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Gene Therapy and Gene Editing

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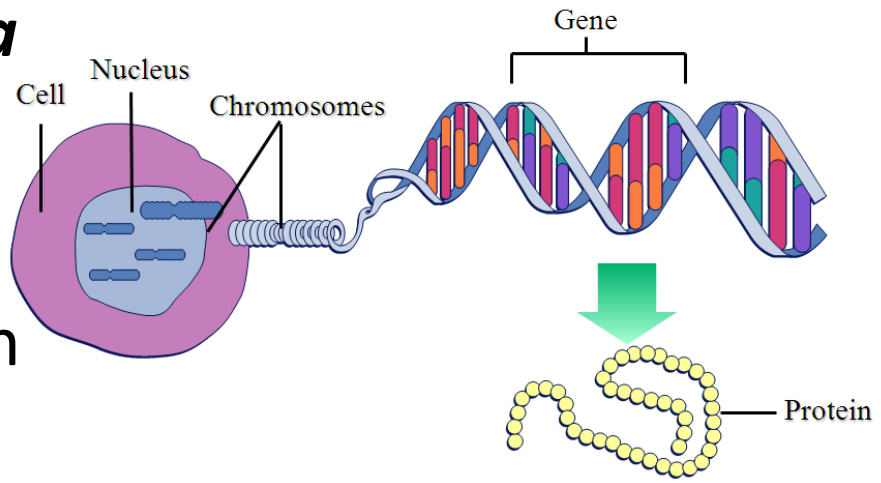
Agenda

1. Background
2. GT Approaches
3. Design an AAV GT Vector
4. Key Takeaways

Basic Definition of Gene Therapy

- **Treatment or prevention of a [genetic] disease *via* introduction of genetic material expected to provide a necessary function**



http://igbiologyy.blogspot.com/2014/03/chromosomes-dna-genes-and-alleles.html

- First developed in 1972 when Theodore Friedmann and Richard Roblin published a paper in *Science* called "Gene therapy for human genetic disease?"
- The first patient to be treated with gene therapy was a four-year-old girl treated at the NIH Clinical Center in 1990
  - She had a congenital disease called adenosine deaminase (ADA) deficiency which severely affects immunity and the ability to fight infections
  - Treatment was considered successful (*ex vivo*)

Recent History of Gene Therapy

- **>1100 GT trials have been initiated across the 3 most common delivery vehicles**
- **4 approved Adeno-associated Virus (AAV) products**
  - Glybera (Uniqure) – Lipoprotein Lipase (LPL) deficiency
    - Withdrawn (EU)
  - Luxterna (Spark) – Leber's congenital amaurosis (RPE65 deficiency)
    - Cost = $425,000 per eye, at launch
  - Zolgensma (AveXis/Novartis) – Spinal Muscular Atrophy (SMN1)]
    - Cost = $2.125M, at launch
  - Hemgenix (CSL Behring/UniQure) – Hemophilia B (FIX)
    Cost = $3.5M, at launch
- **Approved Retro/Lenti products**
  - Zynteglo (Bluebird Bio) – Beta-thalassemia (ex-vivo Lentivirus)
    - Cost = $2.1M
  - Strimvelis (GSK) – Adenosine deaminase deficiency (ADA-SCID)
  - Kymriah (Novartis) – Acute lymophoblastic leukemia (ALL)
    Ex vivo CAR-T
  - Yescarta (Kite/Gilead) – Large B cell lymphoma (Gammaretrovirus)

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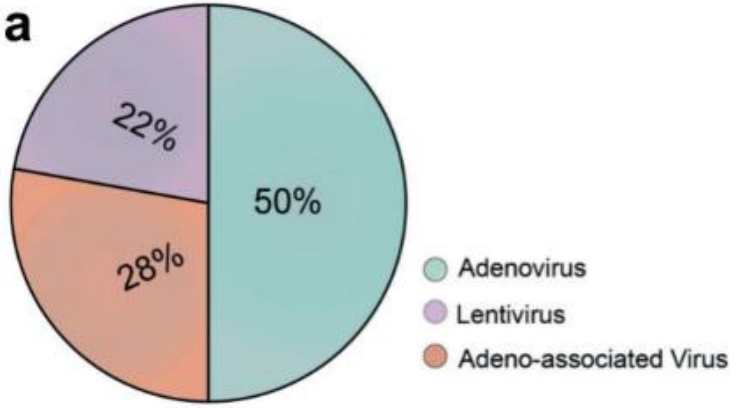

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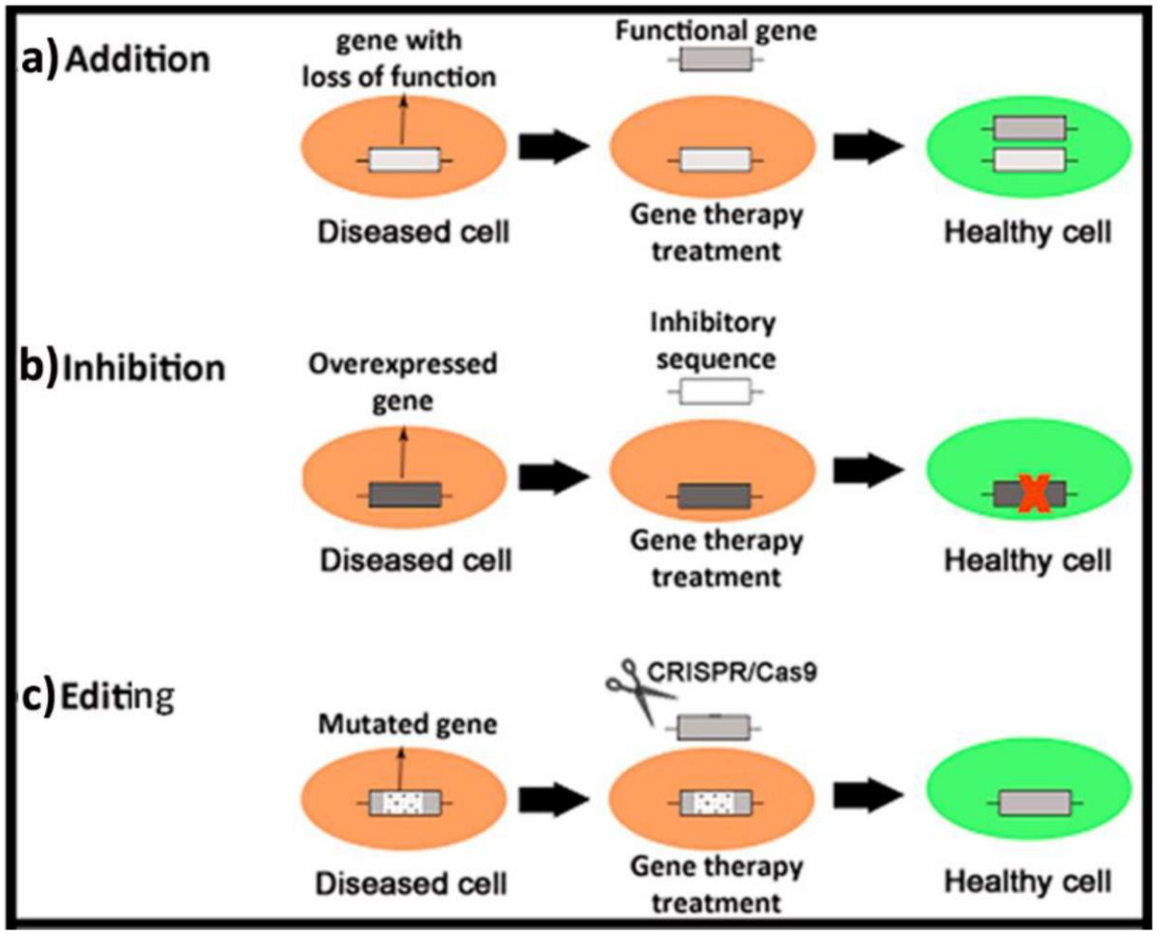
Vectors	Number of clincal trials
Adenovirus	575
Adeno-associated Virus	250
Lentivirus	315
Total	1140

Signal Transduction and Targeted Therapy (2021)6:53

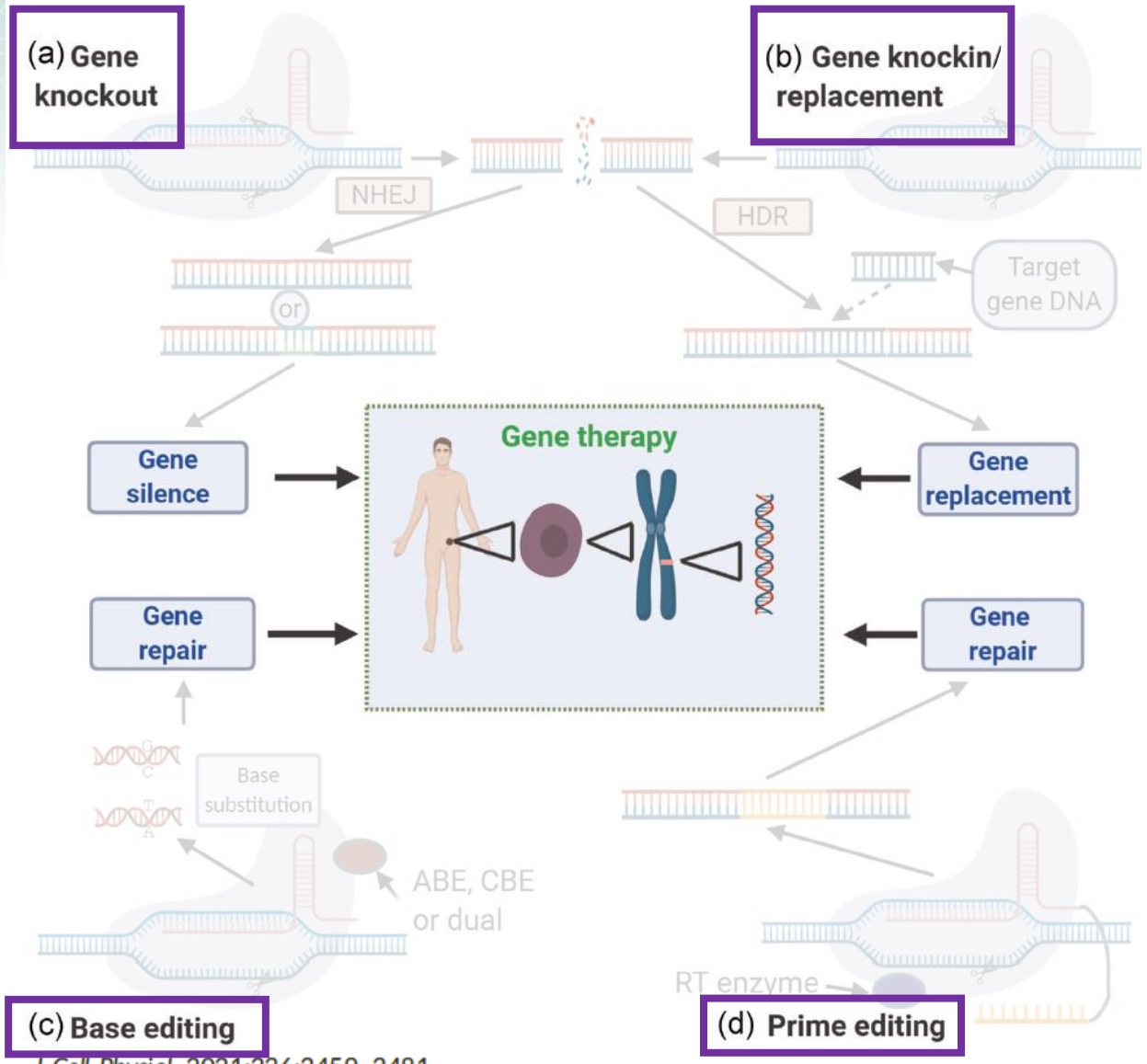
Gene Therapy Approaches



Int. J. Mol. Sci. **2021**, *22*, 7545. https://doi.org/10.3390/ijms22147545

- **Gene therapy approaches include the delivery of a package/cargo designed to:**
  - Add back a functional or wildtype copy of a mutated or missing gene that is causing disease
  - Inhibit, inactivate, or "knock out," a mutated or overexpressed gene that is functioning improperly
  - Edit a mutated gene back to a functional or wildtype copy of that gene

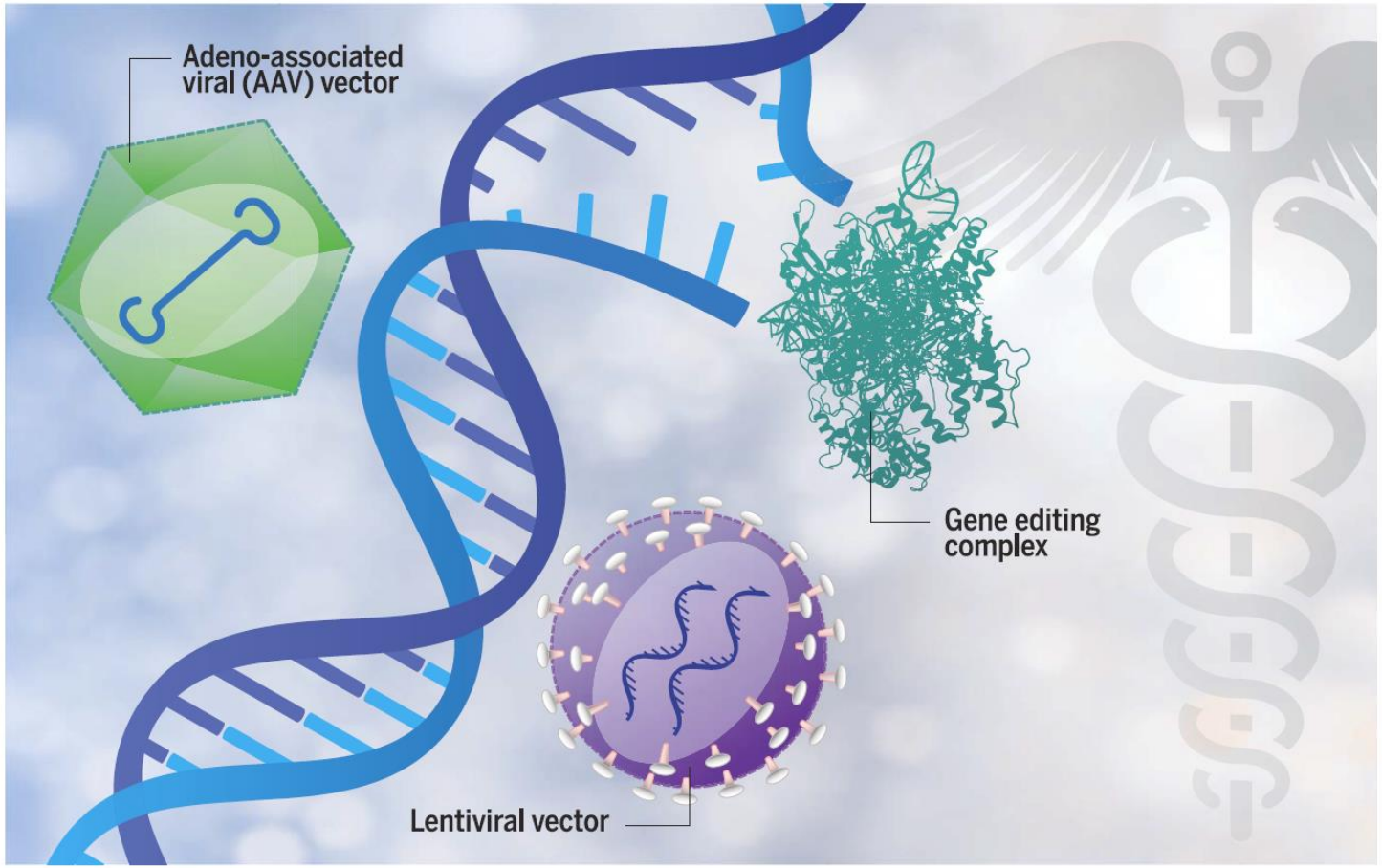
Major Strategies for CRISPR/Cas9-based Gene Therapy



J Cell Physiol. 2021;236:2459–2481.

