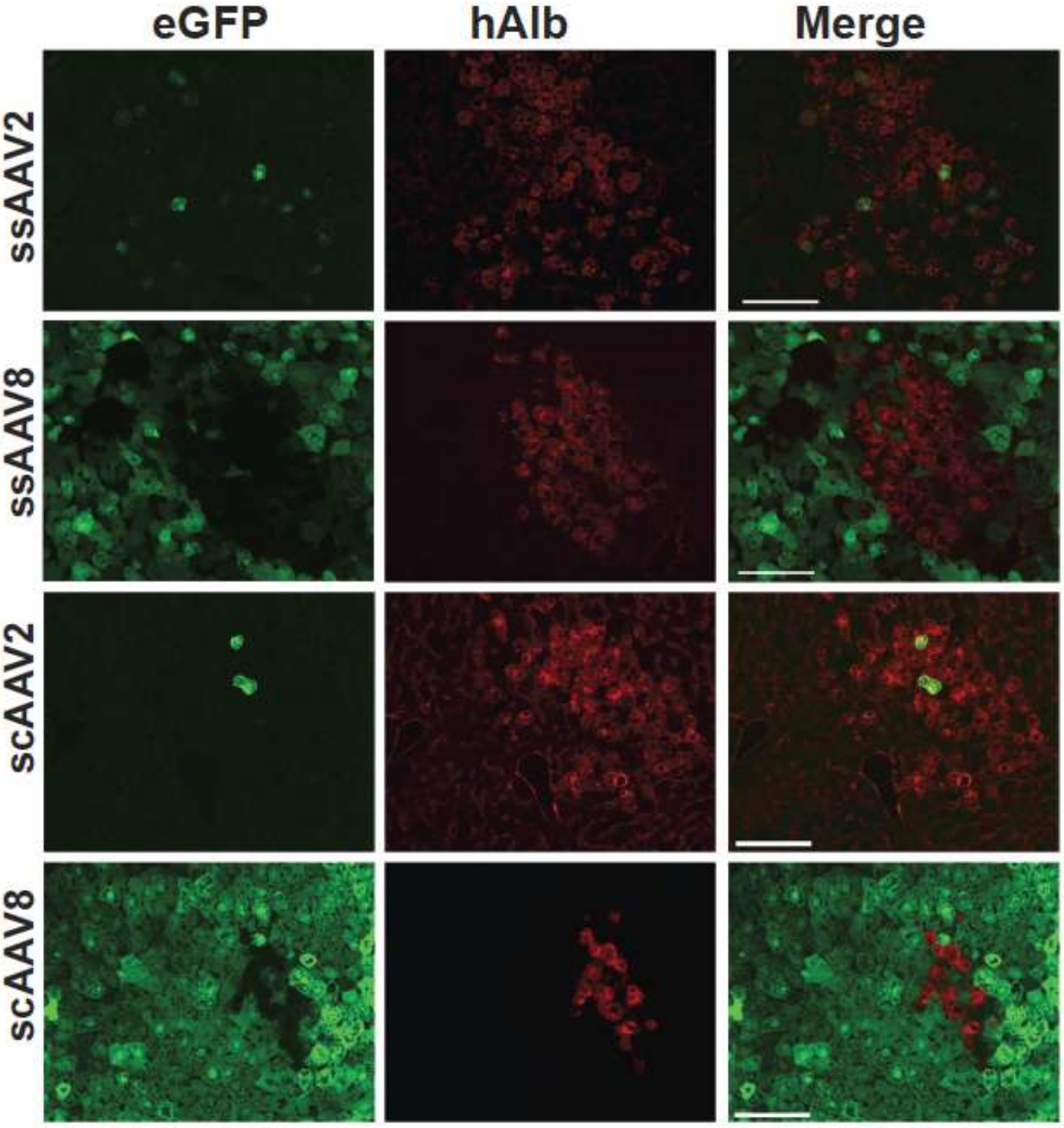
NATURE BIOTECHNOLOGY VOLUME 25 NUMBER 8 AUGUST 2007





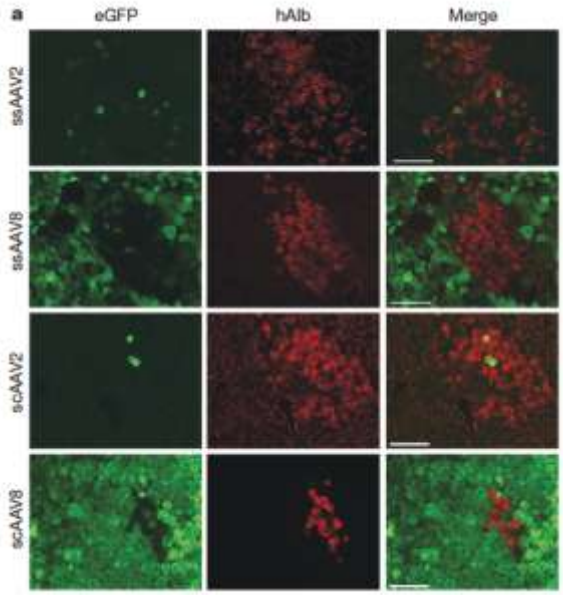


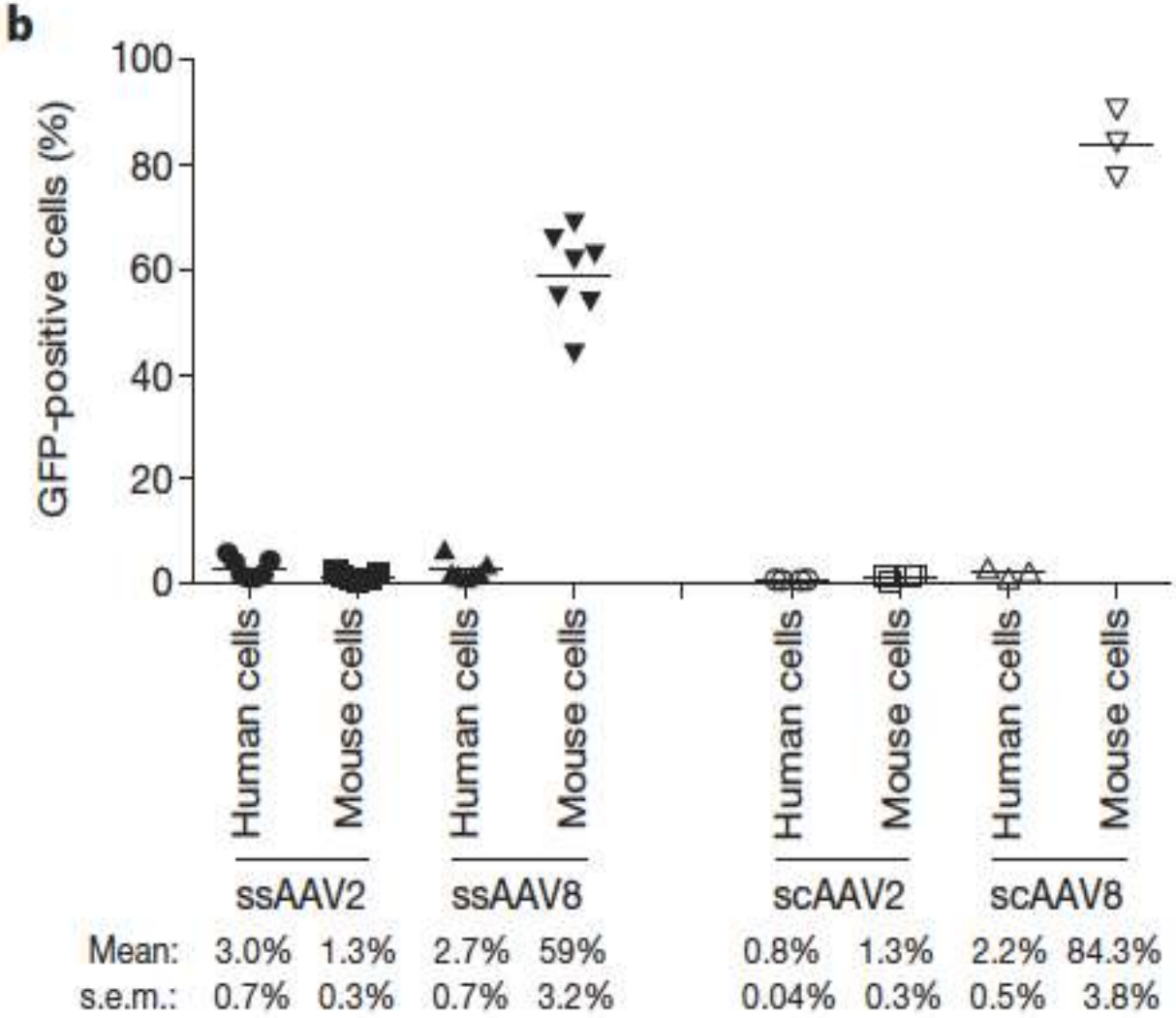
rAAV8 and rAAV2 provide similar levels of transduction in human but not Murine hepatocytes in chimeric humanized mouse liver model



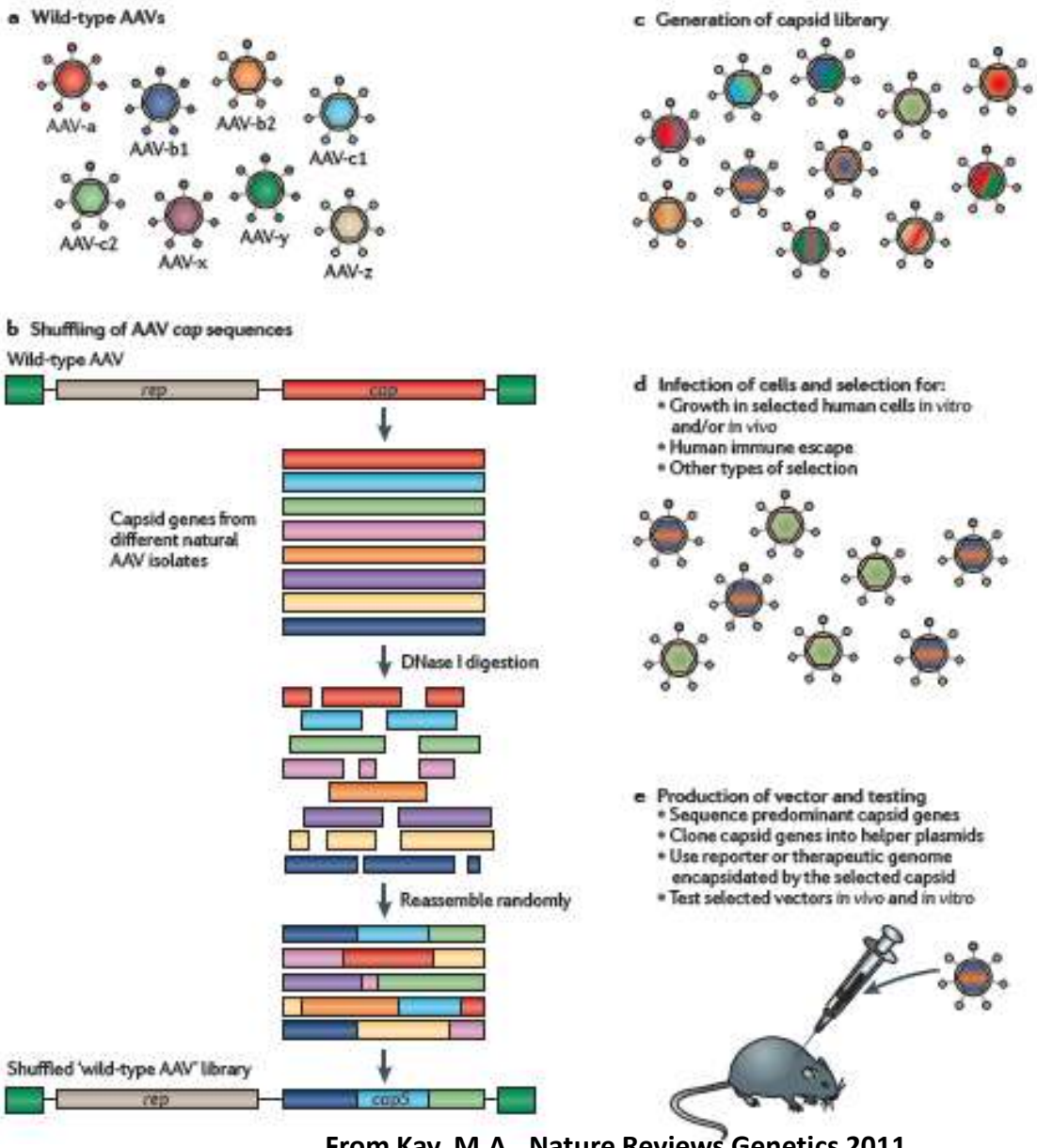
Lisowski et al.,
Nature 2013

rAAV8 and rAAV2 provide similar levels of transduction in human but not murine hepatocytes in a chimeric humanized mouse liver model



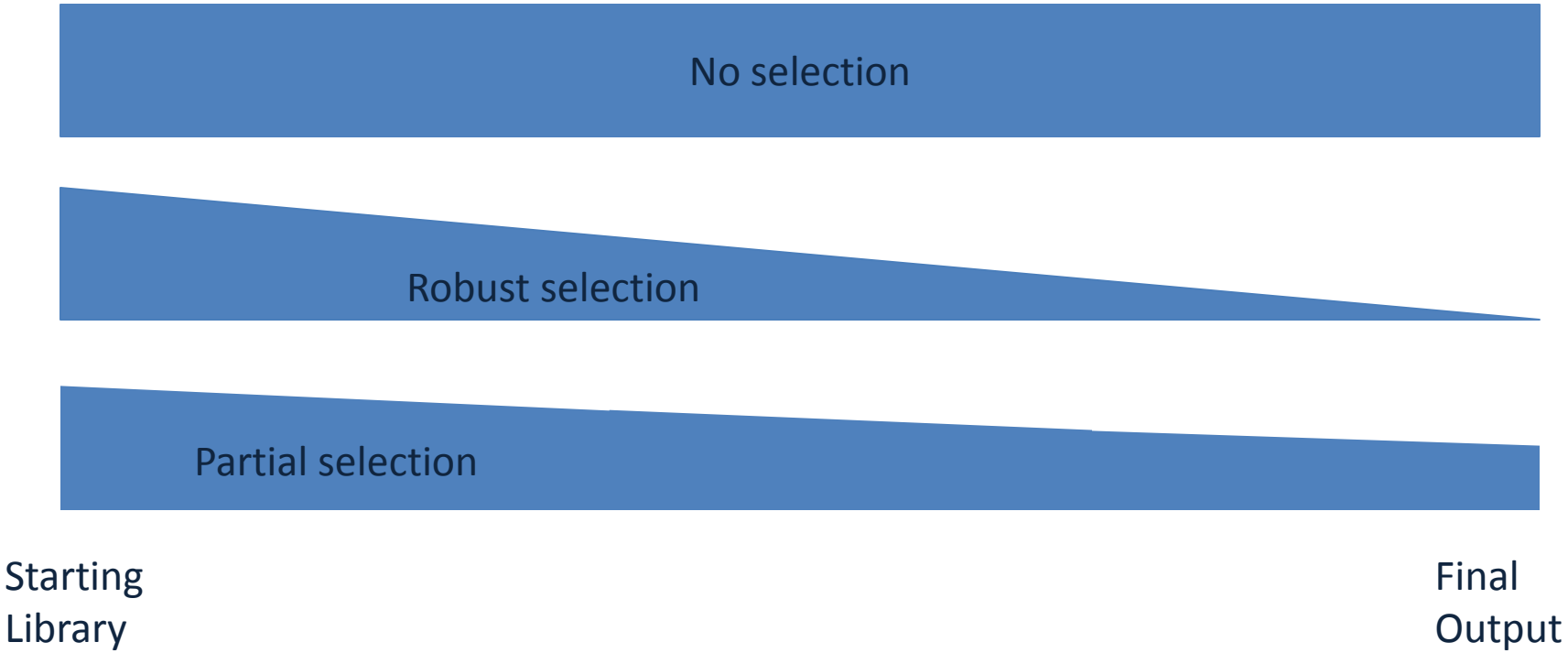


Molecular shuffling and evolution of new viruses



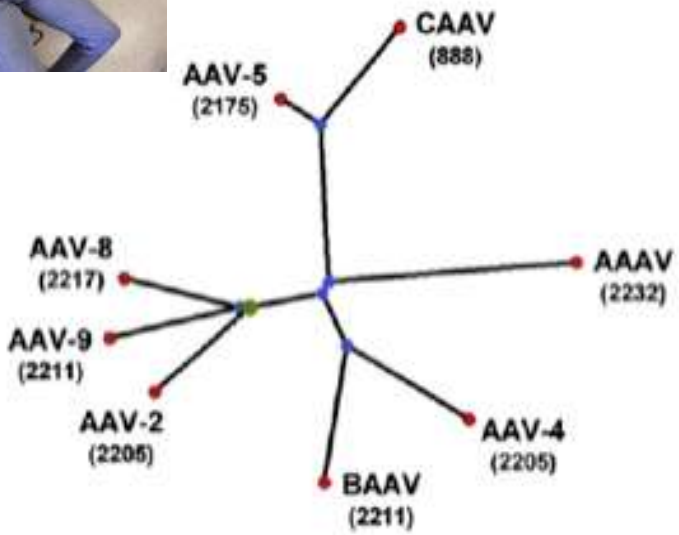
From Kay, M.A. Nature Reviews Genetics 2011

Expected Results if Positive Selection

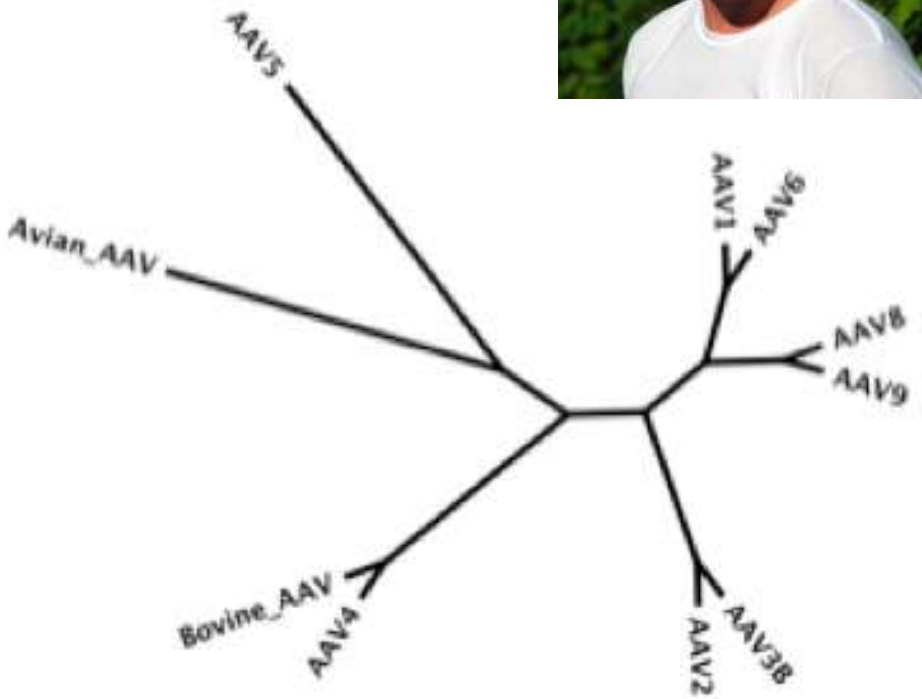


Molecular Evolution Approaches for Identifying New AAV Vectors

Starting Capsids For Library

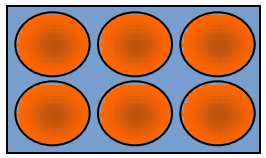


Grimm et al. 2008 J. Virol



Lisowski et al. Nature 2014

AAV shuffle library selection in Tissue Culture Cells



TC cells

AAV library (MOI range)
hAd5 (fixed
predetermined MOI)

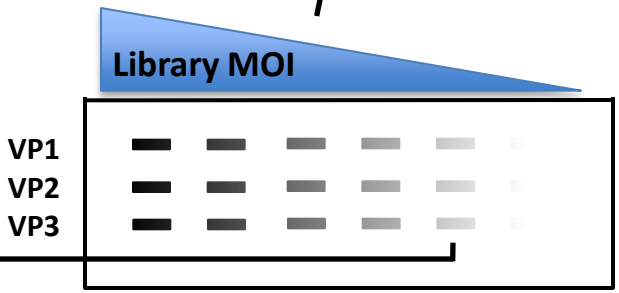
Harvest supernatant
on day 3 post infection

Human IVIG negative selection

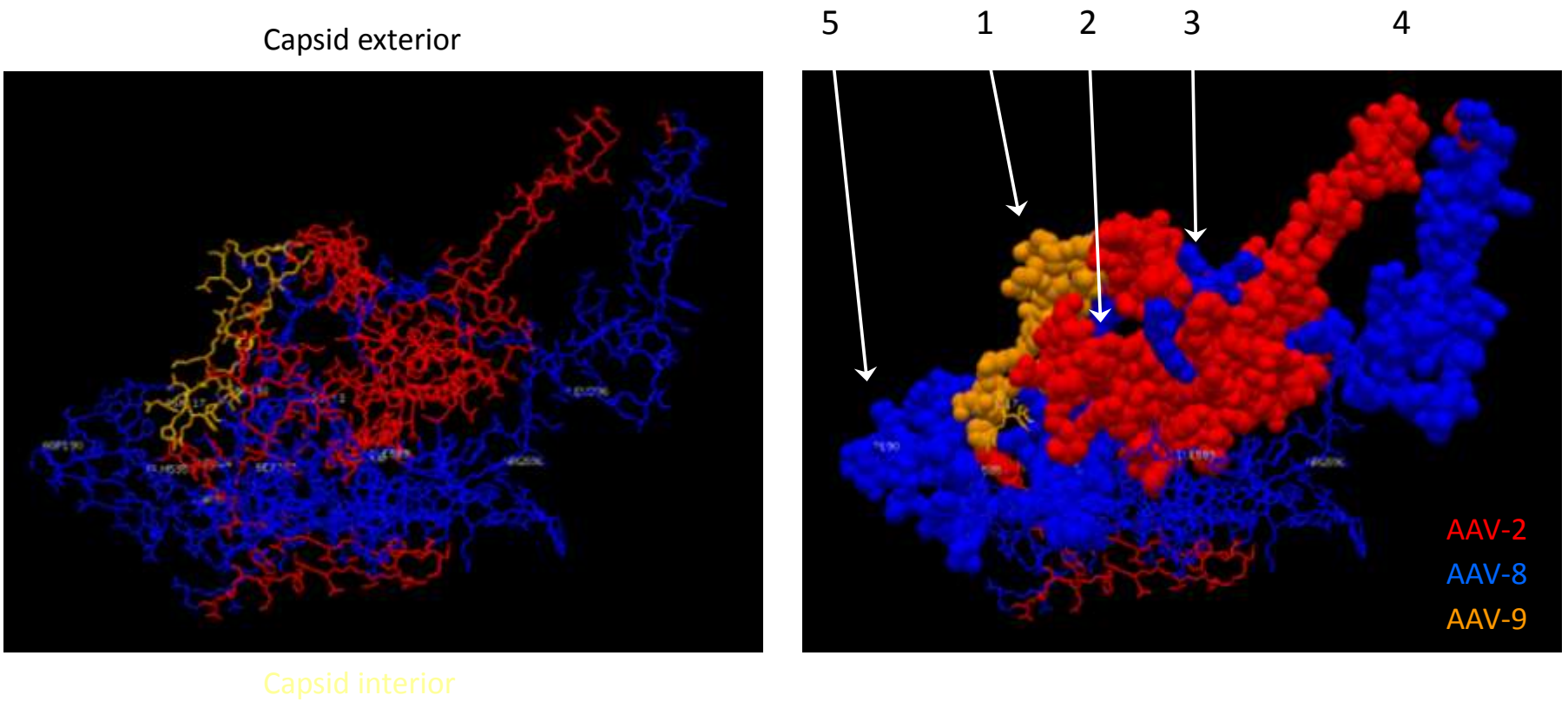
Western Blot
analysis

- Vector DNA extraction
- PCR amplification of CAP gene
- TOPO subcloning
- Sequencing