- Delivery *via* lentivirus, AAV, extracellular vesicles or lipid nanoparticles possible
- *Ex vivo* applications likely to be more successful with current state of technology
- "Dead" Cas9 variants also available to upregulate or downregulate gene expression
- Off-target effects are a source of concern
- Targeting strategy must be tailored to specific DNA sequences, which could be affected by the variation of patient mutations
- Expected approval of Exa-cel (Vertex/CRISPR Therapeutics) forthcoming [2023?] for treatment of Sickle Cell Disease and Beta-Thalassemia
  - Cost not yet established
  - Range expected $1.9M-$4M

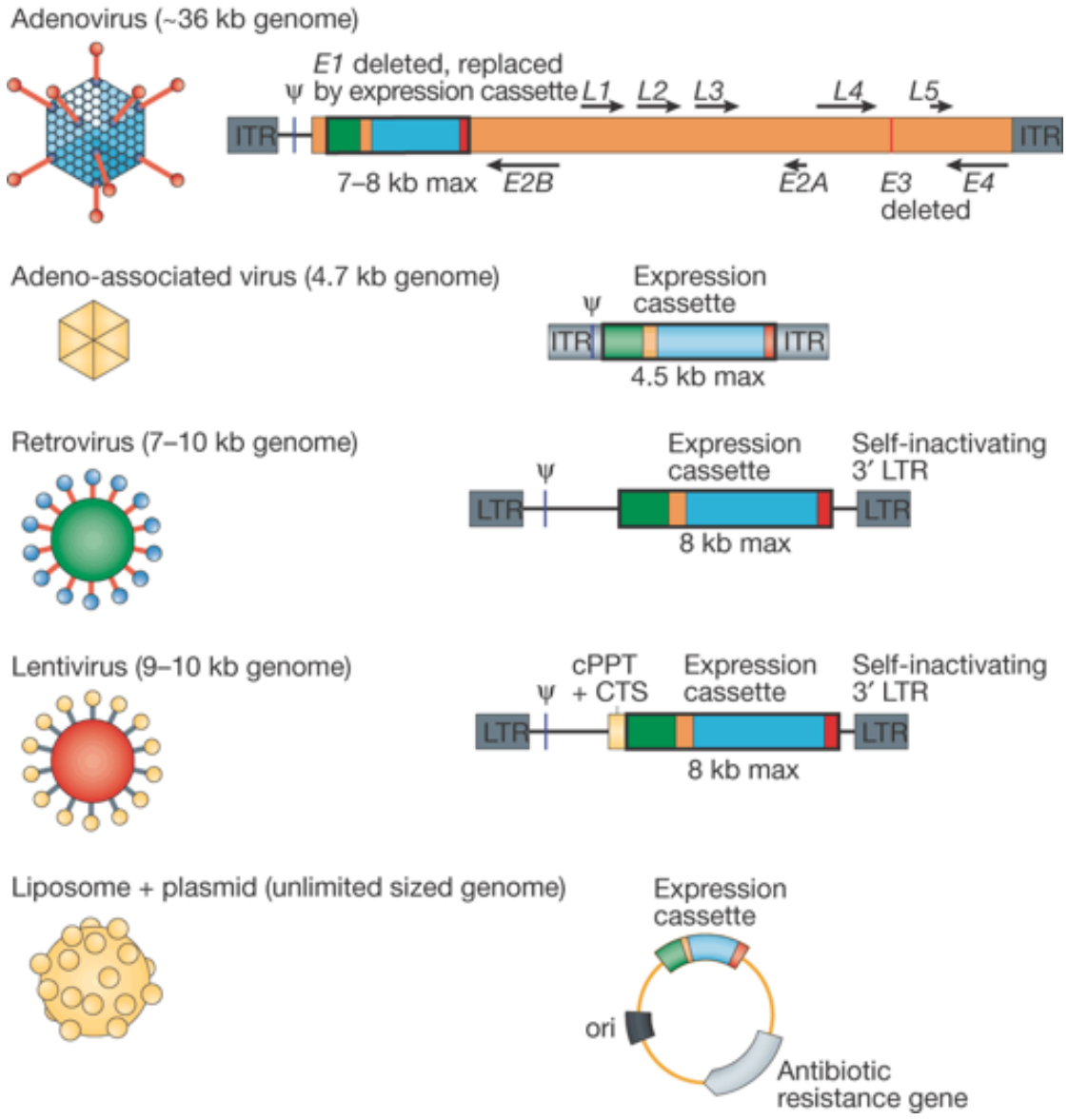
Basic Gene Therapy Tools



Dunbar *et al.*, *Science* **359**, 175 (2018)

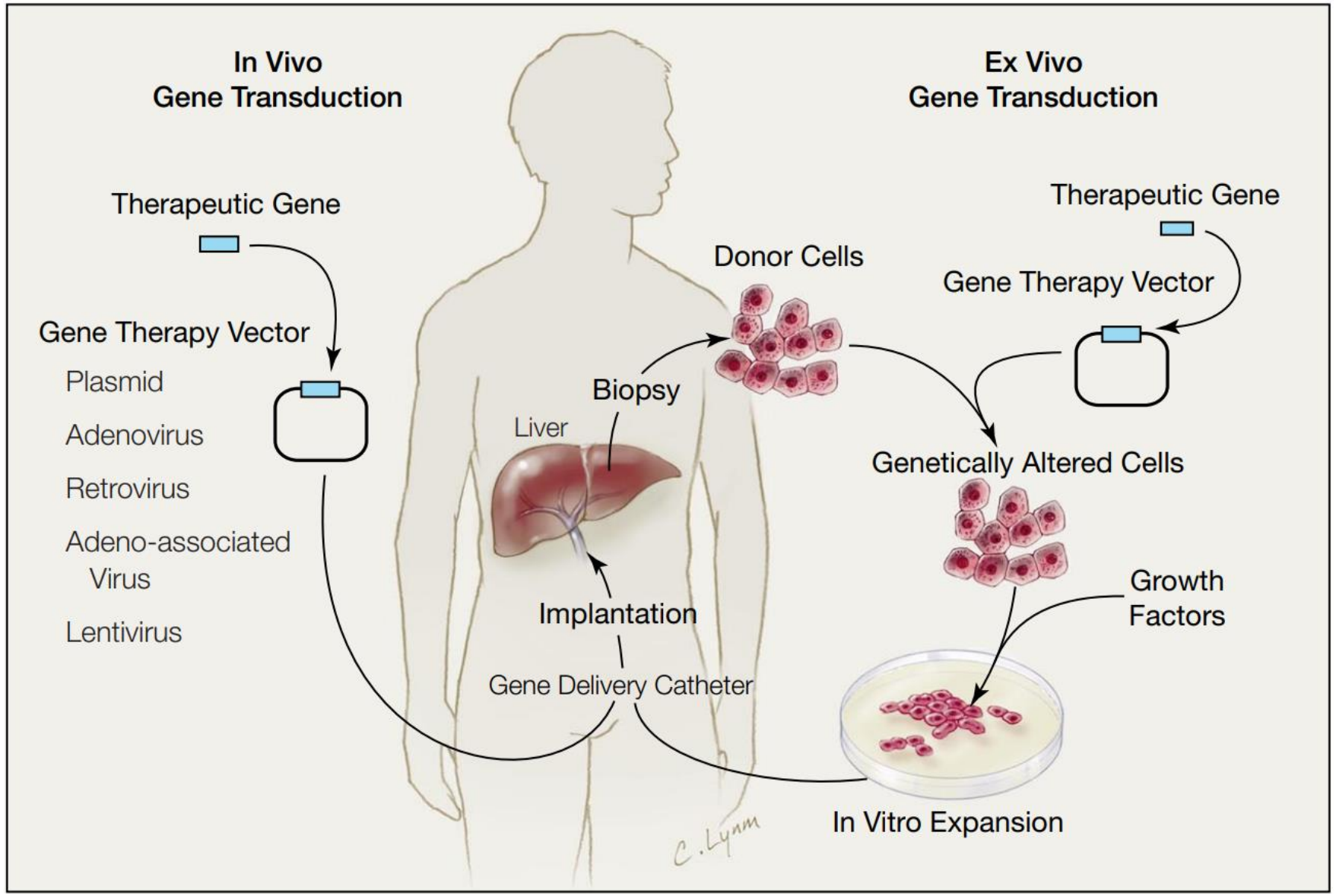
- **The 3 most basic tools currently utilized for Gene Therapy are:**
  - **Adeno-associated virus (AAV)**
    - Delivery Vehicle and Cargo
  - **Lentivirus**
    - Delivery Vehicle and Cargo
  - **Gene Editing complex**
    - Cargo
    - New technology*
    - Delivery vehicles vary

Extended Toolbox of Gene Therapy Vectors



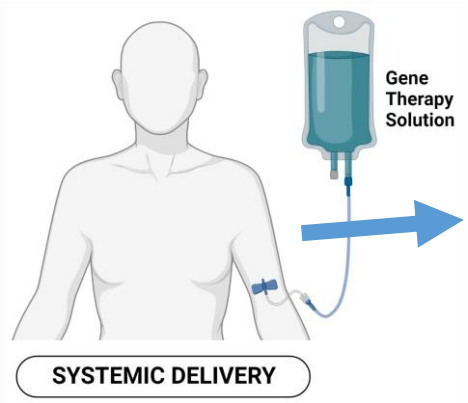
- **Adenovirus**
  - Antiviral vaccines; Anticancer therapy; Larger transgene size; Potential immune response to vector
- **AAV**
  - Ideal for targeting non-dividing cells; Ideal for *in vivo* delivery; Ideal for gene replacement; Smaller transgene size
- **Retrovirus**
  - Require active cell division for infection (target dividing cells); Lower safety profile than lentivirus due to higher rate of oncogenesis
- **Lentivirus**
  - Ideal for targeting non-dividing cells; Ideal for *ex vivo* delivery; Larger transgene size than rAAV, less than Adenovirus
- **Liposome + Plasmid**
  - Very large transgene size; Complicated manufacturing and delivery

In Vivo versus *Ex Vivo* Gene Delivery

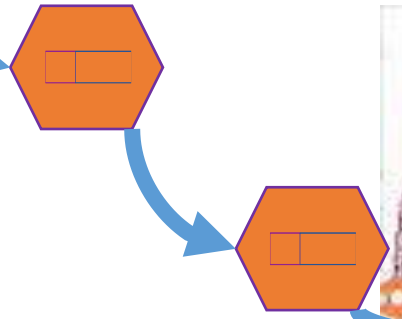


JAMA. 2001;285(5):545-550. doi:10.1001/jama.285.5.545

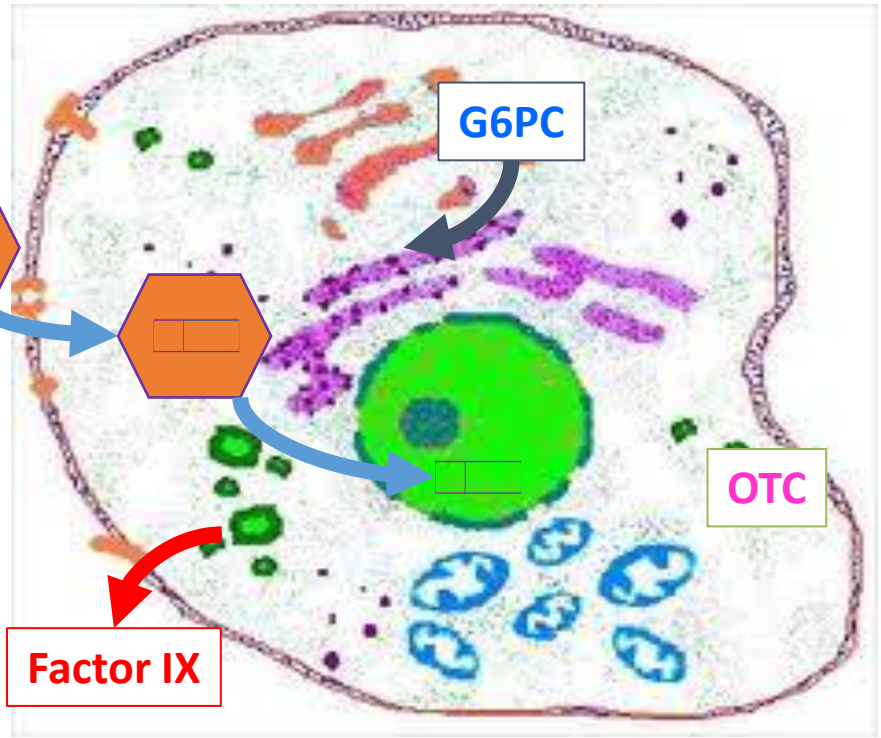
Gene Therapy Delivery is a Complicated, Multi-Step Process



Liver Promoter



Hepatocyte



1. The Vector must get <u>to</u> the target tissue/organ
2. Then <u>into</u> the target cell, and
3. Deliver the vector genome <u>into</u> the nucleus
4. Where the therapeutic gene <u>must</u> be expressed

What Makes a "Good Candidate" for Gene Therapy?

- **Does your transgene fit?**
  - AAV maximum packaging capacity is ~5 kilobases DNA
  - Lentivirus maximum packaging capacity is ~9 kilobases DNA
  - Note – This maximum capacity is not for transgene alone, but requires consideration of *required* viral and regulatory elements as well

- **Can the delivery vehicle (AAV/Lentivirus) infect and transfer genetic material to the target cell(s) important for correcting the disease?**
- **Are the target cell(s) amenable to the gene therapy approach/cargo you are using? (addition vs. inhibition)**

What Is the Best Vehicle for my Gene Therapy?

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AAV

- **Advantages**
  - Ideal for *in vivo* delivery
  - Ideal for targeting nondividing cells (reduced risk for oncogenesis)
  - Very low integration rates
  - Multiple capsid choices tailor cellular tropism
  - Academic and industry manufacturing experience
- **Challenges**
  - Small genome size
  - Expensive to manufacture
  - Higher potential for integration with CRISPR components
  - Single dose limitation*
  - Immune reactions to treatment in small number of patients not well understood

Lentivirus

- **Advantages**
  - Ideal for *ex vivo* delivery
  - Ideal for targeting nondividing cells
  - Infect wide variety of cell types
- **Challenges**
  - Lentiviral integration poses higher oncogenic/genotoxic risk than AAV
  - Potential activation of neighboring genes post-integration

* The field is attempting to address this with IdeS (https://www.nature.com/articles/s41591-020-0911-7)

Why is Gene Therapy a Compelling Therapeutic Modality?

- *Potential* for halting, treating *or* curing disease using a one-time* treatment
- Broad experience in global clinical trials
- Increased awareness and acceptance due to recent product approvals and compelling late-stage clinical results
- Regulators and payers becoming more familiar with therapeutic paradigm

* Limited by lifetime/dilution of treated cells

Keys to Recent Successful Outcomes

- Solid basic and clinical science
- High unmet medical need
- Good cellular/tissue target choice
- Increased platform understanding by Health Authorities

What limitations exist?

- Commercial CMC
- Continued stringency of Health Authority CMC requirements
- Extension of clinical success to new targets
- Safety concerns with high dose AAV delivery (>1-2x10^{14} vg per kg)
  - Zolgensma (AveXis/Novartis) – SMA AAV9
  - AT132 (Audentis/Astellas) – XLMTM AAV8
- Continued study of immunological reactions to gene therapy

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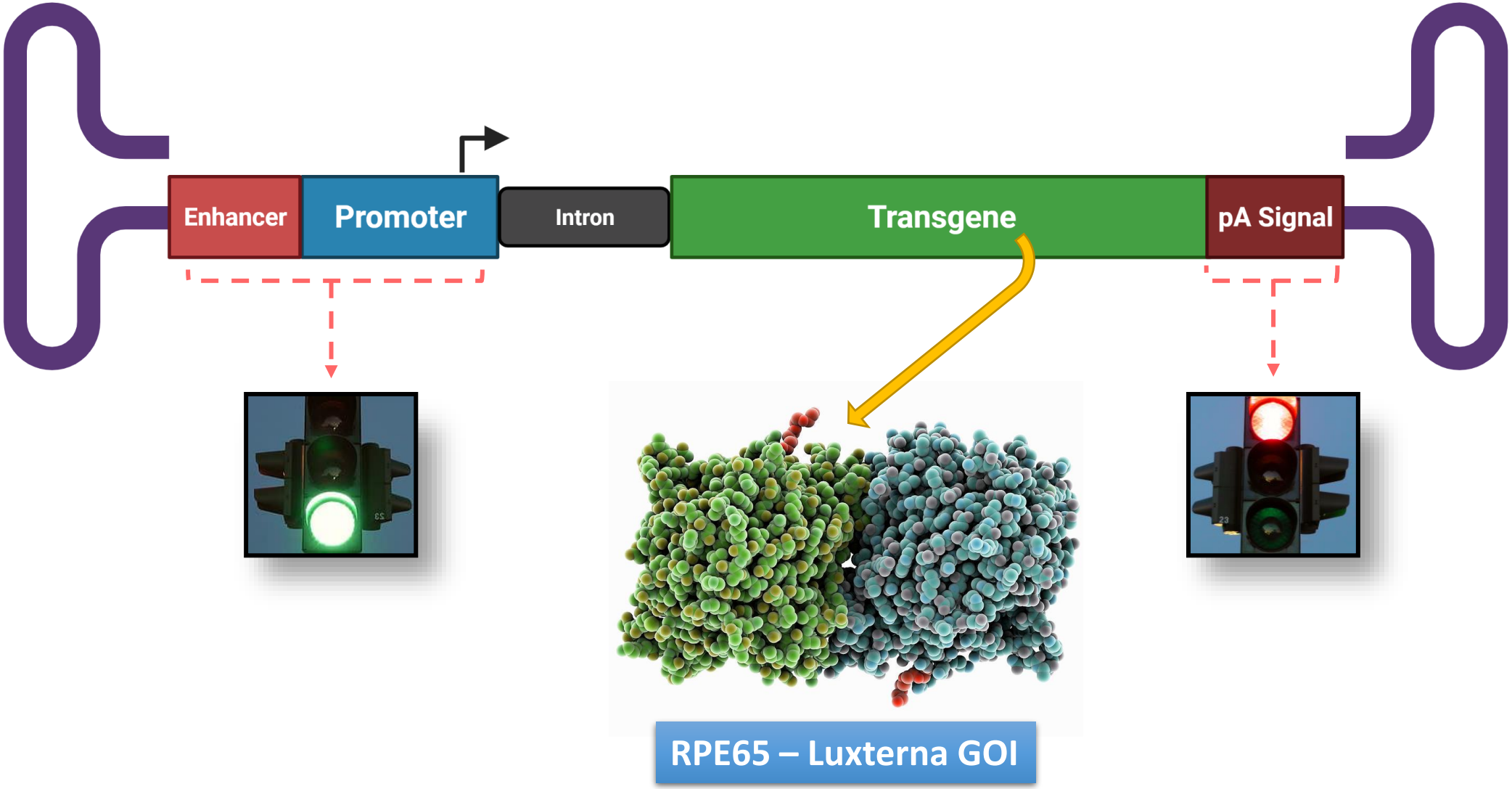
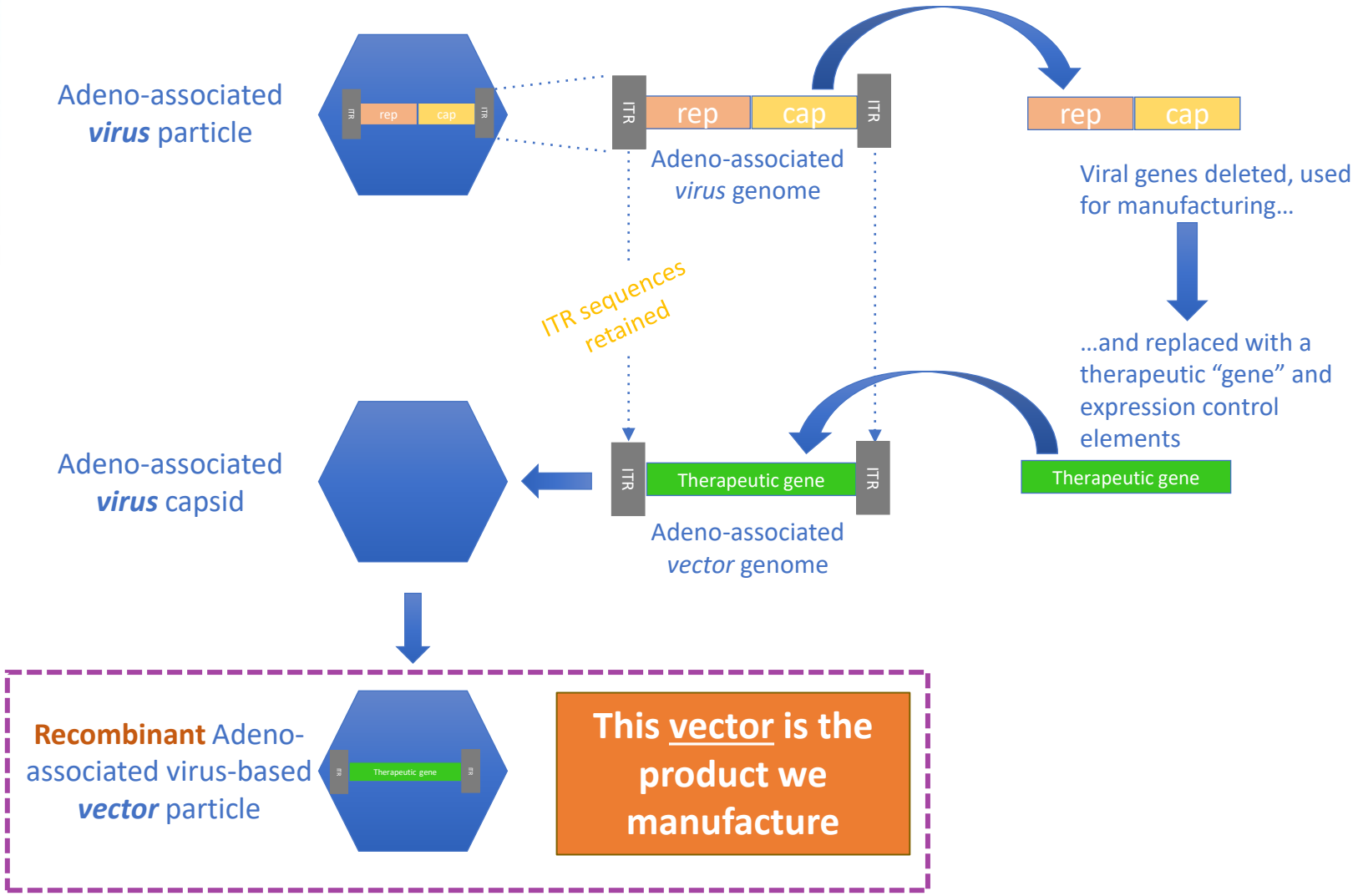
How to Design and Generate an AAV GT Vector

Fundamental Design of a Recombinant AAV



Basic recombinant AAV Molecular Engineering



Step 1: "Gut" wildtype AAV virus

Step 2: Insert gene of interest (GOI)

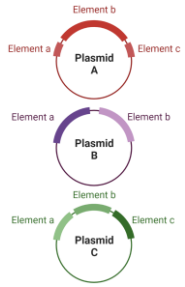
Step 3: Manufacture recombinant AAV

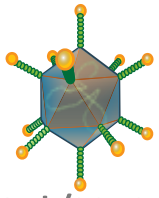
AAV Vector Manufacturing – Overview

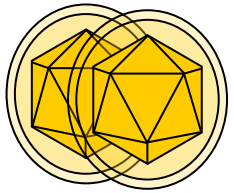
AAV rep+cap functions and helper functions must be provided

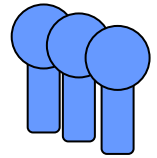
Commercial success requires bioreactor production platform and scale

Scalable operations must be designed to purify the product at acceptable yield and quality, while removing contaminants

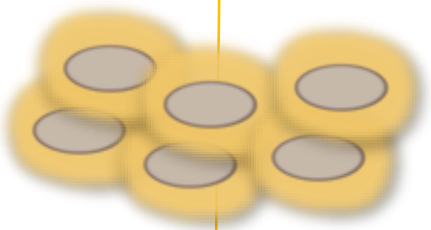






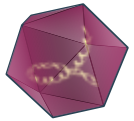


Plasmid Ad/HeLa HSV Baculo

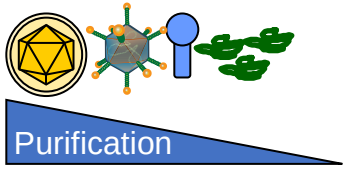


Human host cell except for baculo

AAV vector with contaminant stream

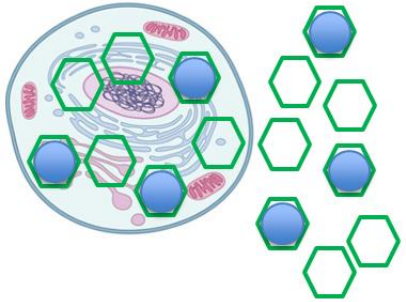


AAV

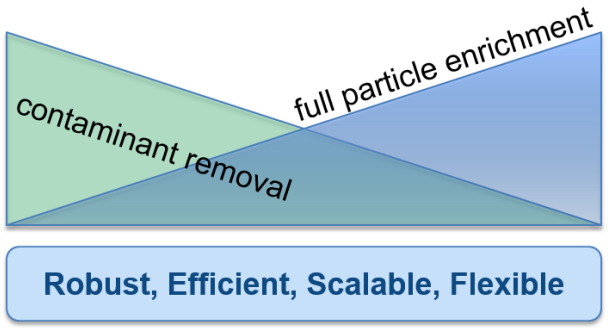


AAV Vector Purification

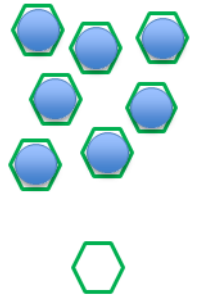
Upstream Cell-based Platform



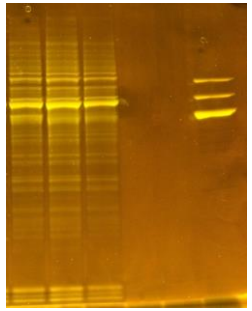
Downstream Purification Platform



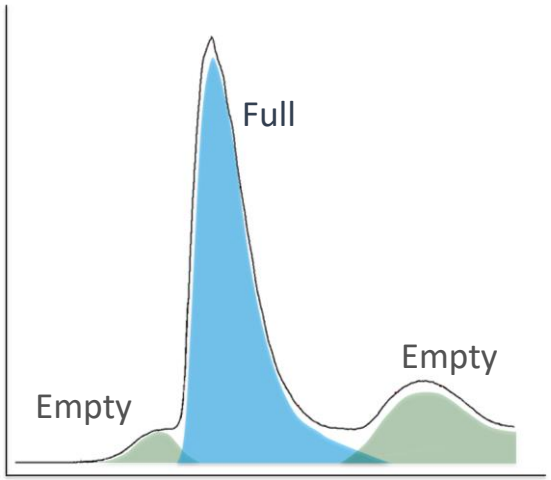
Final Product
highly enriched for active DNA containing vector



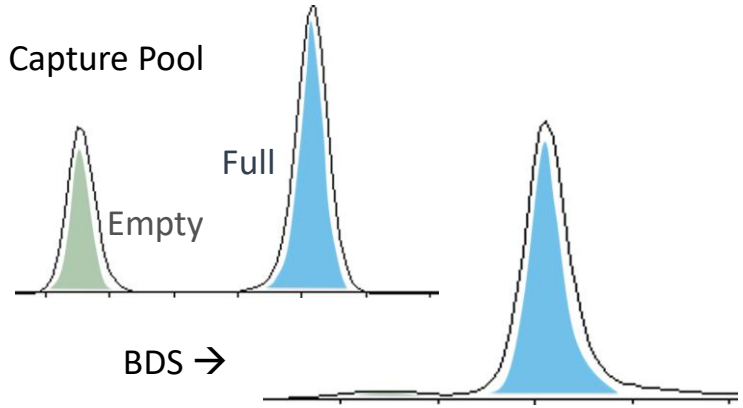
Affinity Capture



Polishing Step



Analytical Ultracentrifuge



Key Takeaways

- **Gene therapy has the p*otential* for halting, treating *or* curing disease using a one-time* treatment**
  - There are successful, FDA approved examples *via* recombinant AAV and Lentiviral vectors
- **Success is *critically dependent* upon solid basic and clinical science knowledge, a targetable cellular/tissue target choice, and the ability to manufacture the vector**
- **Each gene therapy strategy and delivery vehicle has pros and cons**
  - Strategy and delivery vehicle must be matched to the biology of the disease and target cells
- **Gene therapy manufacturing and reimbursement are *expensive***
  - The field is continually working improve manufacturability and reduce costs
  - Broader adoption of gene therapy treatments and continued understanding of treatment paradigm by regulators and payers should lead to reduced costs over time

Thank You!