Select dilution with the **lowest**
expression of AAV proteins



Predicted capsid structure of AAV-DJ



AAV-DJ is as good as AAV-8 at transducing mouse liver in vivo

One property which was inadvertently selected for……

Grimm et al., J. Virol 2008

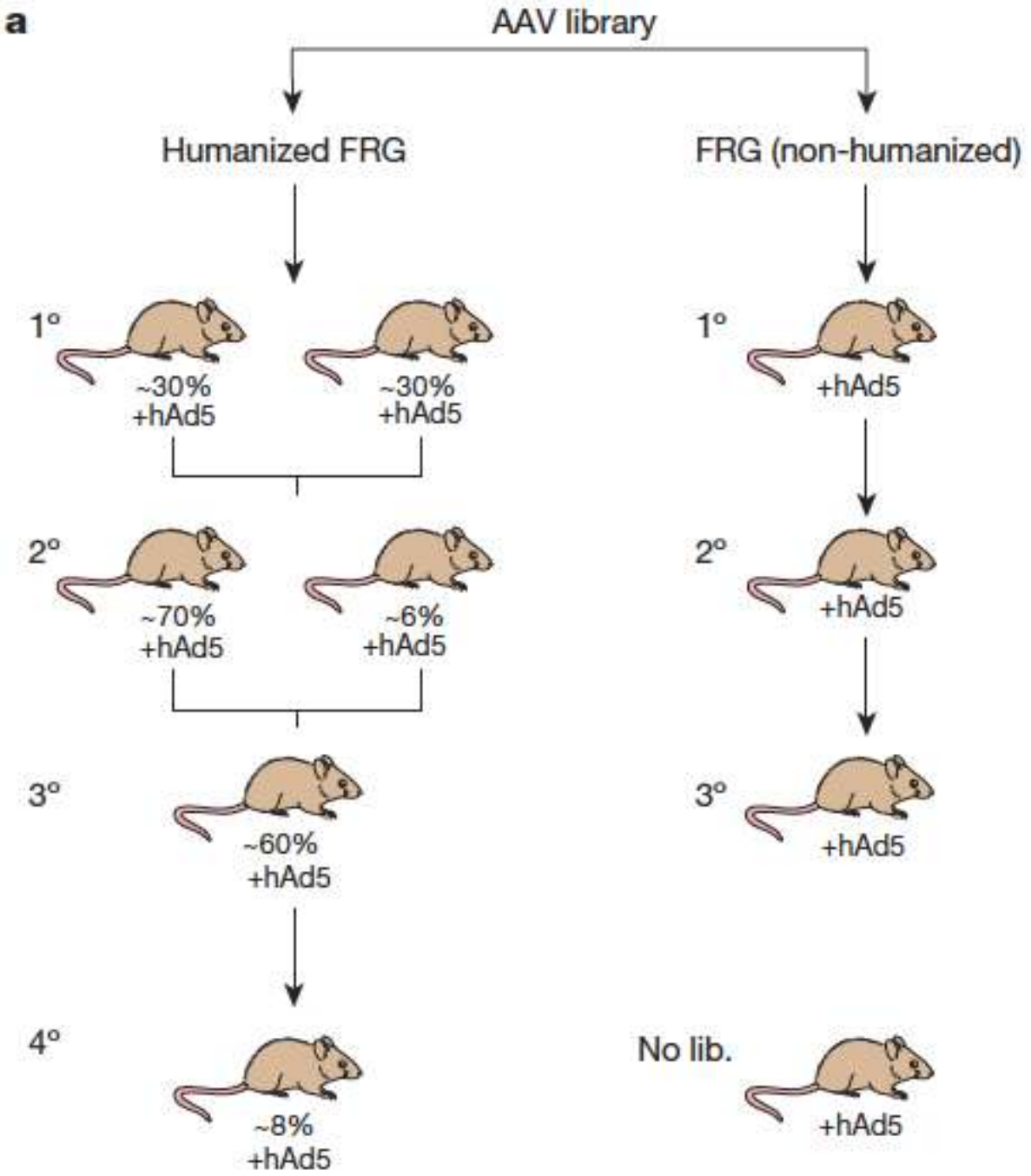
In vitro infectivity of AAV-DJ and wildtype vectors

		AAV vectors									
Cell line	Tissue	1	2	3	4	5	6	8	9	DJ	DJ/8
Huh-7	hu liver	4e3	5e2	2e4	2e6	4e5	5e3	7e4	7e6	1e2	3e5
293	hu kidney	2e3	5e2	2e4	7e5	4e5	1e4	7e4	7e5	1e2	2e5
HeLa	hu cervix	7e4	2e3	1e5	2e6	3e4	2e5	1e6	2e6	3e2	1e6
HepG2	hu liver	2e6	5e4	3e5	2e7	3e6	1e6	2e7	nd	4e3	1e7
Hep1A	mu liver	1e4	2e3	1e6	2e5	2e6	2e5	1e6	2e7	5e2	2e6
911	hu retina	6e3	1e3	9e3	5e5	7e5	6e3	1e6	nd	2e2	4e5
CHO	ha ovary	1e4	1e4	7e4	7e5	3e3	2e4	1e5	1e6	4e1	2e5
COS	si kidney	3e3	1e3	3e3	3e4	2e4	7e3	5e4	2e5	2e2	3e5
MeWo	hu skin	2e3	2e2	1e3	7e4	3e3	2e3	2e4	1e5	7e0	2e4
NIH3T3	mu fibrobl.	2e5	2e4	7e5	7e5	7e6	2e5	7e6	nd	4e3	2e7
A549	hu lung	7e4	1e4	5e4	nd	2e6	1e5	2e6	7e6	1e3	2e7
HT1180	hu fibrobl.	5e4	1e4	1e5	7e6	3e6	3e4	2e6	1e7	3e3	5e6

rAAV has not historically been a good vector for ex vivo approaches

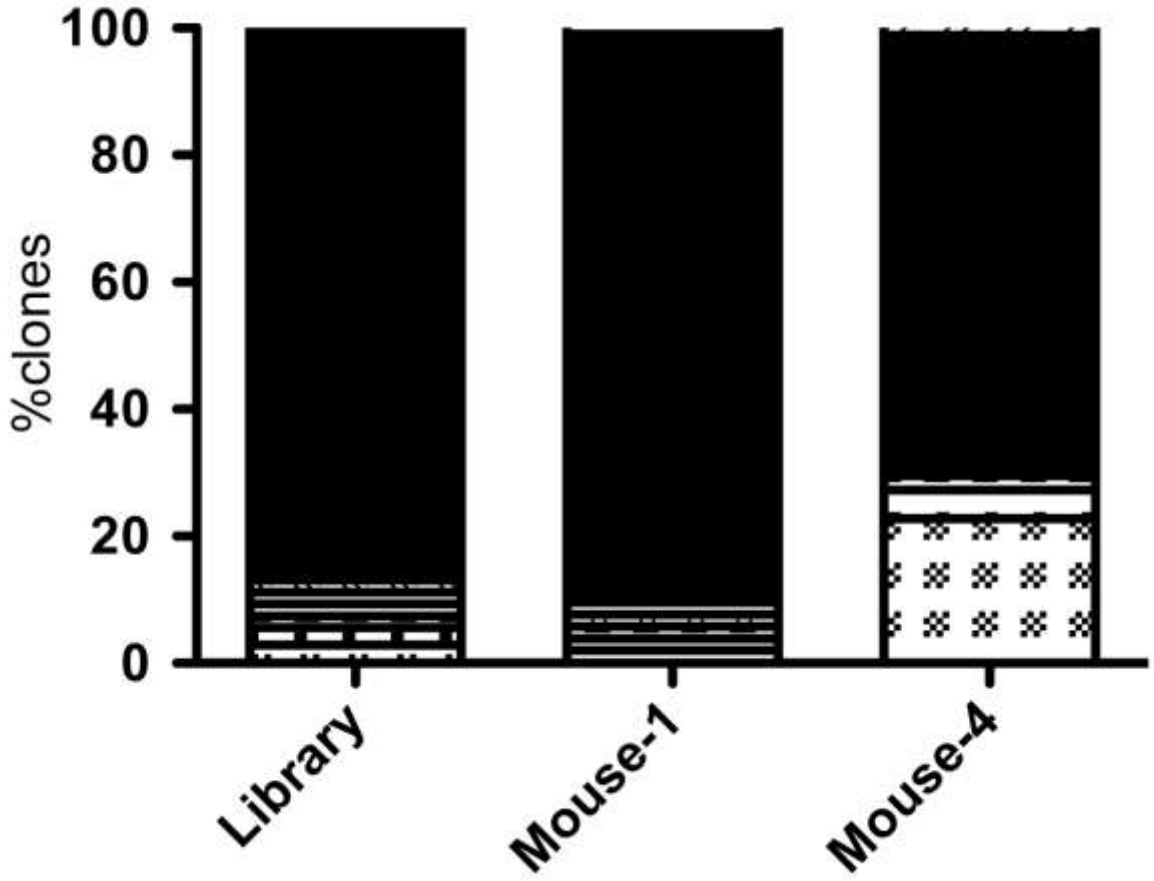
rAAV-DJ is being used for genome editing of ES, iPS, and mature somatic cells in culture