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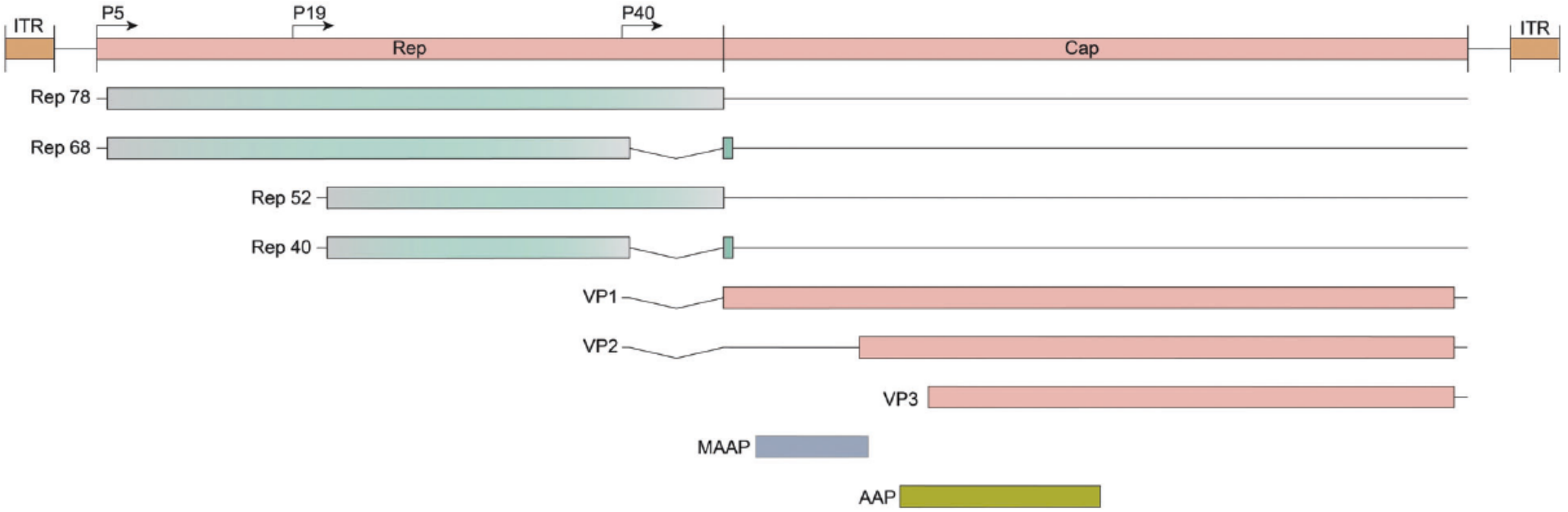
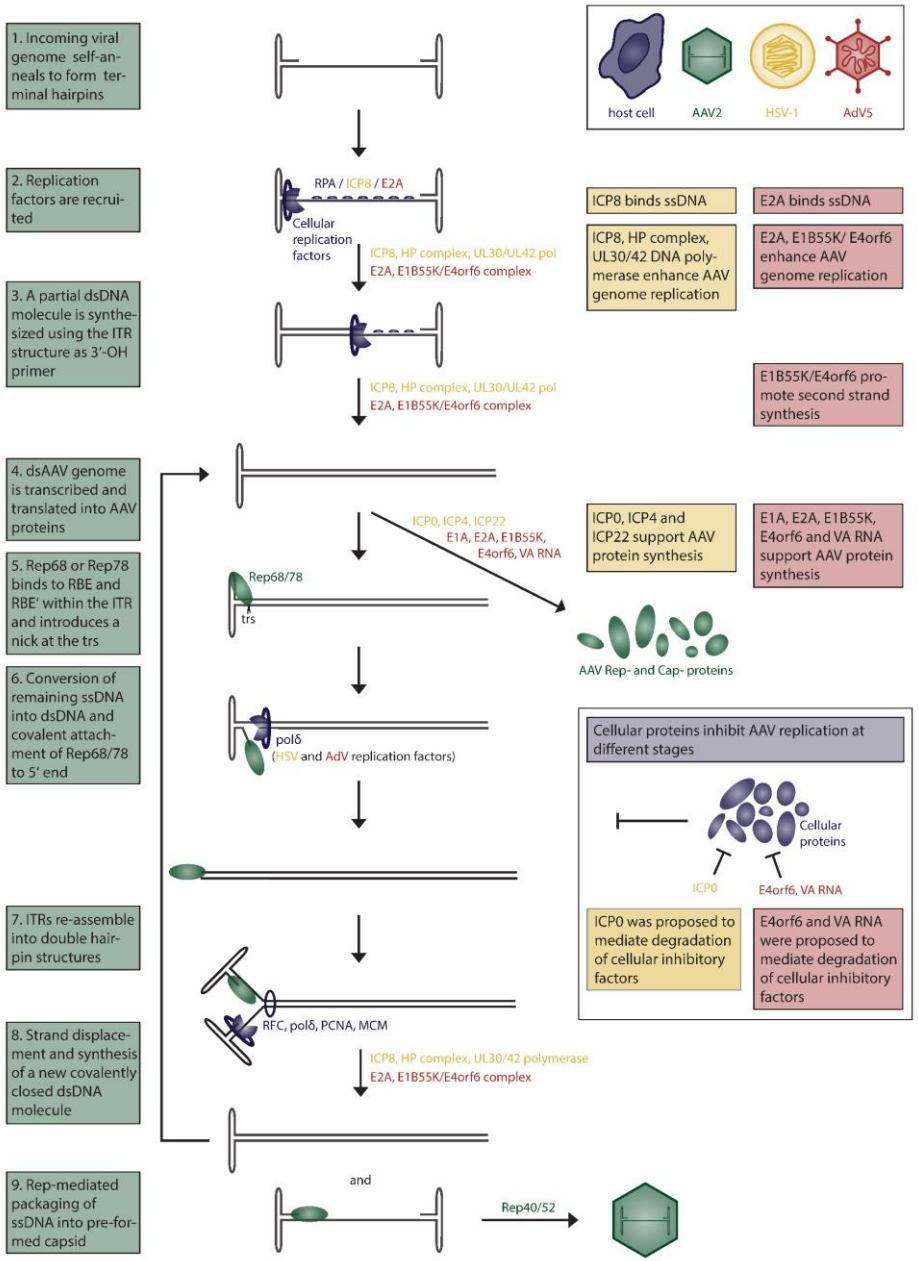
A bit more detailed information on AAV

AAV Protein Expression



- AAV “Large Rep” proteins provide a nicking function required for genome replication [Rep78 and Rep68]
- AAV “Small Rep” proteins are involved in packaging the genome into the preformed capsid [Rep52 and Rep 40]
- AAV Capsid proteins (“Cap”) form the structure that the genome is packaged into and is the delivery mechanism of gene therapy [VP1, VP2, VP3]

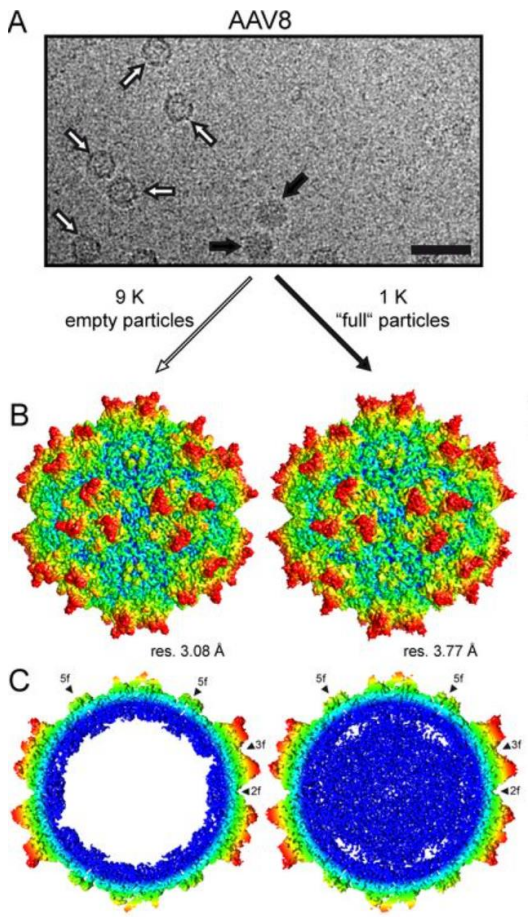
AAV Replication – Helper Virus



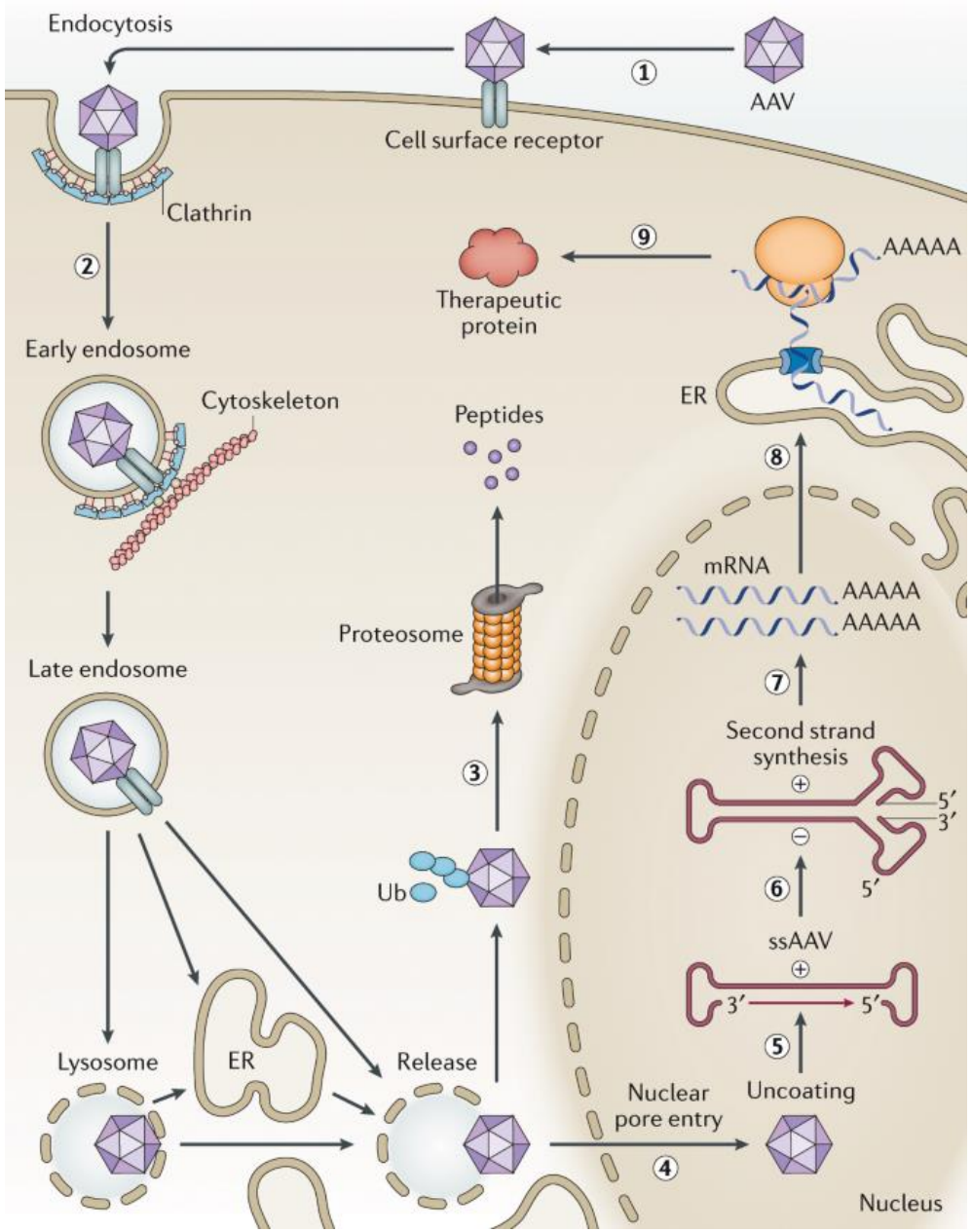
- AAV co-opts both cellular and viral factors to facilitate completion of its life cycle (genome replication and packaging)
- Cellular factors are used to replicate the AAV genome
- Helper virus factors are used to support replication of the AAV genome
- AAV Rep proteins are used to facilitate replication and packaging of the AAV genome
- AAV Cap proteins associate to form the capsid

AAV Capsid Selection

AAV serotype	Origin of isolation	Primary receptor	Co-receptor	Tissue tropism	Condition (ClinicalTrials.gov identifier)	Approved drug
AAV1	Monkey	Sialic acid	AAVR	Muscle, CNS, heart	Muscle diseases (NCT01519349)	None
					Heart failure (NCT01643330)	
					AAT deficiency (NCT01054339, NCT00430768)	
AAV2	Human	Heparin	Integrin, FGFR, HGFR, LamR, AAVR	Liver, CNS, muscle	Eye diseases (NCT00643747)	Luxturna for Leber congenital amaurosis
					Haemophilia (NCT00515710)	
					CNS diseases (NCT00400634)	
					AAT deficiency (NCT00377416)	
AAV3	Human	Heparin	FGFR, HGFR LamR, AAVR	Muscle, stem cells	No trials underway	None
AAV4	Monkey	Sialic acid	Unknown	Eye, CNS	Eye diseases (NCT01496040)	None
AAV5	Human	Sialic acid	PDGFR, AAVR	CNS, lung, eye	Haemophilia (NCT03520712)	None
					Eye diseases (NCT02781480)	
					AIP (NCT02082860)	
AAV6	Human	Heparin, sialic acid	EGFR, AAVR	Muscle, CNS, heart, lung	Haemophilia (NCT03061201)	None
					CNS diseases (NCT02702115)	
AAV7	Monkey	Unknown	Unknown	Muscle, CNS	No trials underway	None
AAV8	Monkey	Unknown	LamR, AAVR	Liver, muscle, pancreas, CNS	Eye diseases (NCT03066258)	None
					Haemophilia (NCT00979238)	
					Muscle diseases (NCT03199469)	
AAV9	Human	Galactose	LamR, AAVR	Every tissue	CNS diseases (NCT02122952)	Zolgensma for spinal muscular atrophy
					Muscle diseases (NCT03362502)	
AAV10	Monkey	Unknown	Unknown	Muscle	No trials underway	None
AAV11	Monkey	Unknown	Unknown	Unknown	No trials underway	None
AAV12	Human	Unknown	Unknown	Nasal	No trials underway	None



AAV Transduction



- AAV capsid interacts with the external cellular receptor and is endocytosed
- AAV capsid interacts with internal endosomal receptor
- Endosome matures from early to late stage, pH change induces conformational change in AAV capsid externalizing VP1
- VP1 phospholipase (PLA) activity opens the endosome allowing capsid escape
- Capsid traffics to the nucleus, disassembles and releases the single-stranded DNA
- WT virus expresses viral proteins and replicates
- Recombinant AAV concatamerizes and resides as an episome loosely associated with cellular chromatin (forms circular DNA molecule)

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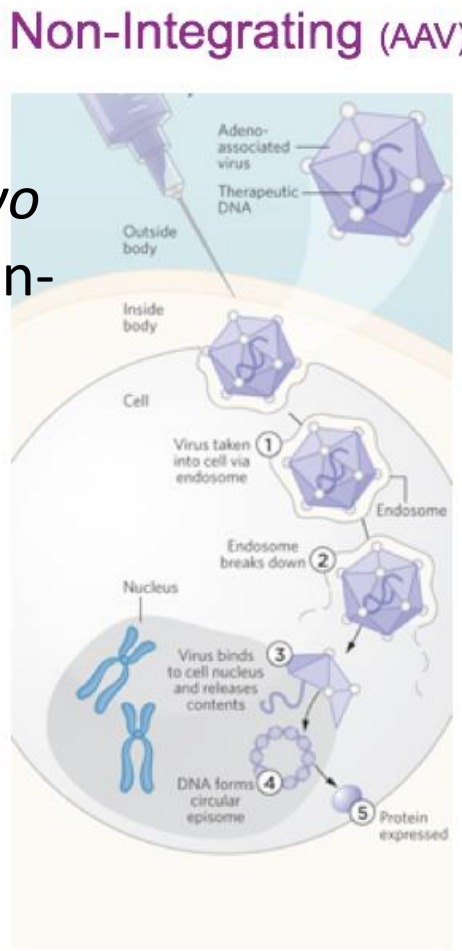
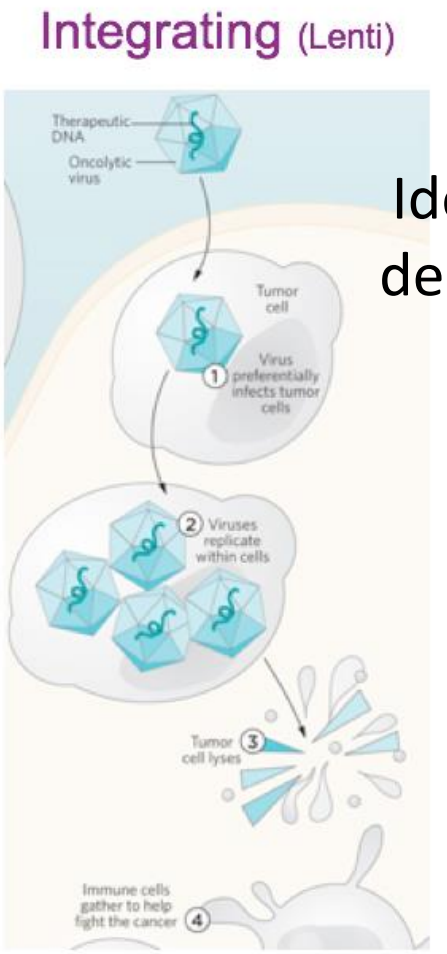
Transition Slide

Appendix

GT Overview: Introducing Genetic Material via Viruses

Ideal for *in vivo* delivery to non-dividing cell targets





Ideal for *ex vivo* delivery to stem cell targets

Introducing Genetic Material via Viruses

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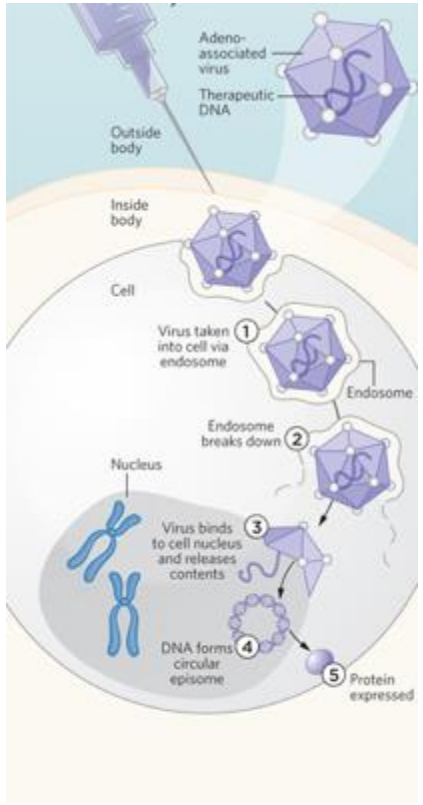
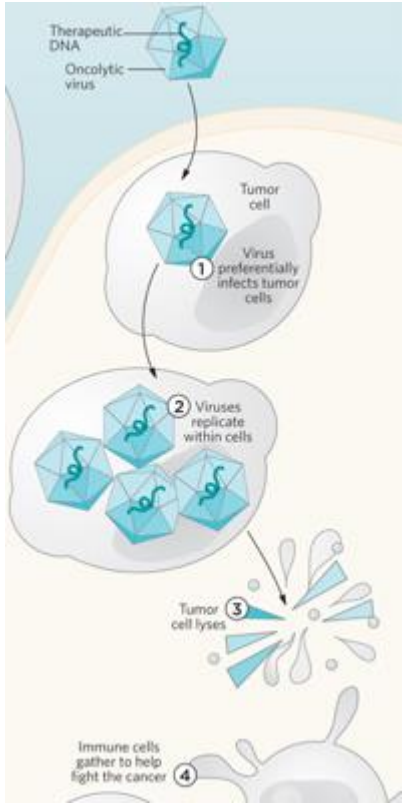
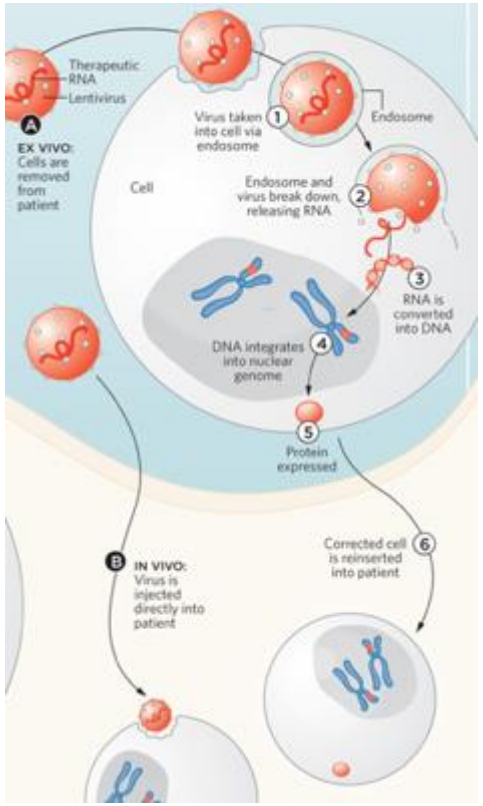
Non-Integrating



Integrating



Oncolytic



Terminology

- Capsid – the protein shell of a virus; essential component involved in cell binding, internalization, and trafficking within the targeted cell
- Genes – the building blocks of inheritance
- Genetic disorder – results when genes don’t produce the right proteins or don’t produce them correctly
- Transgene – the gene or genetic material that is being transferred to the cell
- Vector – delivery vehicles that encapsulate therapeutic genes for delivery to the cell; include genetically disabled viruses, such as adeno-associated virus

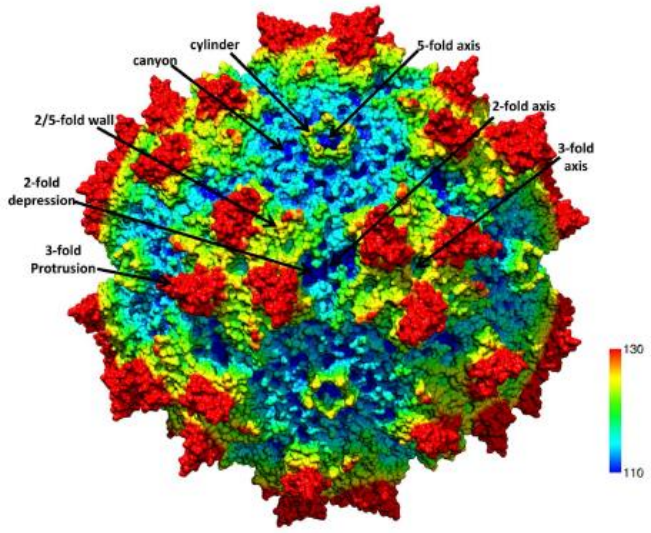
Why AAV Gene Therapy?

- Non-integrating - reducing oncogenic potential
- Multiple capsid types allowing for tailored tropism
- Expertise and experience to manufacture commercial scale product
- Leverages biopharma protein manufacturing experience

AAV: Family Parvoviridae, Genus Dependovirus

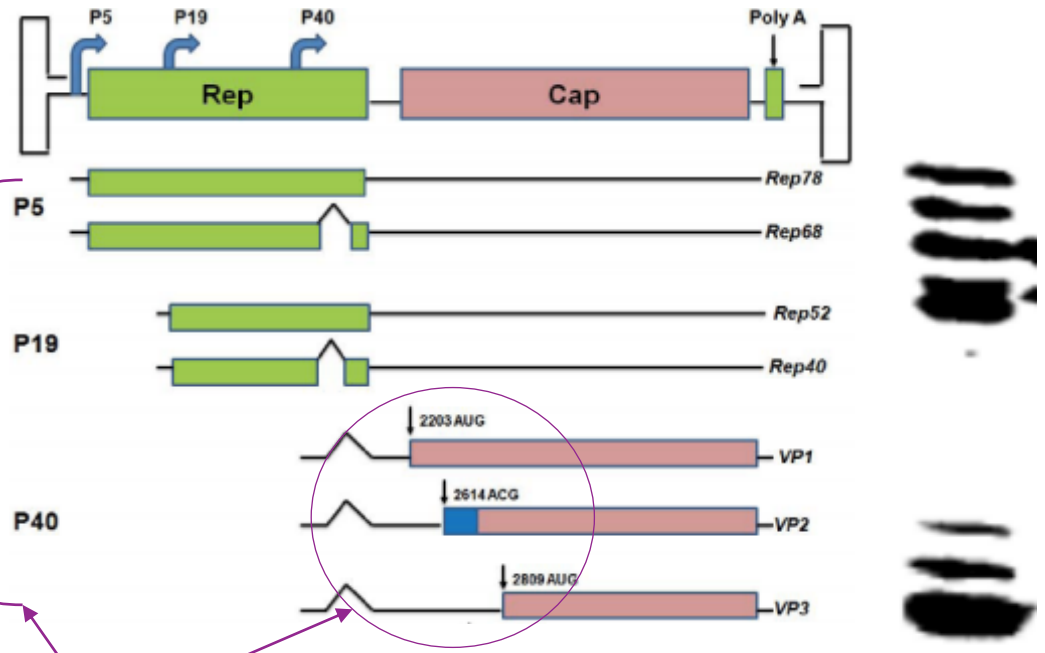
Scientific Platform

- AAV discovered in early 1960s
- Wild type AAV is not associated with disease
- Variable seropositivity in human population depending on capsid serotype



- 20 nm non-enveloped icosahedral capsid
- Virion extremely stable
- Single-stranded genome of 4,680 nt
- Three capsid proteins (VP1,2,3)
- Multiple capsid structural variants available

Dependovirus Genome Structure – Implications for Manufacturing

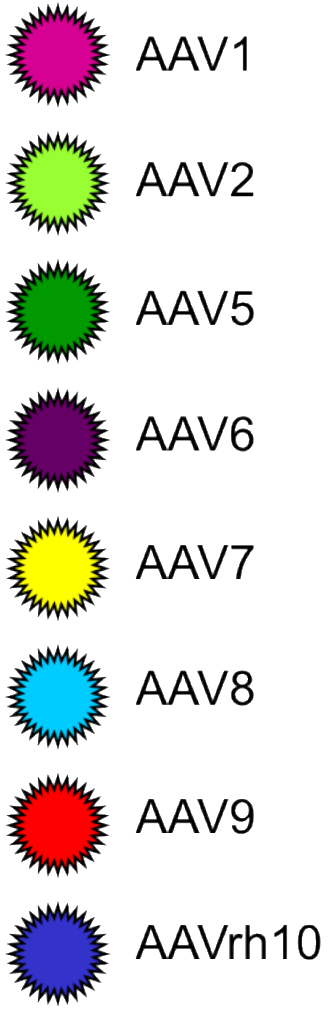


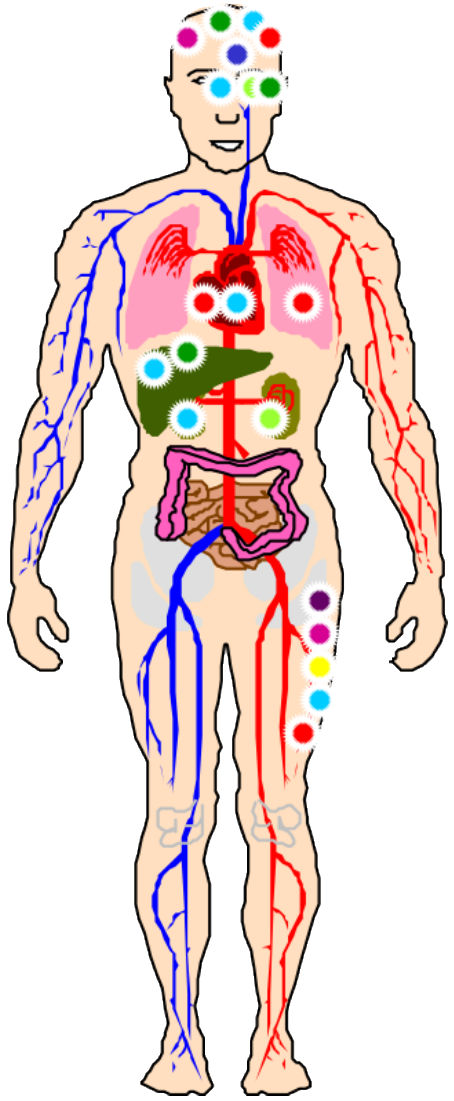
Alternative splicing

And alternative translation initiation

- Mammalian platform leverages existing molecular biology
- Baculovirus platform needs to engineer around this biology

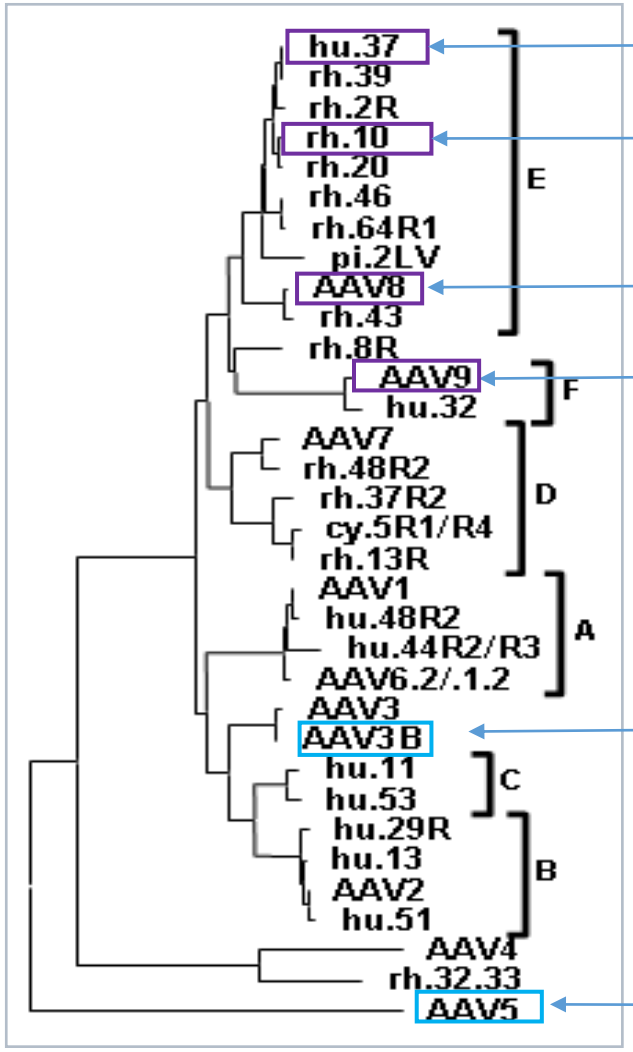
Different AAV Capsids Have Different Tropisms