Passage of rAAV library in chimeric human-mouse chimeric liver



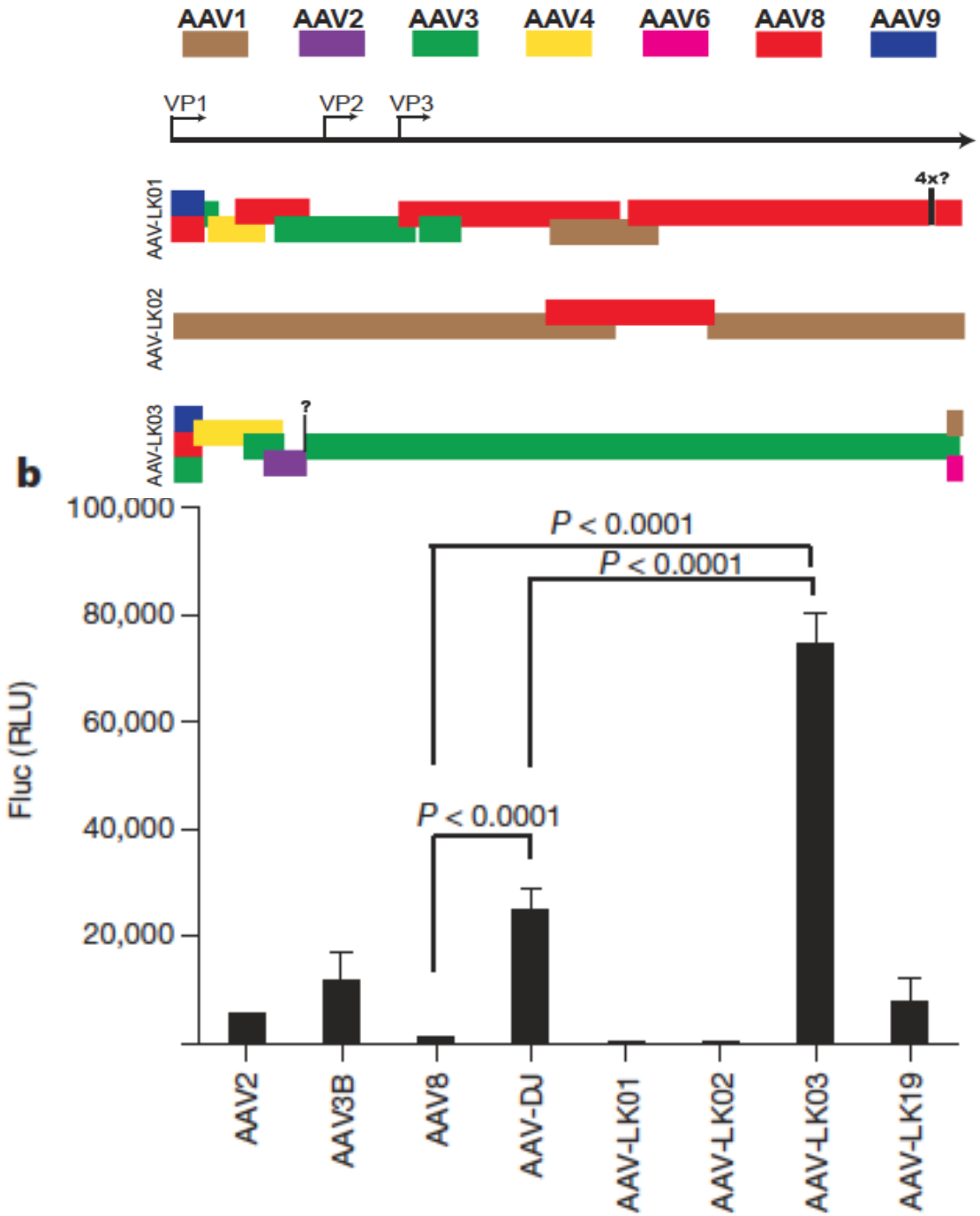
AAV shuffle library selection

amino acid sequence

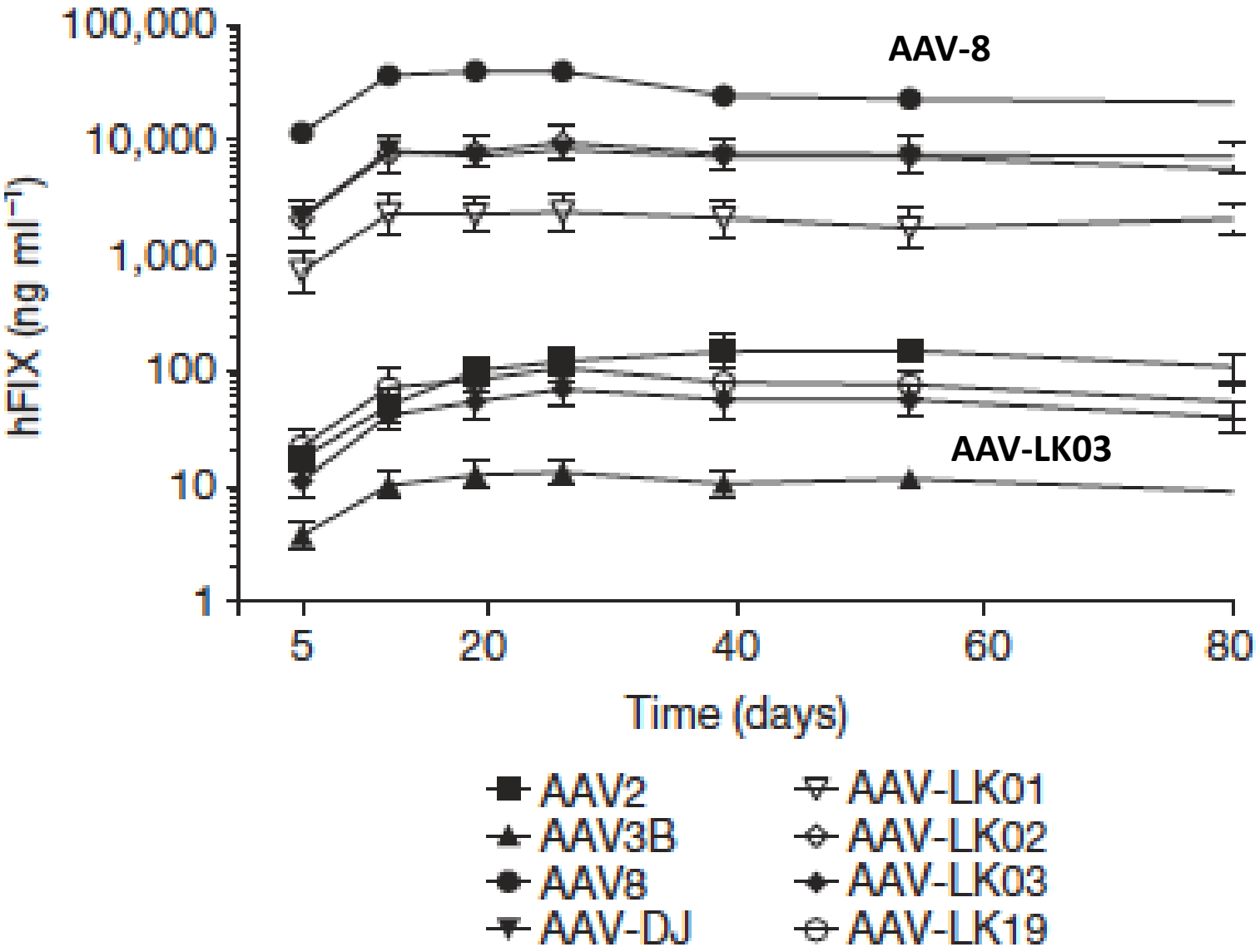


N=100-150 / round

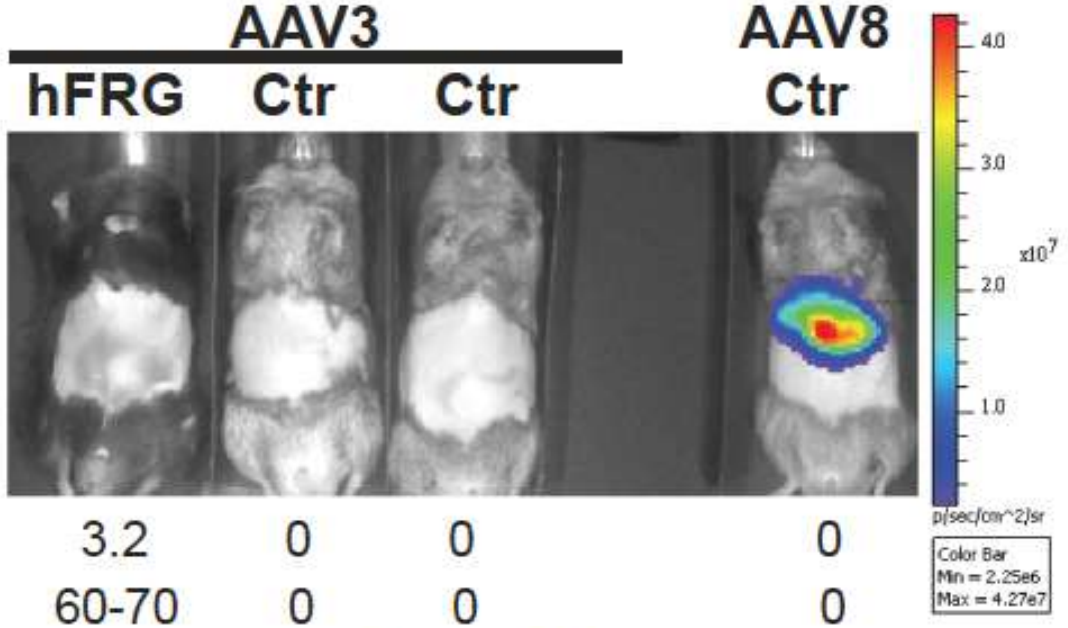
Vectorization of AAV capsids obtained from in vivo screen

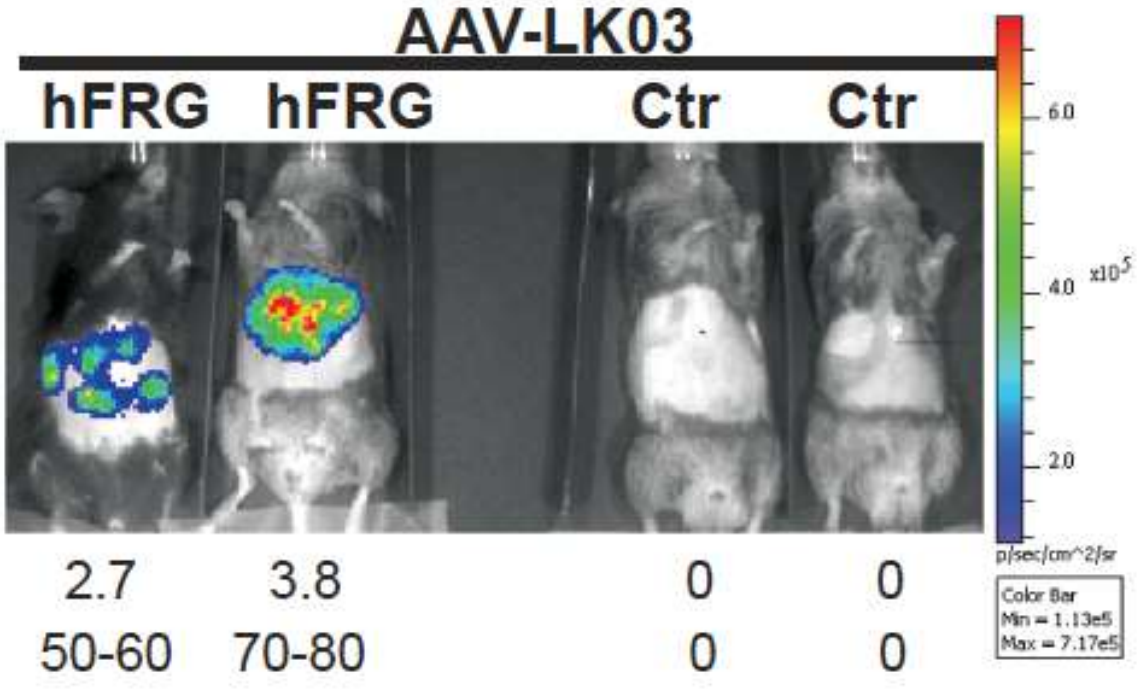


Performance of Selected Vectors in Mouse Liver

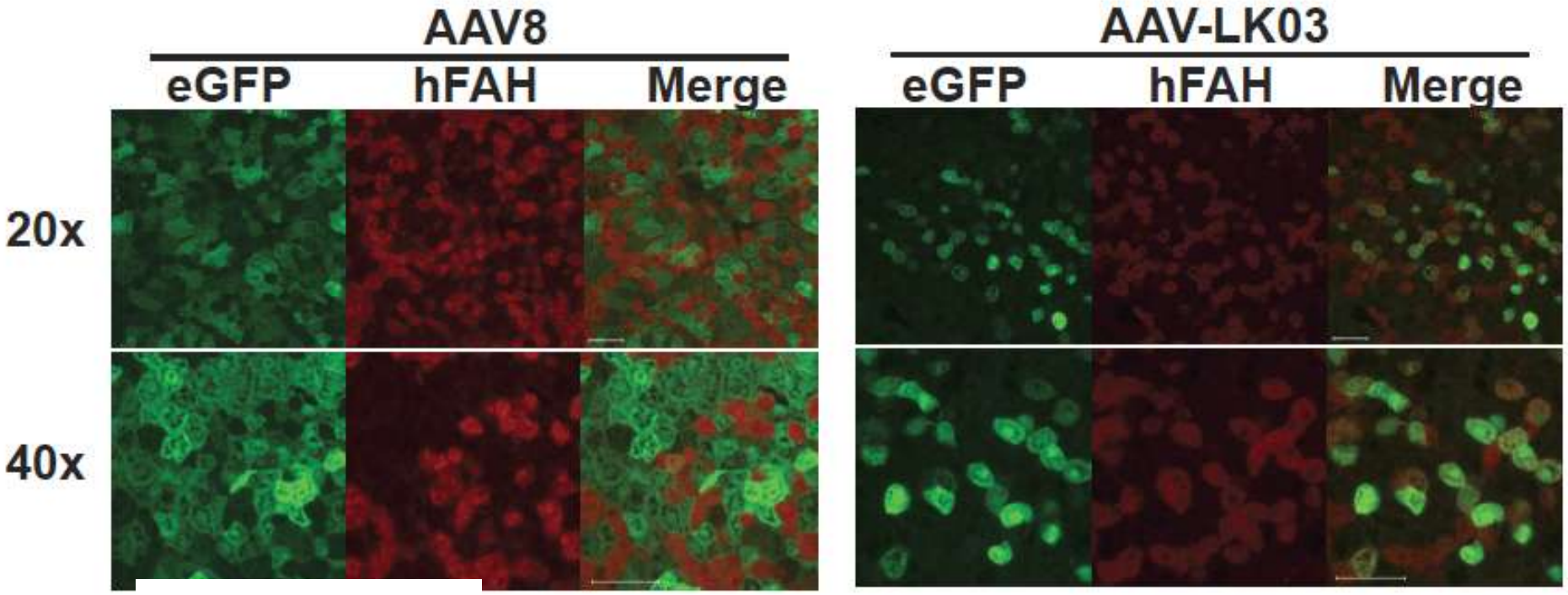


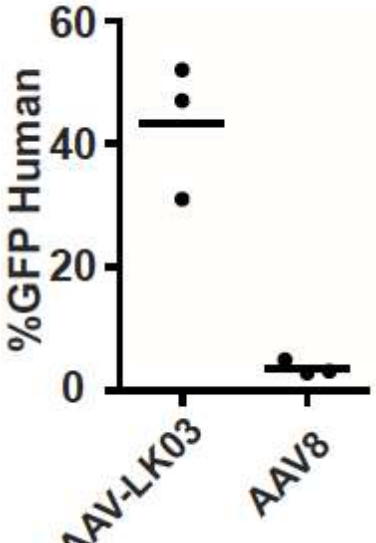
Differences In human hepatocyte transduction AAV-3B, AAV-LK03, and AAV8