- Tropism is not absolute
- Route of administration can overcome inherent tropism
- Prevalence of anti-capsid antibodies is a major consideration
  - Lack of precision on differences between capsids
  - Extremes are AAV2 with high antibody prevalence and AAV5 with low antibody prevalence

Current Products Based on Clade E Family Capsids



DTX201, AAVhu37

DTX101, AAVrh10

UX301, 401, AAV8

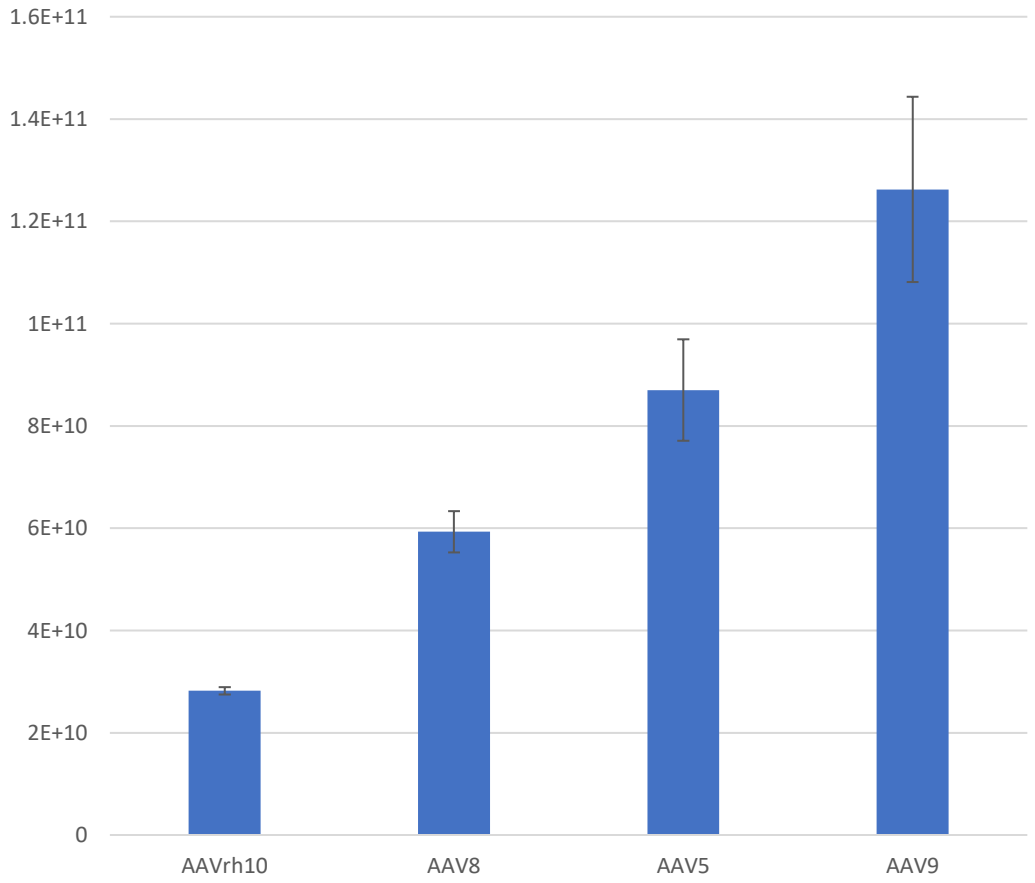
Undisclosed, AAV9

Ultragenyx Programs

Spark

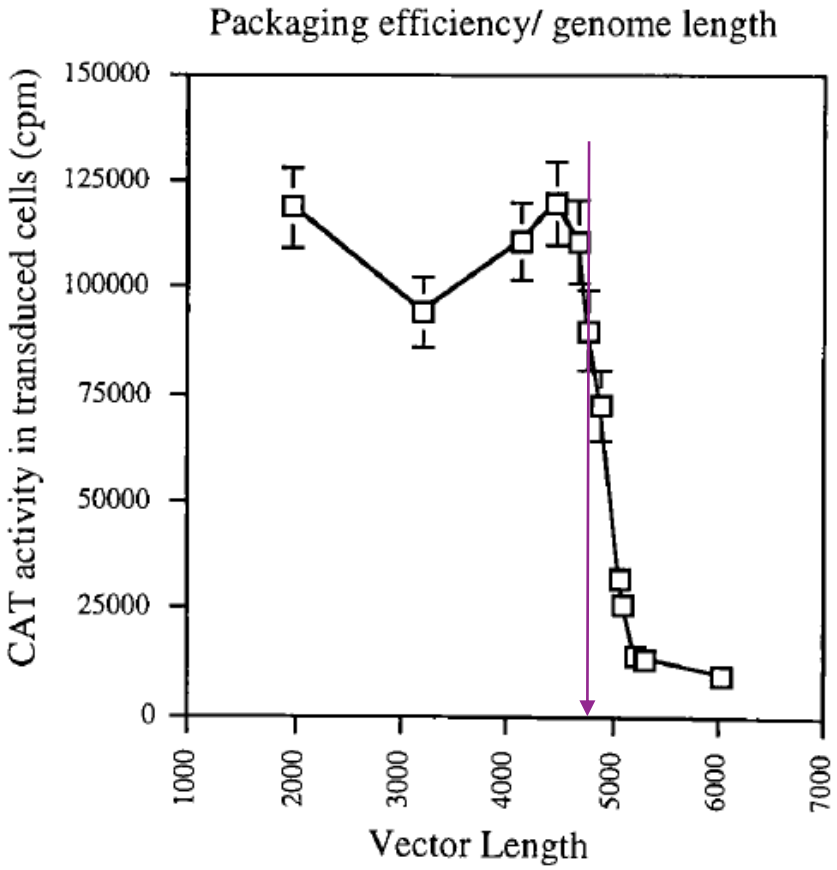
uniQure, BioMarin

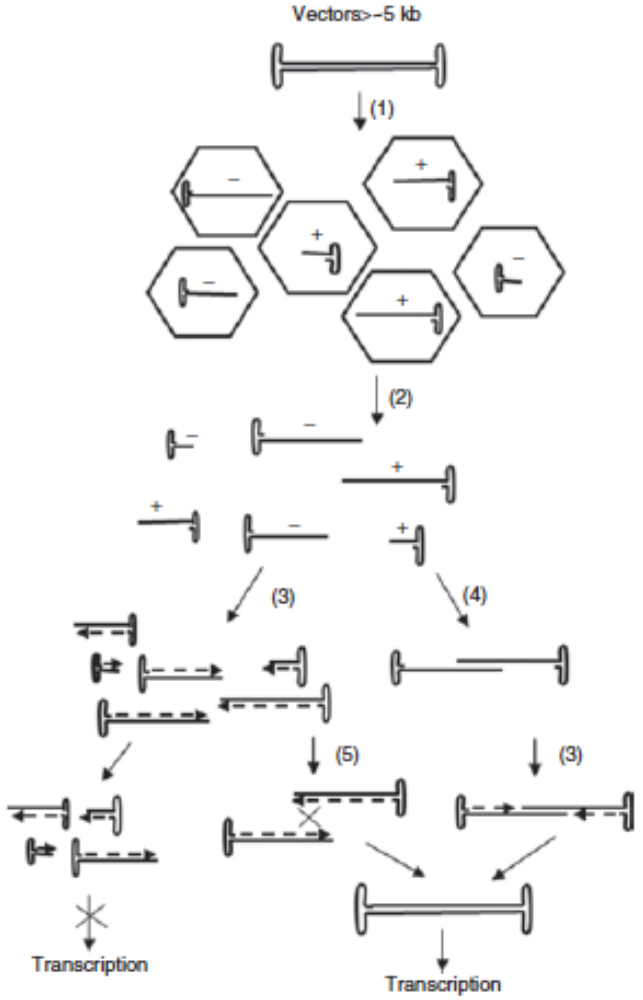
Different Capsids May Have Manufacturing Advantages



- Packaging of 4 different vector genomes evaluated in 4 different capsids
- Certain downstream unit operations are common in vector purification, others are related but are fine-tuned

AAV Genome Forms – Reduced Packaging Efficiency & Expression Efficiency for Oversized Genomes





CNS AAV Gene Therapy

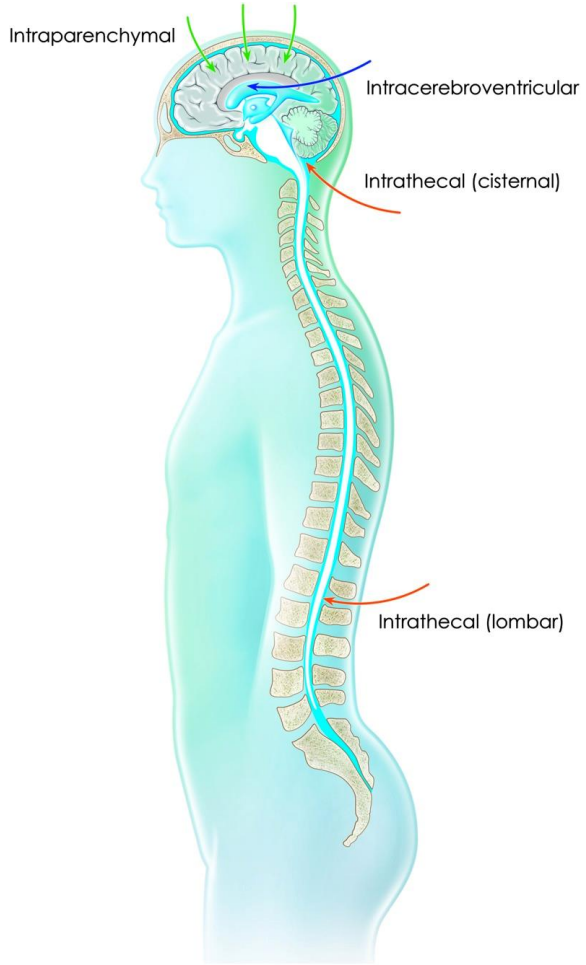
A number of viral vector-mediated phase I/II clinical trials have been initiated to treat neurologic disorders

Table 2
Viral vector-mediated clinical trials for neurological disorders.

Disease	Vector	Transgene	Phase	Trial code
Ex vivo				
Alzheimer's disease	Retrovirus	NGF	I	US-0322
Metachromatic leukodystrophy	Lentivirus	ARSA	I, II	Biffi et al., 2013
Multiple sclerosis	Retrovirus	MBP	I, II	US-0851
Wiskott-Aldrich syndrome	Lentivirus	WASP	I, II	Aiuti et al., 2013
X-linked adrenoleukodystrophy	Lentivirus	ABCD1	I, II	Cartier et al., 2009
In vivo				
AADC deficiency	AAV	AADC	I, II	NCT01395641
Alzheimer's disease	AAV	NGF	I, II	NCT00087789, NCT00876863
Batten disease	AAV	CLN2	I	NCT00151216
Batten disease	AAV	CLN2	I, II	NCT01414985
Canavan disease	AAV	ASPA	I	Leone et al., 2012
Giant axonal neuropathy	AAV	GAN	I	NCT02362438
Glioblastoma	Oncolytic poliovirus	–	I	NCT01491893
Glioblastoma multiforme (GBM), other gliomas	Oncolytic adenovirus	–	I	NCT00805376, NCT01956734, NCT02197169
Glioblastoma multiforme, other gliomas	Retrovirus	CD	I, II/III	NCT01470794, NCT02414165
Glioblastoma, other gliomas	Oncolytic HSV	–	I	NCT02031965
Glioblastoma, other gliomas	Oncolytic HSV	–	I	NCT00028158, NCT00157703
Leber's hereditary optic neuropathy	AAV	MT-ND4	I	NCT02161380
Metachromatic leukodystrophy	AAV	ARSA	I, II	NCT01801709
MPS IIIA (Sanfilippo Disease Type A)	AAV	SGSH, SUMF1	I, II	NCT01474343, NCT02053064
Parkinson's disease	AAV	GAD	I, II	NCT00195143, NCT00643890
Parkinson's disease	AAV	NTRN	I, II	NCT00252850, NCT00400634
Parkinson's disease	Lentivirus	TH, AADC, CH1	I, II	NCT00627588, NCT01856439
Parkinson's disease	AAV	GDNF	I	NCT01621581
Parkinson's disease	AAV	AADC	I, II	NCT02418598
Parkinson's disease	AAV	AADC	I	NCT00229736
Pompe disease	AAV	GAA	I, II	NCT00976352
Pompe disease	AAV	GAA	I	NCT02240407
Spinal muscular atrophy type 1	AAV	SMN	I	NCT02122952

S.R. Choudhury et al., Neuropharmacology, 2017
Review – Viral vectors for therapy of neurologic diseases

Potential delivery sites for CNS AAV gene therapy

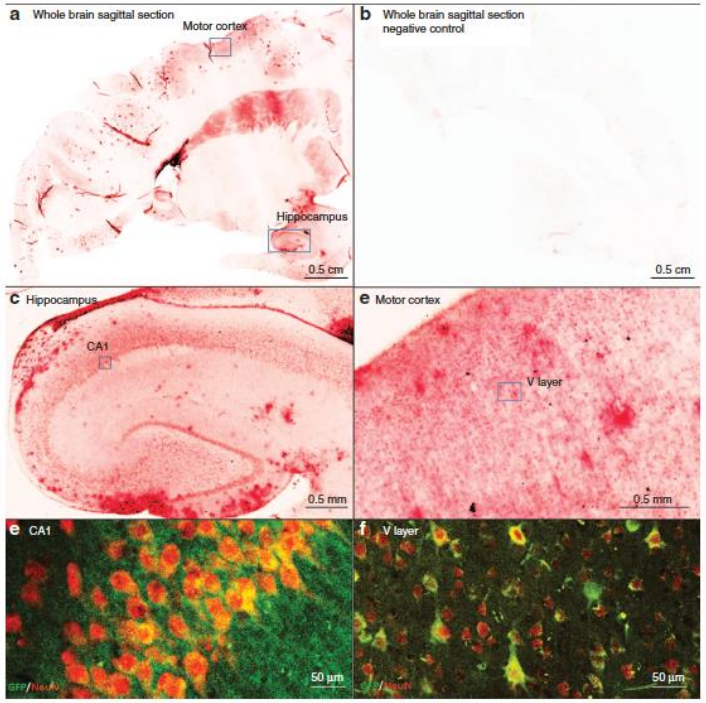


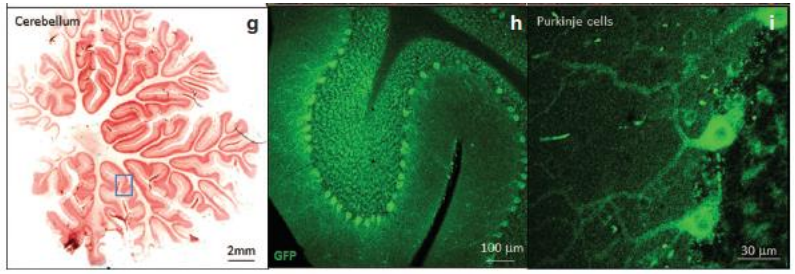
- **Global delivery**
  - IV: easiest delivery, requires high vector doses, and may not target sufficient cells in regions of interest due to low penetration of BBB
- **CSF based delivery**
  - ICV, IT (cisternal or lumbar): potentially challenging delivery method, may not reach deep brain structures, but will target a higher % of neurons compared to IV at a similar dose
- **Intraparenchymal delivery**
  - Potentially target a large percentage of neurons but only in a select area