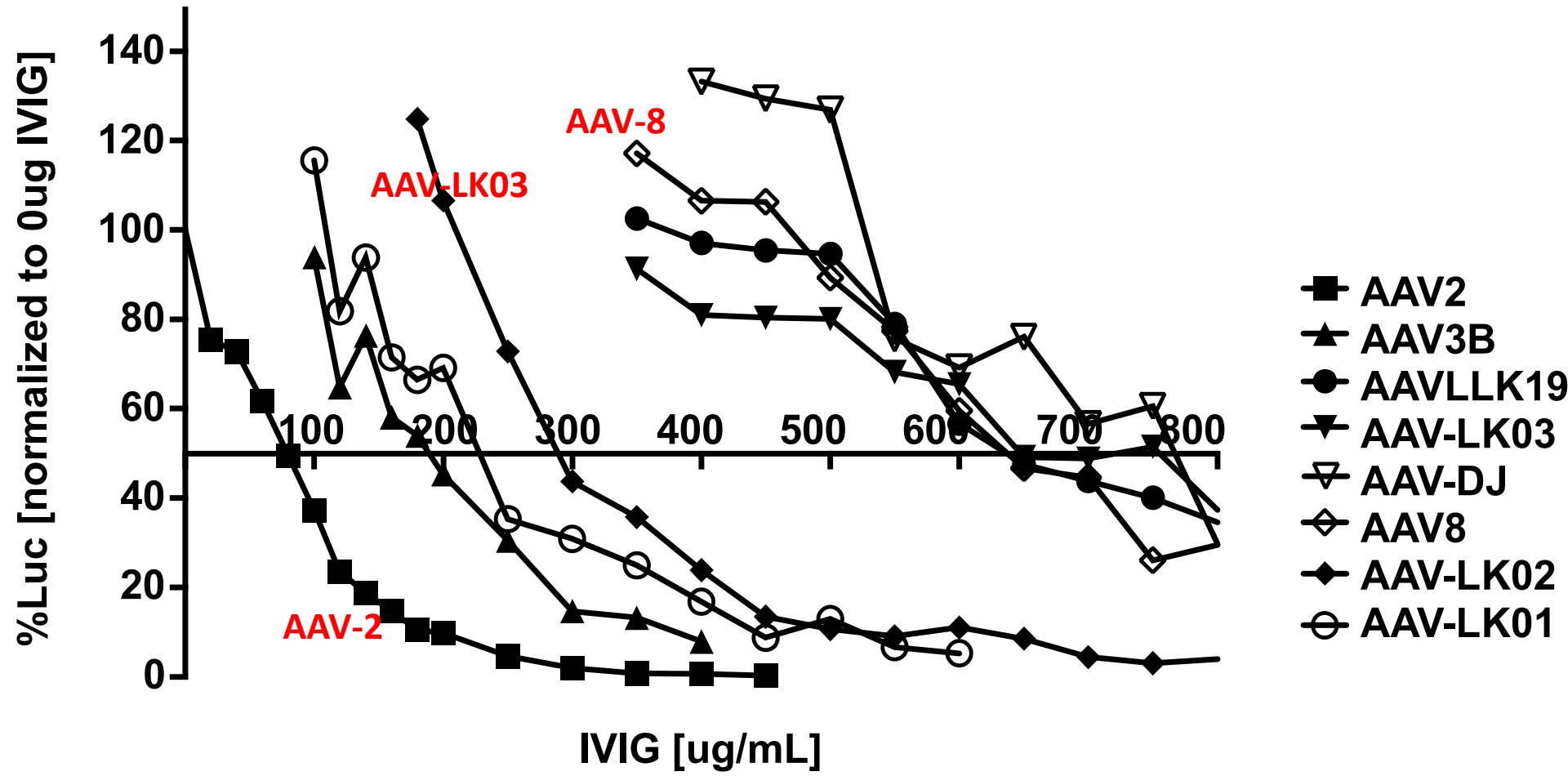
rAAV-8 vs AAV-LK03 in Human Liver





AAV-LK03 may be a better alternative For humans !

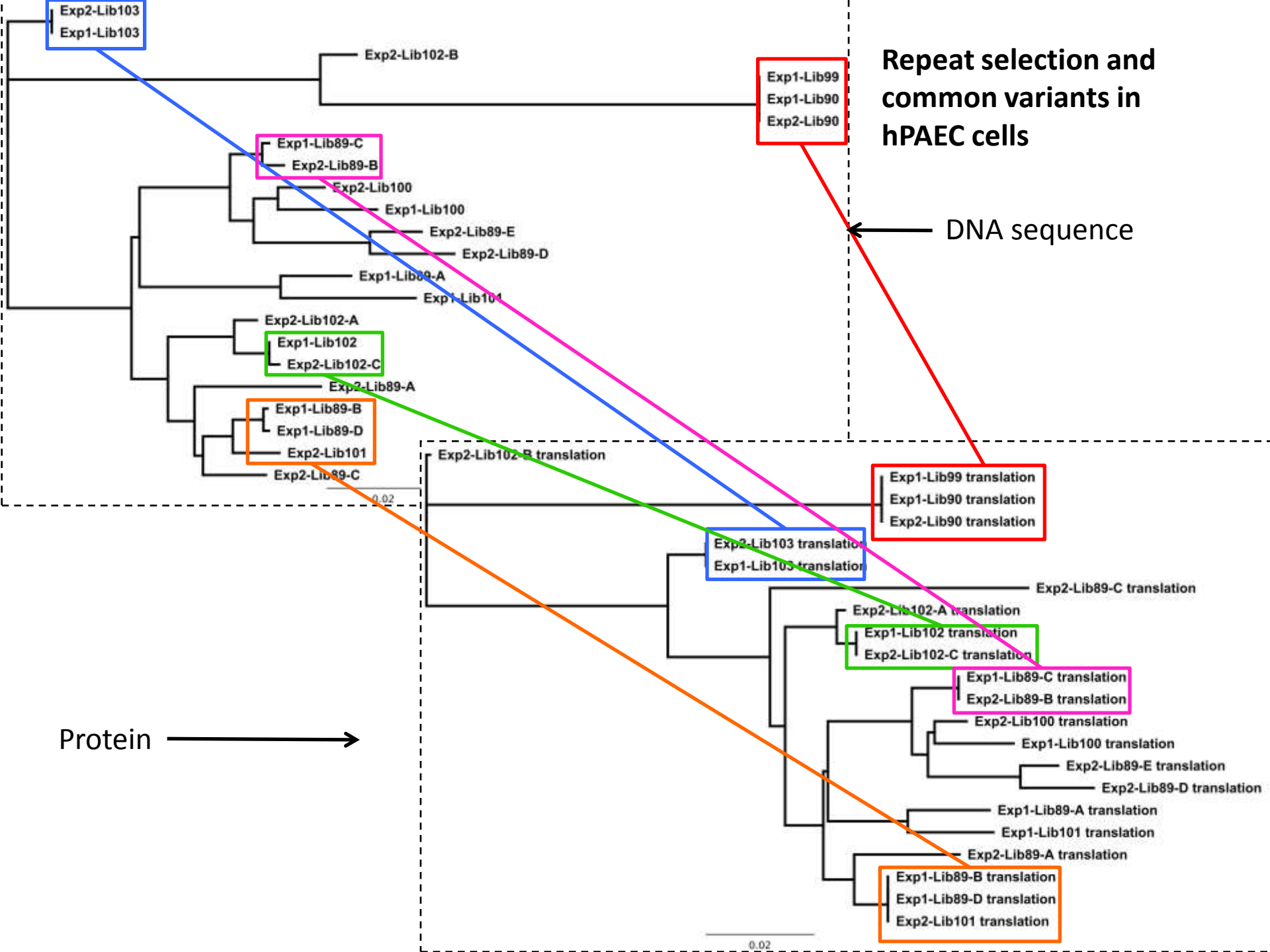
Pre-existing Immunity to Vector Serotypes



Do you find the same capsids if you perform independent library selection?

Human PAEC Selection

- Six rounds of selection on huPAEC cells
- Seven different libraries used
- Two independent experiments
- Ten clones from each fully sequenced

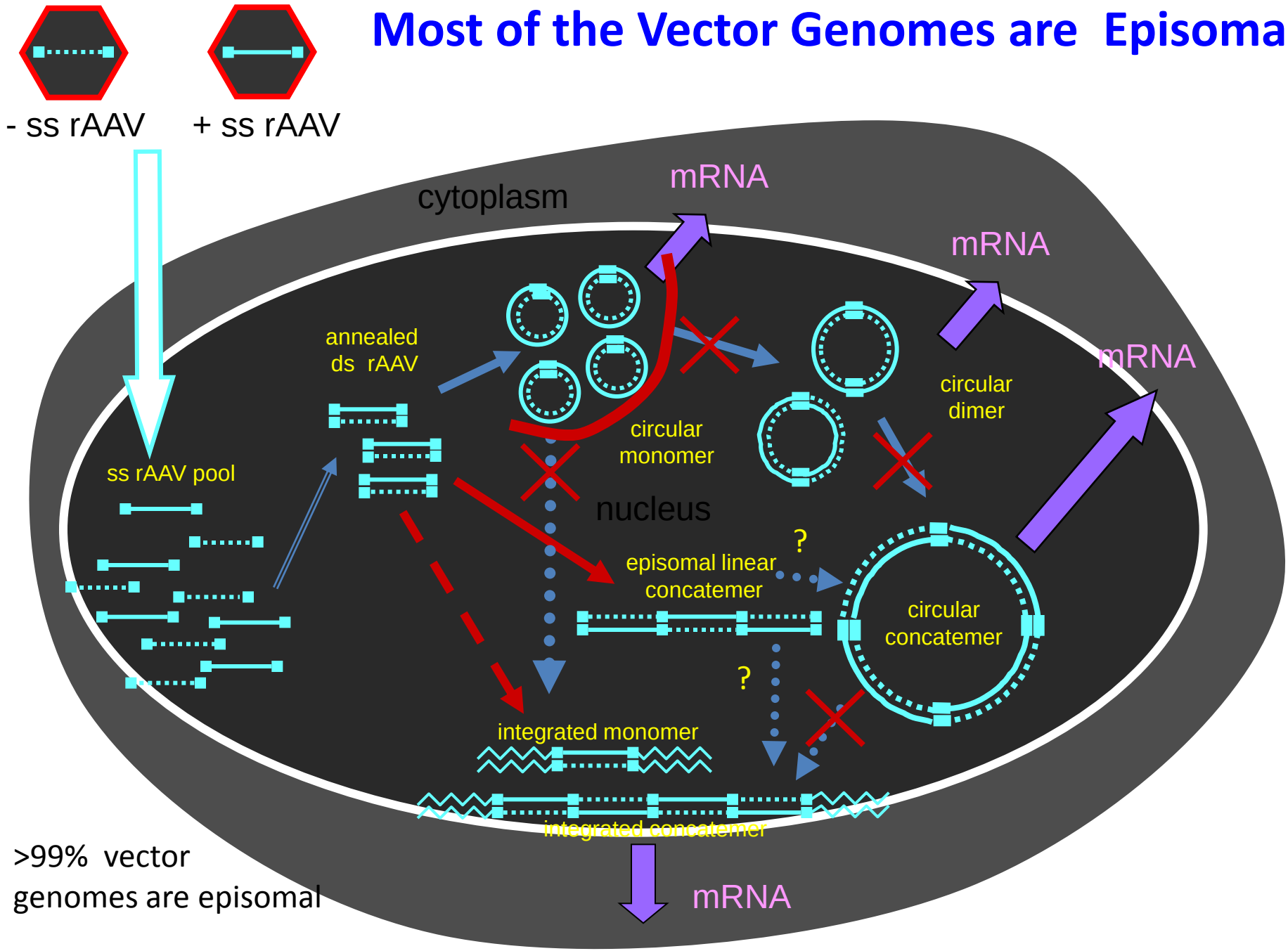

Repeat selection and common variants in hPAEC cells
DNA sequence
Protein
Exp2-Lib103
Exp1-Lib103
Exp2-Lib102-B
Exp1-Lib99
Exp1-Lib90
Exp2-Lib90
Exp1-Lib89-C
Exp2-Lib89-B
Exp2-Lib100
Exp1-Lib100
Exp2-Lib89-E
Exp2-Lib89-D
Exp1-Lib89-A
Exp1-Lib101
Exp2-Lib102-A
Exp1-Lib102
Exp2-Lib102-C
Exp2-Lib89-A
Exp1-Lib89-B
Exp1-Lib89-D
Exp2-Lib101
Exp2-Lib89-C
0.02
Exp2-Lib102-B translation
Exp1-Lib99 translation
Exp1-Lib90 translation
Exp2-Lib90 translation
Exp2-Lib103 translation
Exp1-Lib103 translation
Exp2-Lib89-C translation
Exp2-Lib102-A translation
Exp1-Lib102 translation
Exp2-Lib102-C translation
Exp1-Lib89-C translation
Exp2-Lib89-B translation
Exp2-Lib100 translation
Exp1-Lib100 translation
Exp2-Lib89-E translation
Exp2-Lib89-D translation
Exp1-Lib89-A translation
Exp1-Lib101 translation
Exp2-Lib89-A translation
Exp1-Lib89-B translation
Exp1-Lib89-D translation
Exp2-Lib101 translation
0.02

Conclusions

- AAV Capsid Shuffling- Lots of possibilities
- Xenotransplant Models May Be Better Predictors of Human Outcomes
- LK03- Additional Preclinical studies underway
- LK03 useful for human xenotransplant studies
- Novel Screens- Tissues, DNA carrying capacity



Most of the Vector Genomes are Episomal



>99% vector genomes are episomal

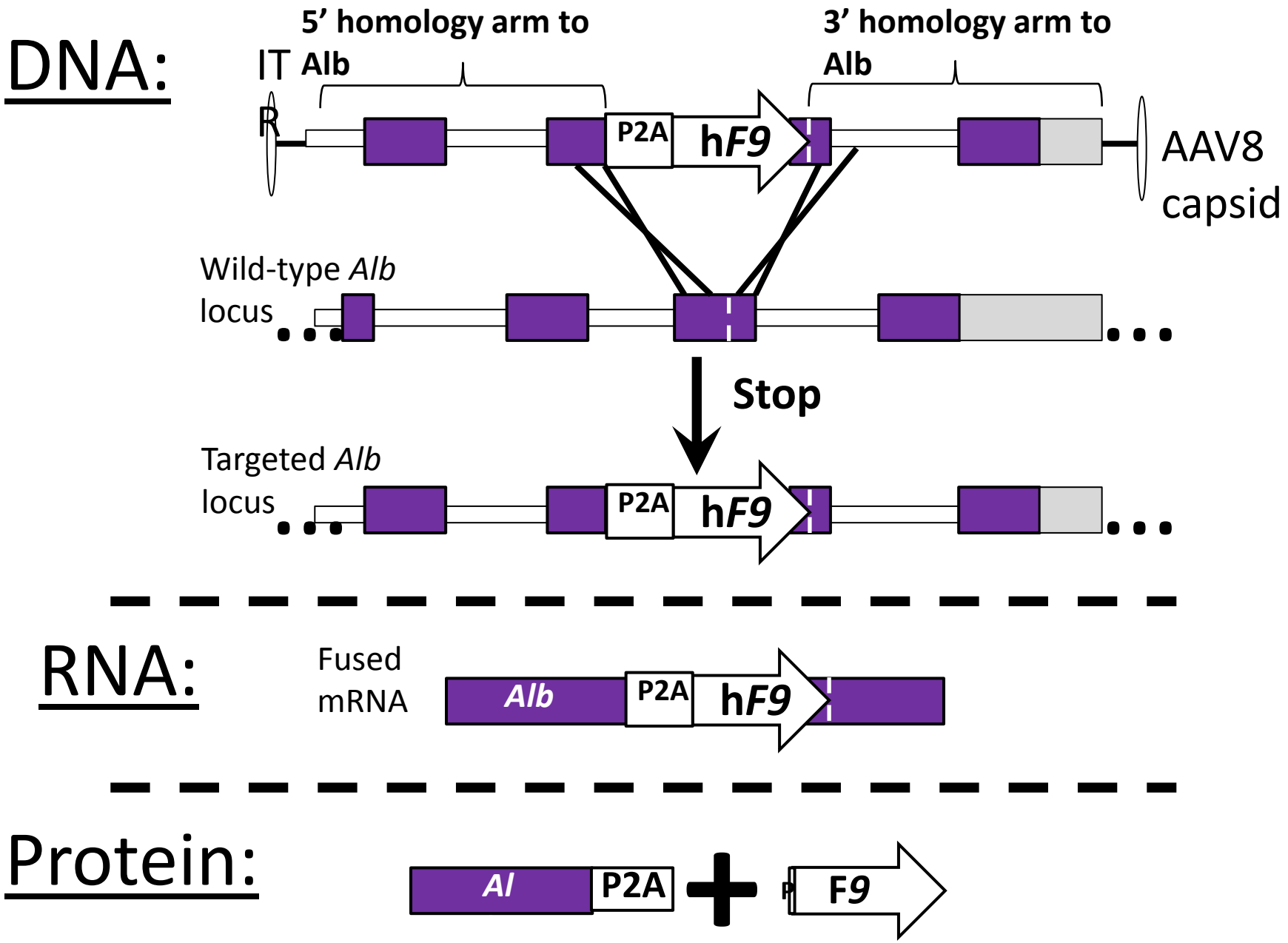
What's better than ZFNs, Talens or CRISPR?

No ZFNs, Talens, or CRISPR

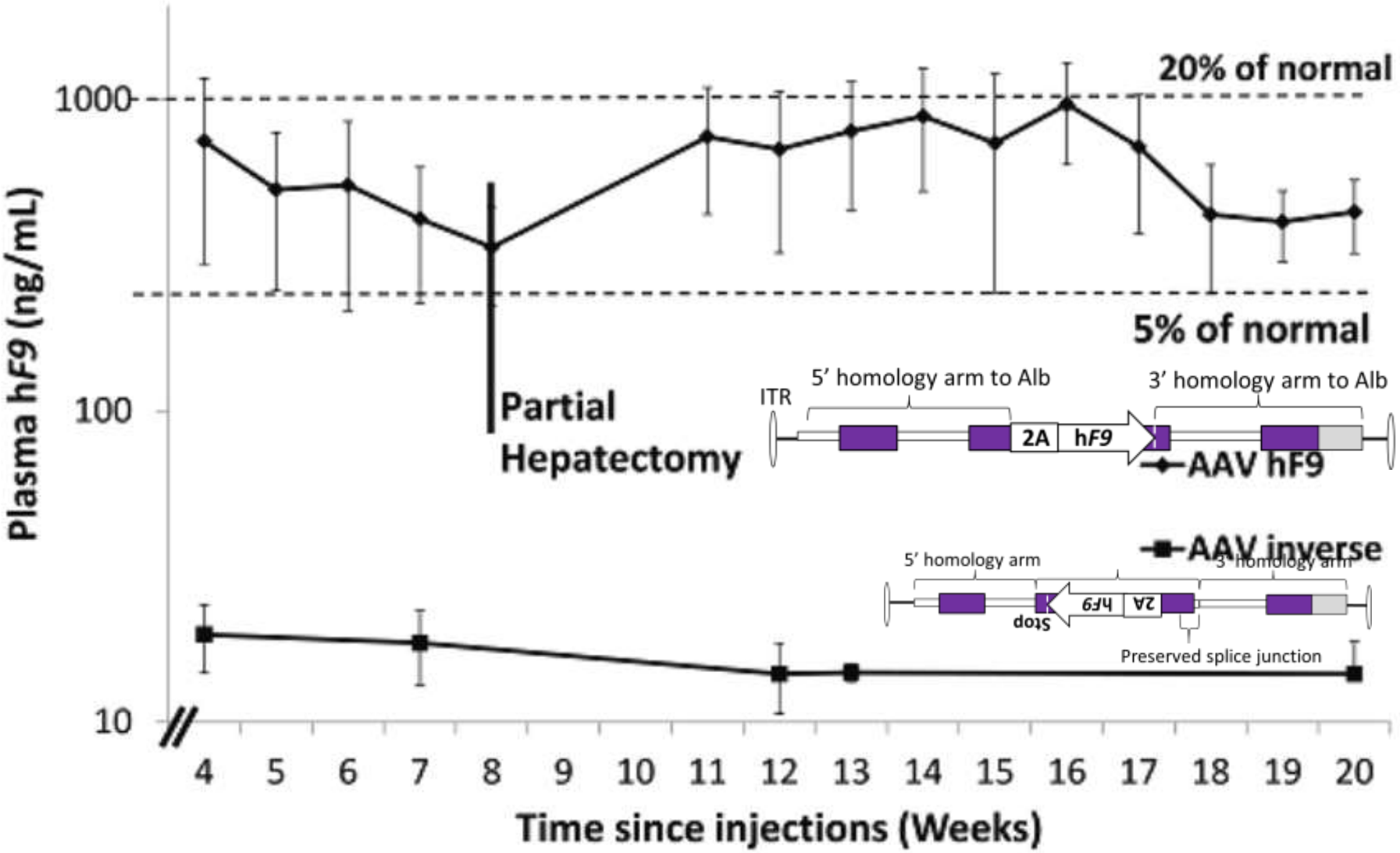
Therapeutic genome editing –vectors without an exogenous promoter

Barzel *Nature* in press

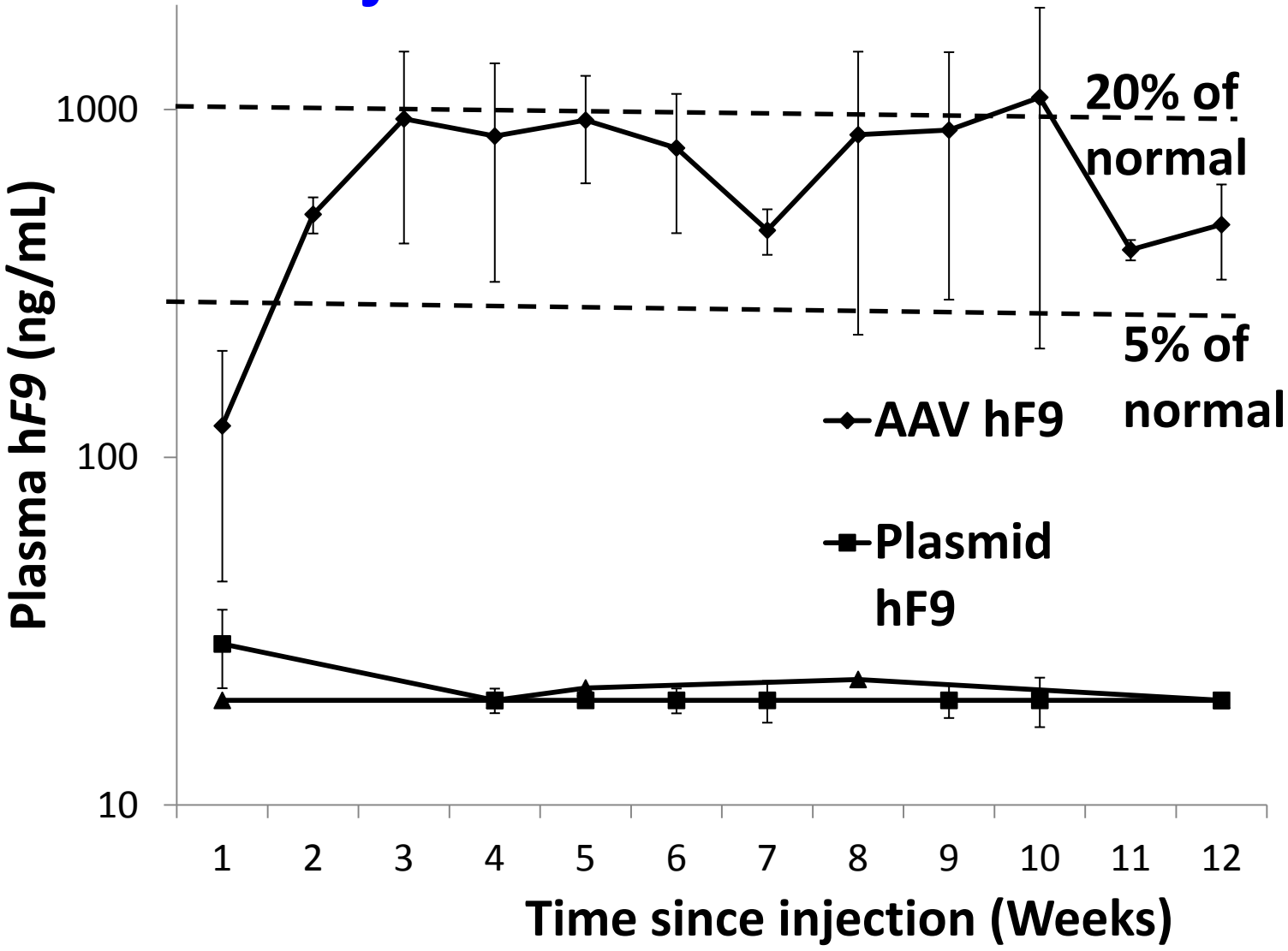
Promoterless Targeting for Site-Specific Transgene Expression



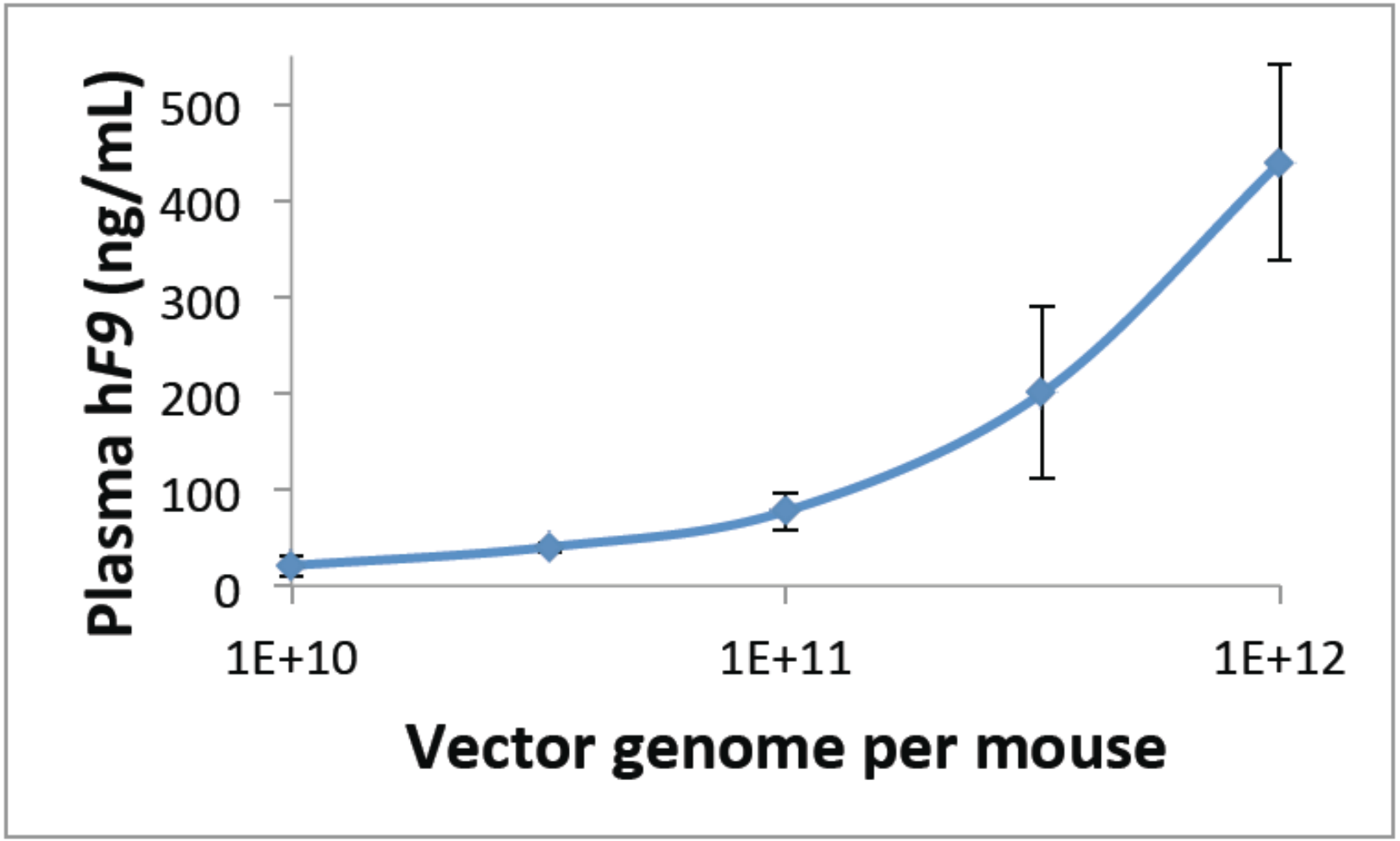
Stable h*FIX* plasma levels after injection into neonates