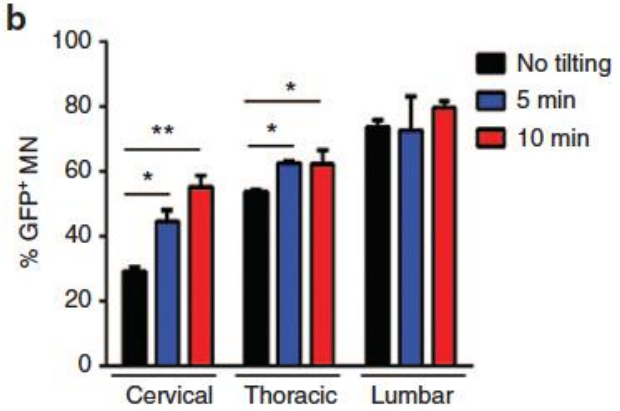
IT delivery of AAV9-GFP results in expression throughout the brain

Meyer et al., 2015. *Molecular Therapy* (Brian Kaspar's lab)

- scAAV9-CBA-GFP was delivered to n=5, **1 yr old NHPs (cynos)** via sacral-IT (**1x10^{13} vg/kg**) – used Trendelenburg position (head tilted down by 15-30 degrees for 10 min following infusion)
- Animals were sacrificed after 2 weeks
- **GFP was noted in all regions of the brain, with particularly strong signal in the hippocampus, motor cortex and cerebellum**



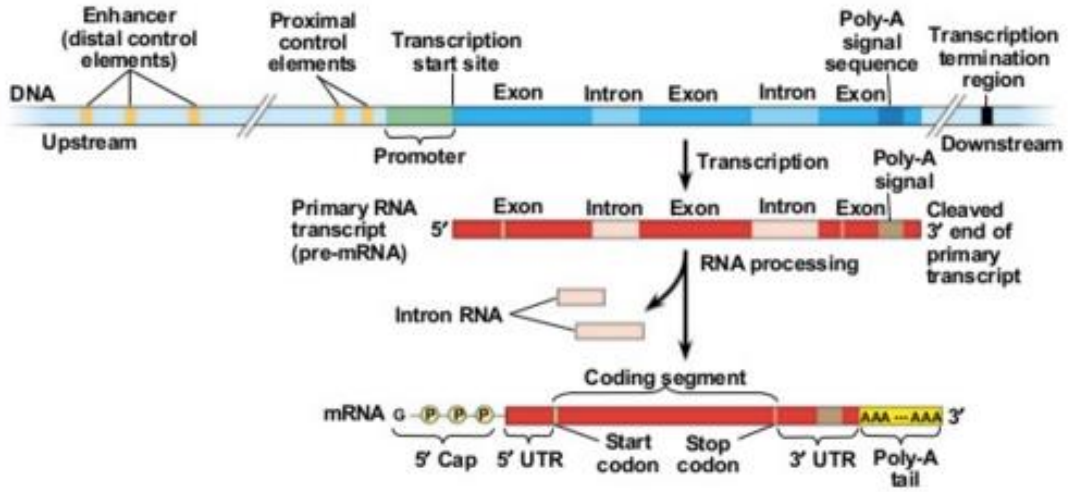




Note: others have reported similar results but with less robust brain delivery

DNA Component: Molecular Engineering to Squeeze Genes into the AAV Genome Limit (5 -5.4 kb)

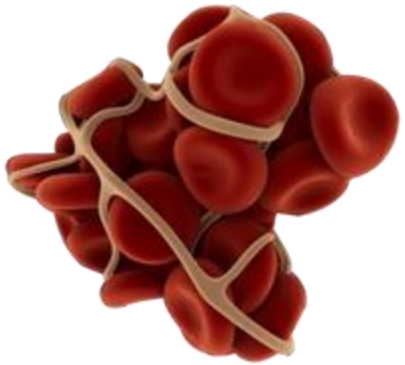
- Most therapeutic gene coding sequences will use their minimal cDNA format
- Overstuffing capsid has negative yield and quality consequences
- Additional elements, such as introns & 5’ and 3’ UTR sequences, can sometimes be included
- Greatest technical challenge is engineering small enhancer and promoter combinations to achieve tissue specific gene expression



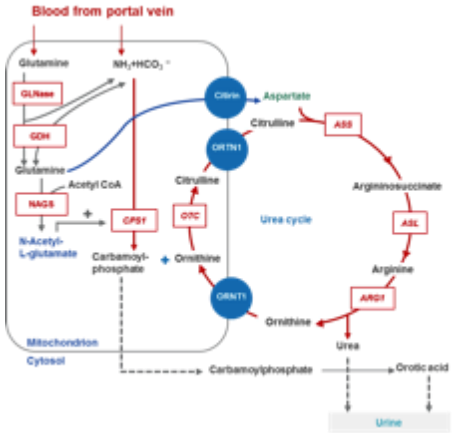
Targeting the Liver – A Master Hub for Rare Diseases