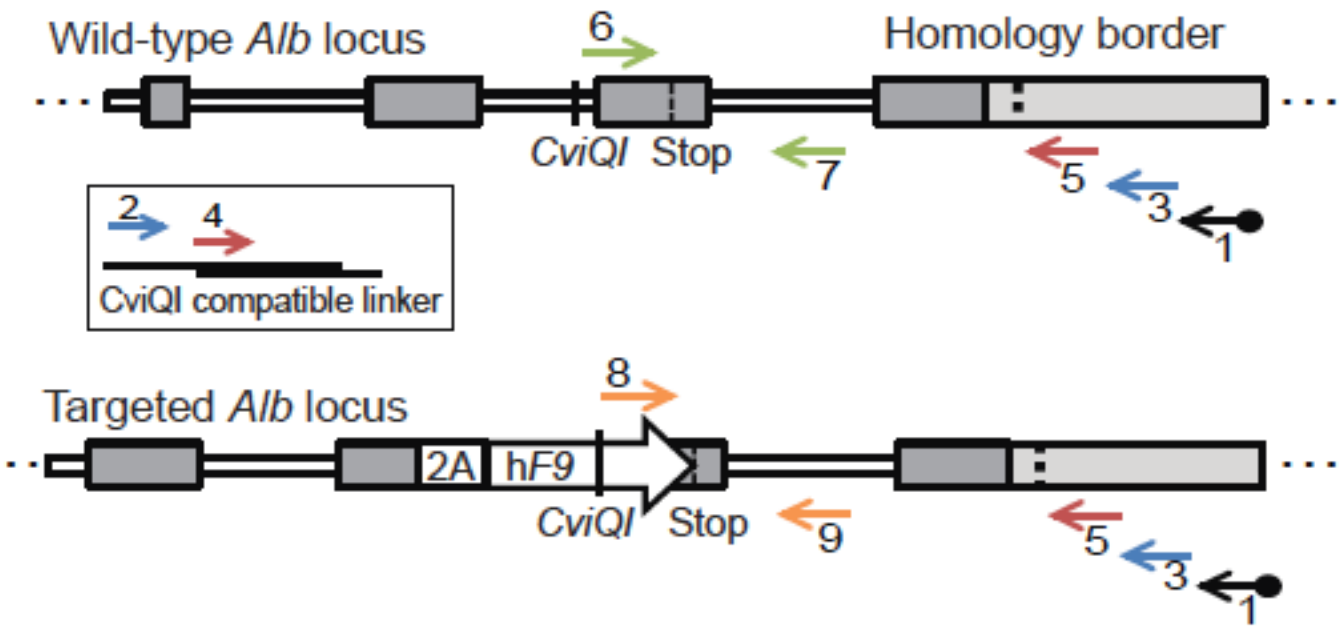
Stable h*FIX* plasma levels after injection into adults



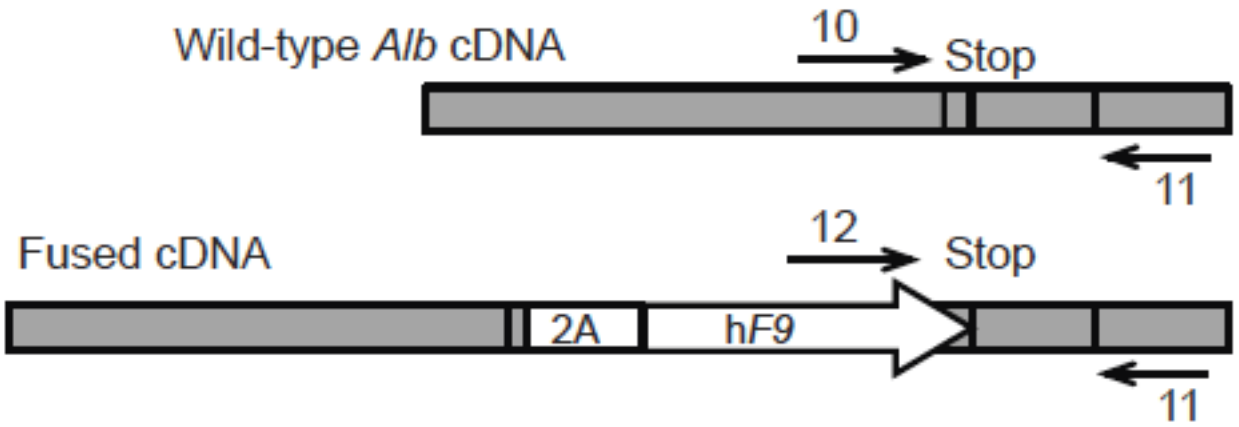
Dose Response in Adult Mice



Establish Allele Targeting Frequency

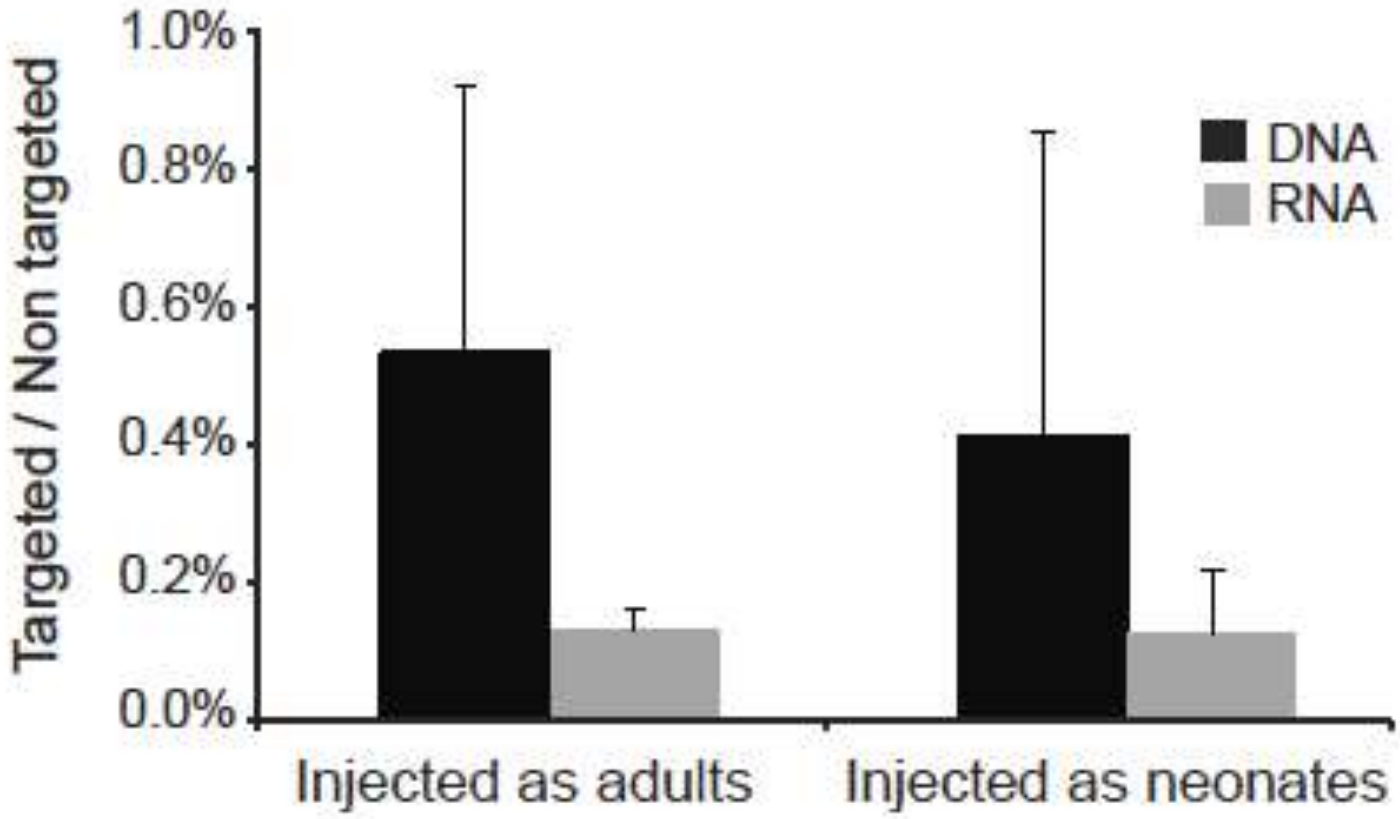


DNA

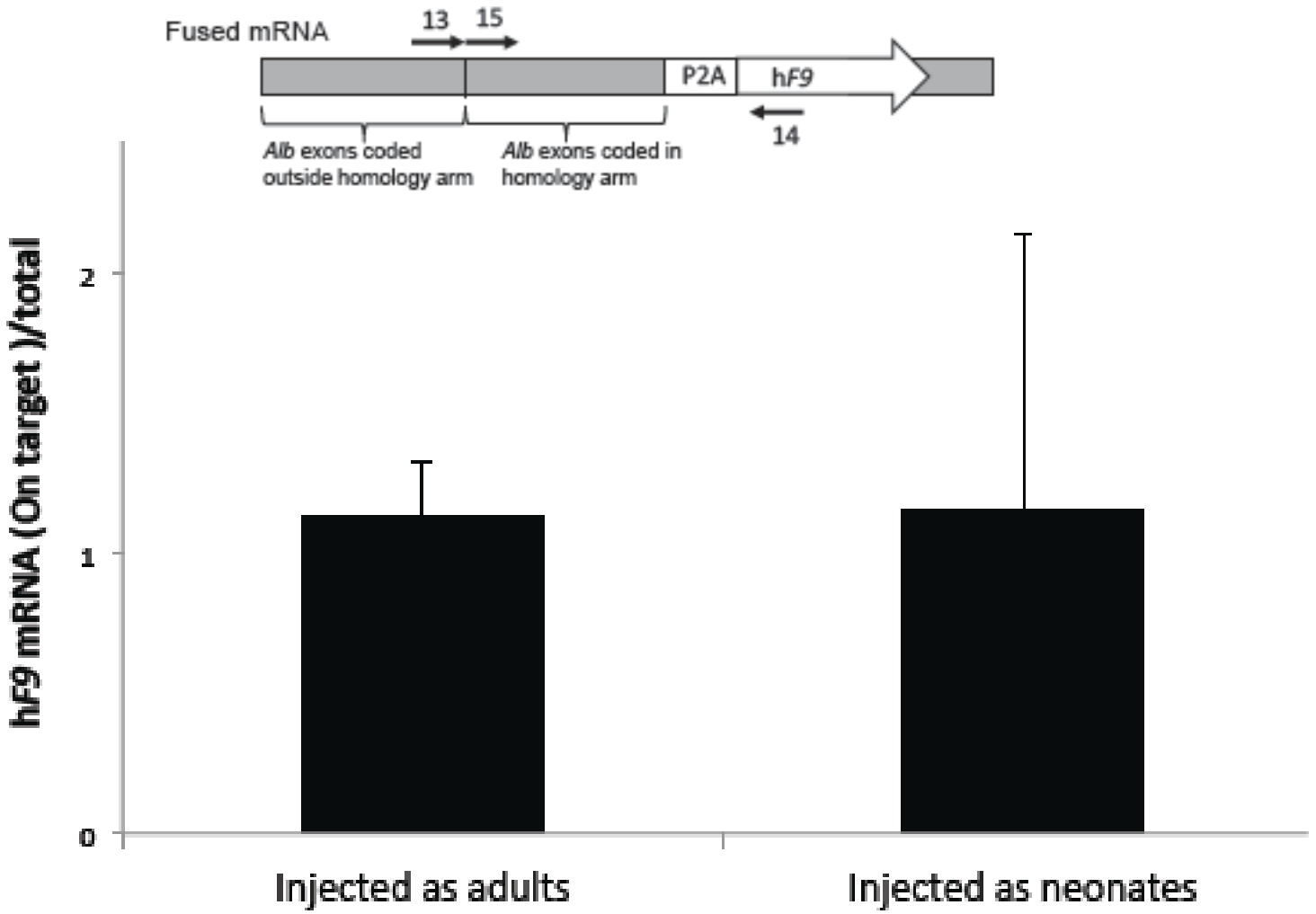


RNA

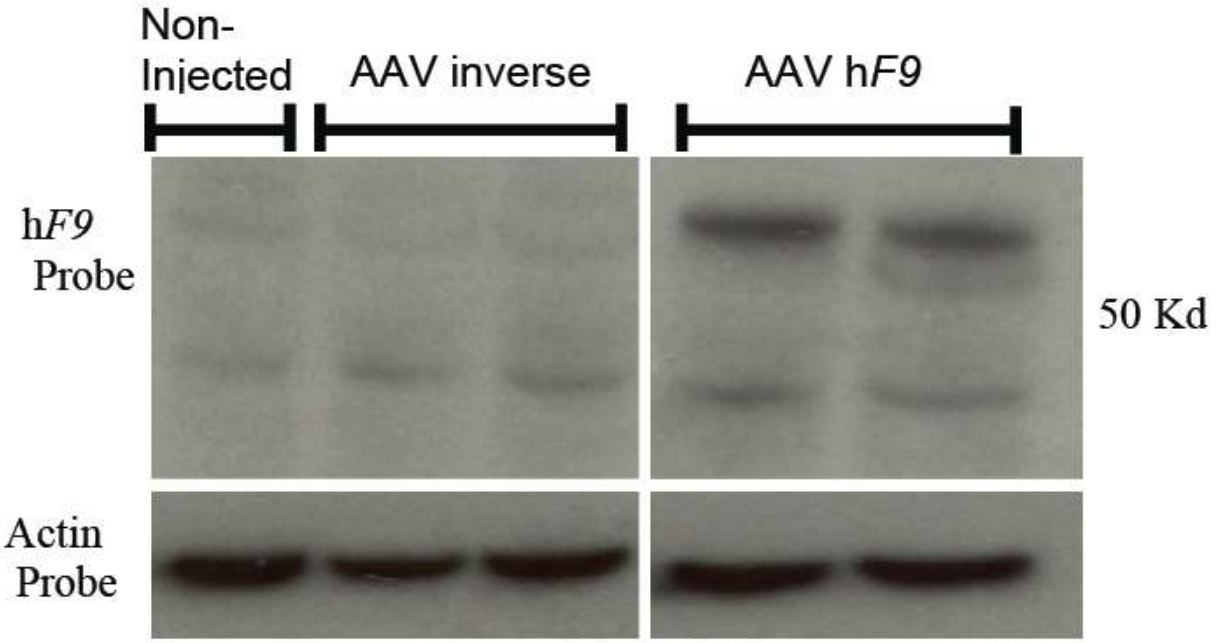
Allele Targeting Frequency

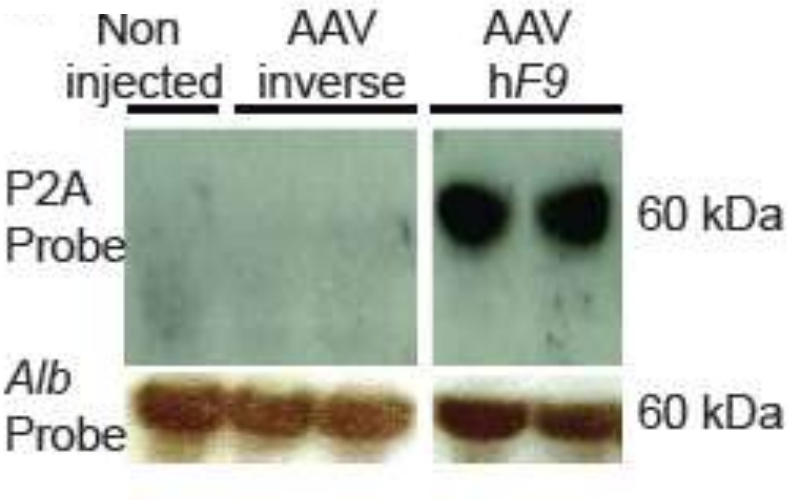


Percentage of hFIX mRNA transcripts derived from *Albumin* locus

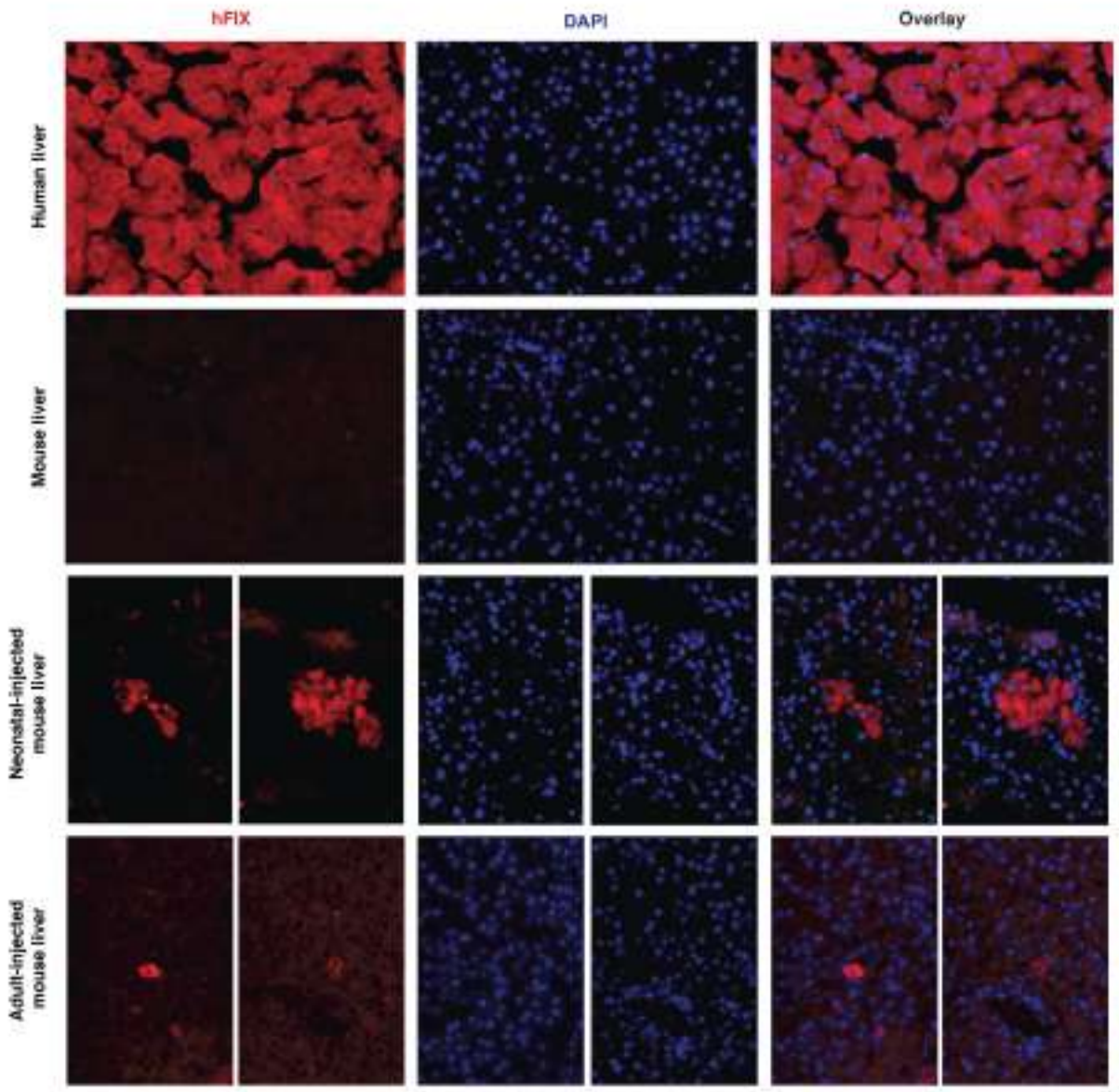


Western Blot Analyses



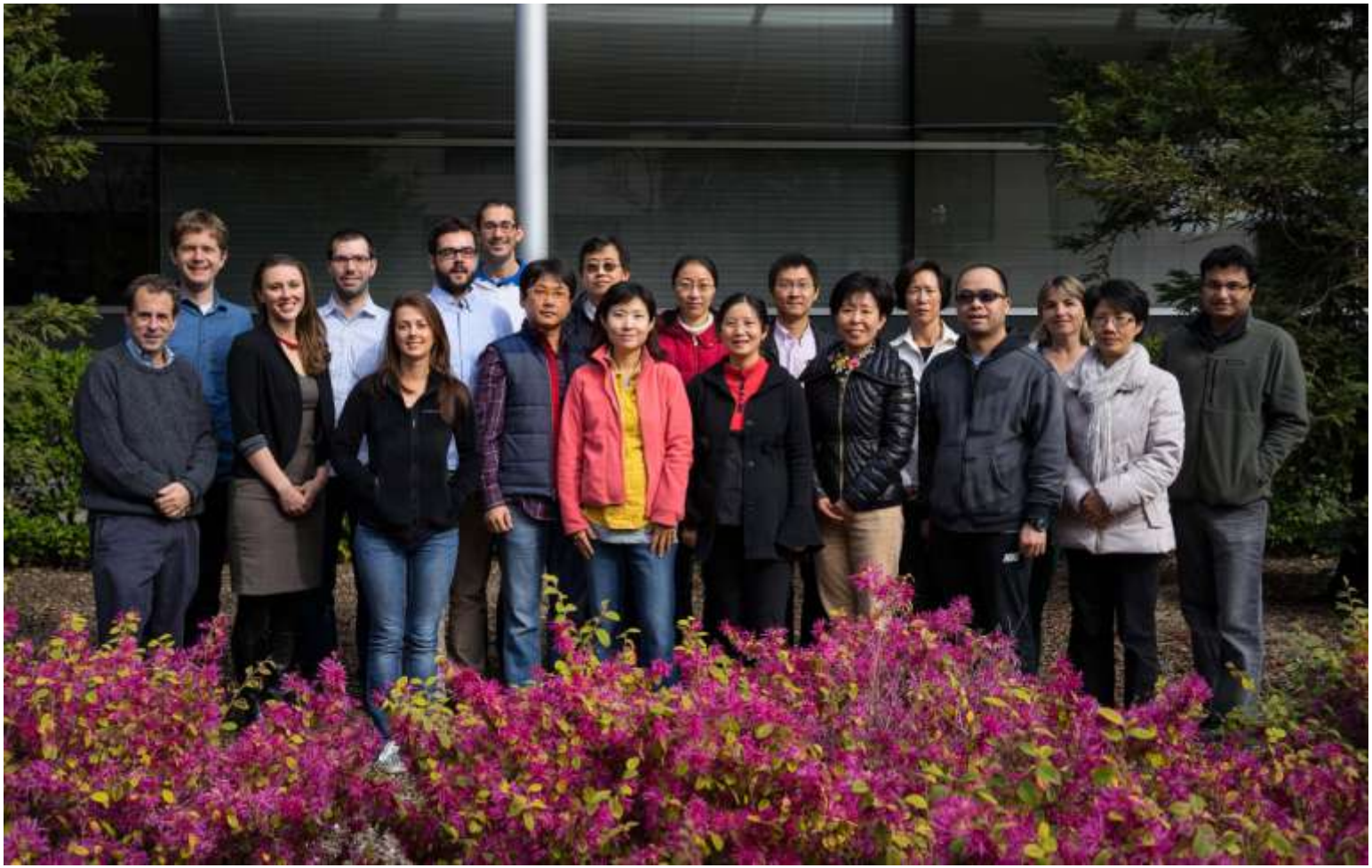


Immunohistochemical staining of hepatocytes



Summary

- Targeting occurs in about 0.5% of albumin alleles in hepatocytes in vivo
- Promoterless AAV recombination based vectors can provide therapeutic transgene expression and promoter(tissue)-specific expression
- Lack of of nuclease (1-vector, toxicity, off-target, immunologic reactions)
- Lack of a promoter means less chance of off-target mediated oncogenesis
- Can be used in dividing cells, liver in neonates?
- For human *Albumin* locus - 2 vectors will be sufficient for 95% of the population



Funding Agency NIH- NHLBI

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