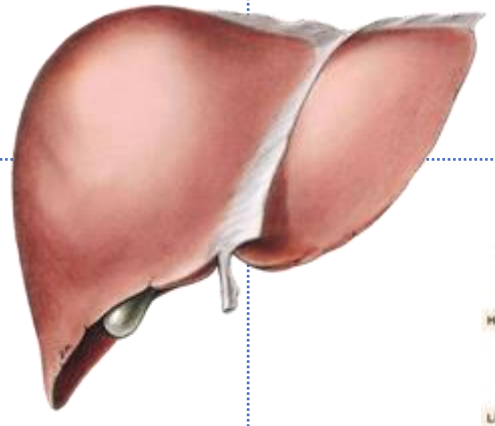
RARE ENTREPRENEUR BOOTCAMP

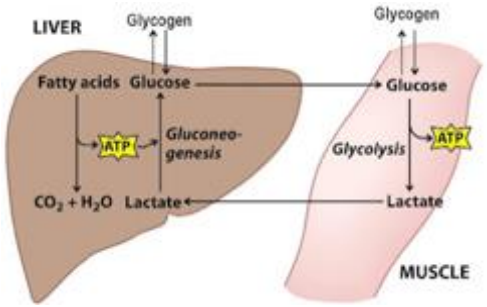
Hemophilia



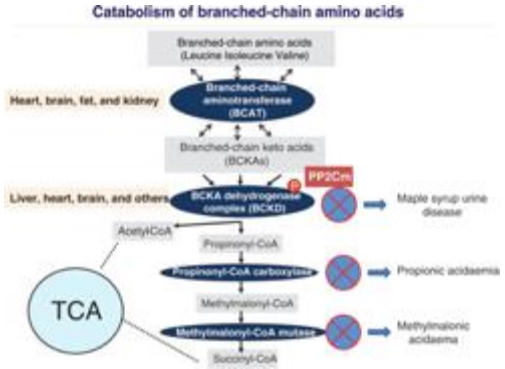
Urea Cycle Disorders





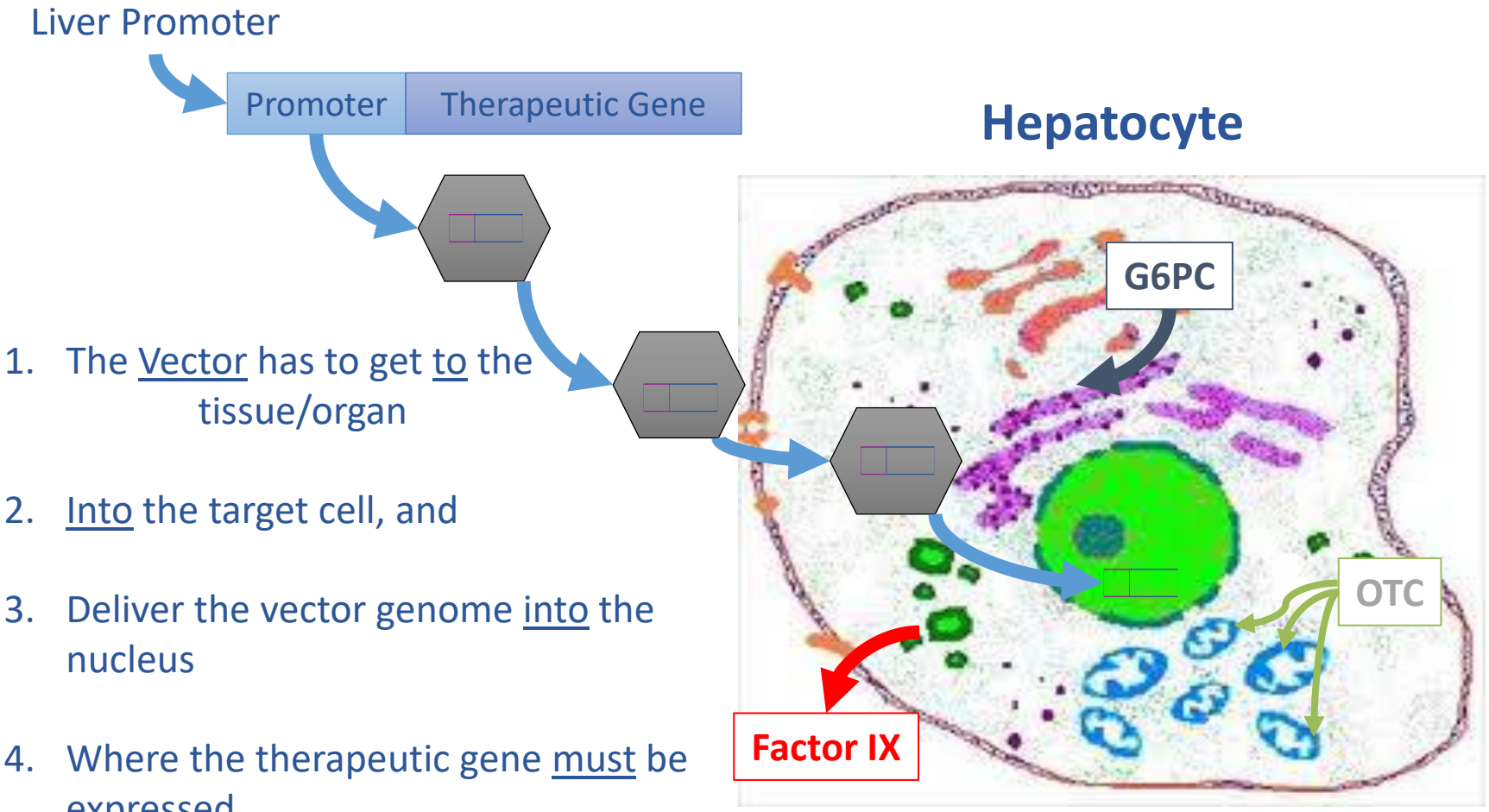


Storage Disorders



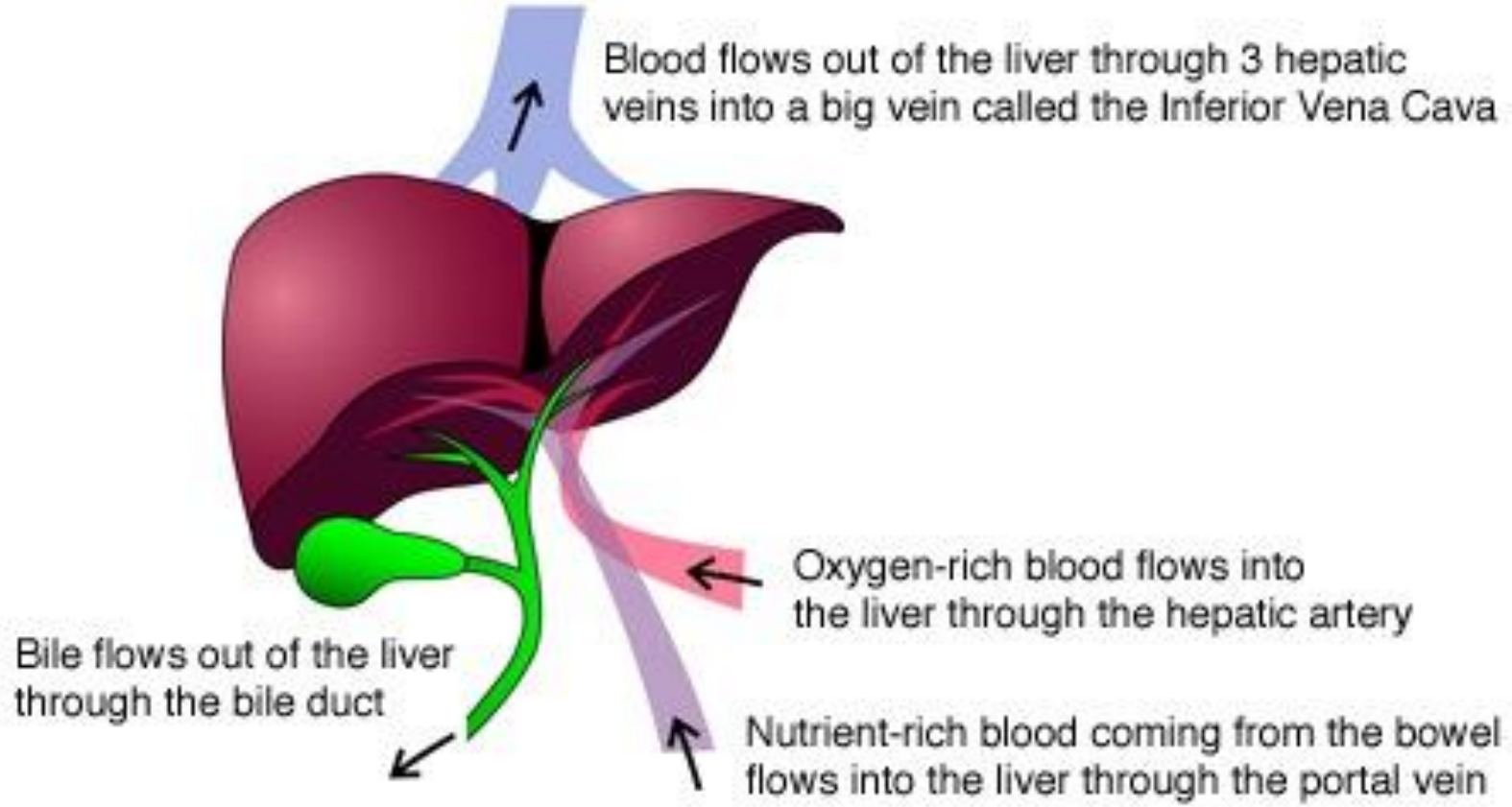
Aminoacidopathies

Gene Therapy is a Multi-Step Process

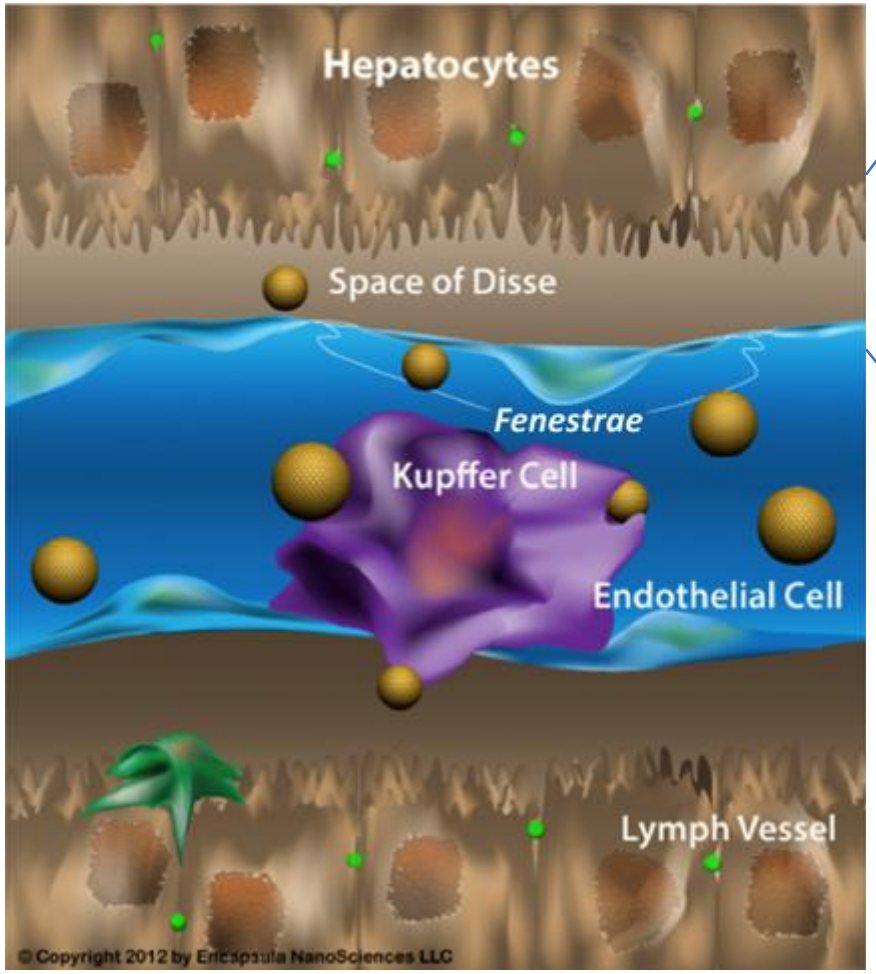


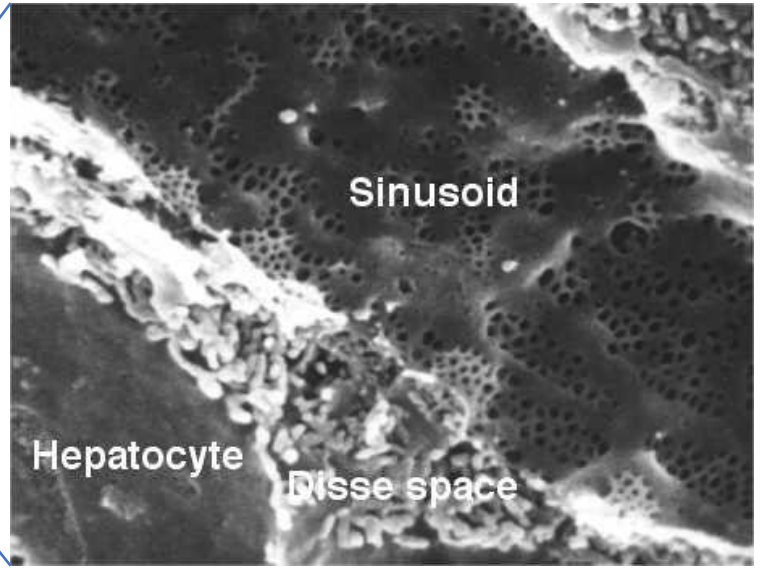
1. The Vector has to get to the tissue/organ
2. Into the target cell, and
3. Deliver the vector genome into the nucleus
4. Where the therapeutic gene must be expressed

Blood Flows Through the Liver at 1.5L per min



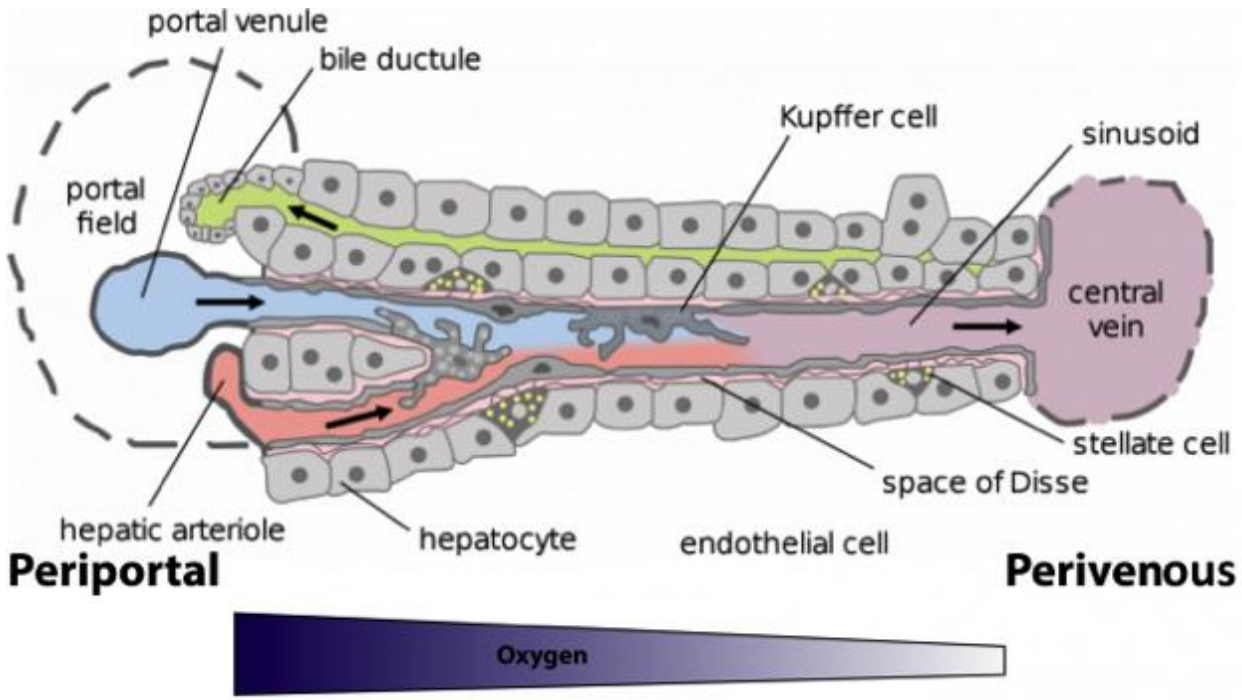
Liver Sinusoidal Endothelium Is Fenestrated



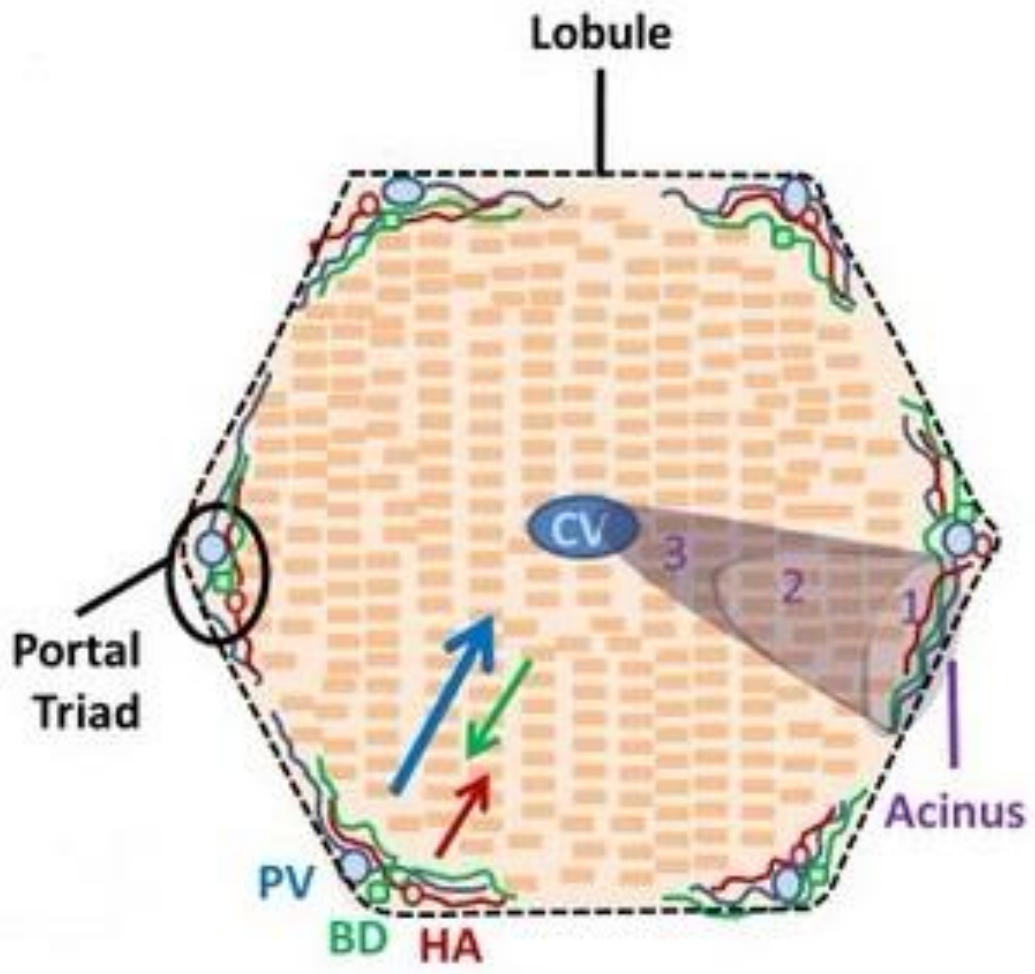


Hepatocytes Are Divided Into Functional Zones

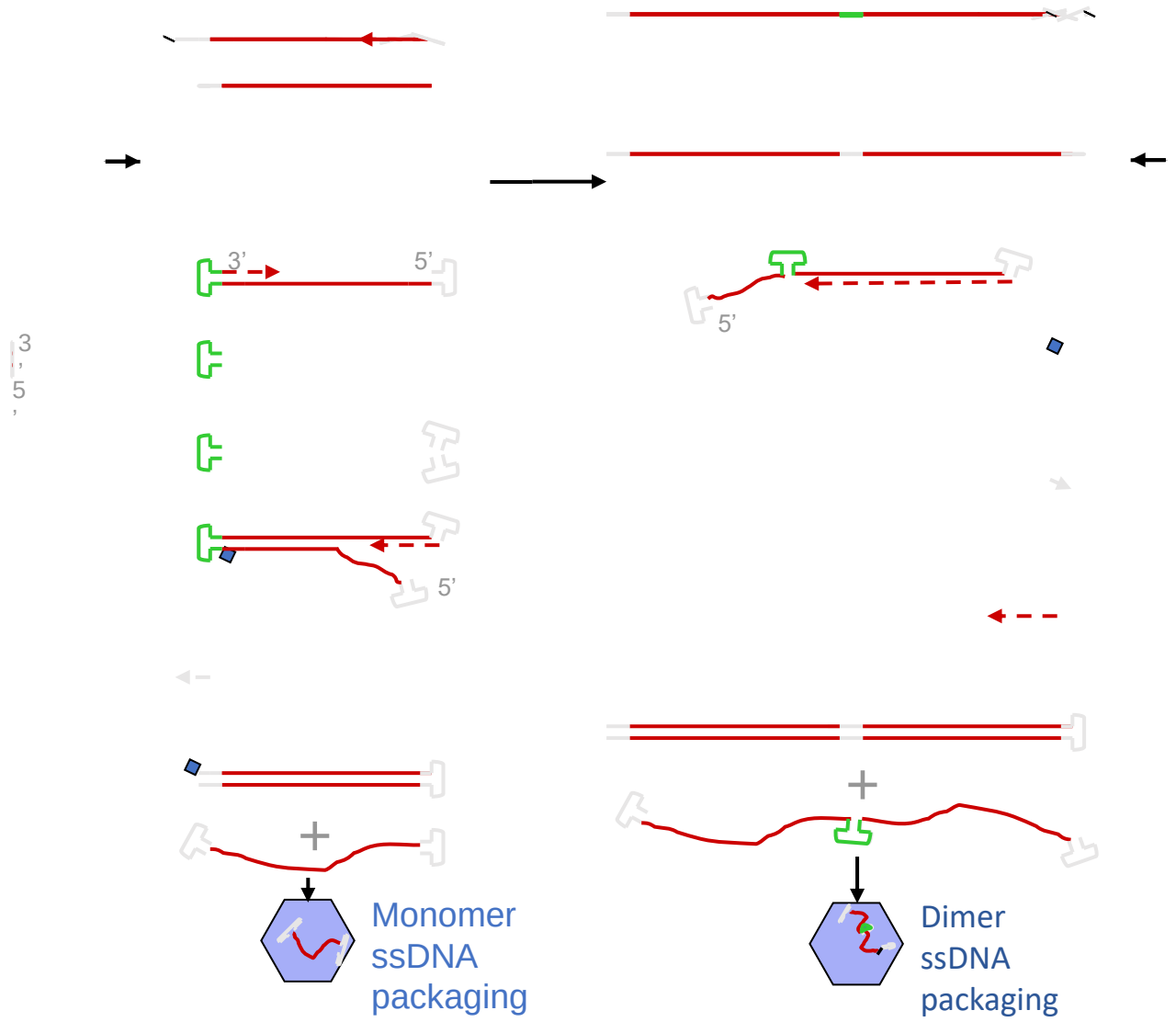
High O2 processes **Low O2 processes**



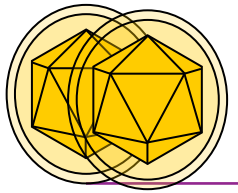
Functional Zones of the Liver (the big picture)



AAV Genome Forms – Single Strand versus Self Complementary

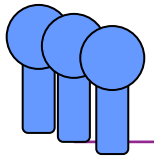


AAV Vector Manufacturing – HSV Advantages and Limitations

	Scalability	Serum Free	Product Yield
HSV 	Bioreactor format enabled, scale unknown (100 to 200L ??)	Yes/No	Good on per cell basis, moderate on per batch basis

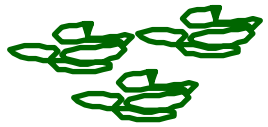
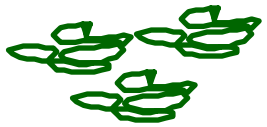
- Comparability to other mammalian platforms good
- Key features/bugs – Good product quality, easy removal of rHSV/Limited by stability of rHSV, requires 2 rHSV vectors as complex GMP raw material supply train
- Alternate bioreactor format would be required to implement at Woburn; different format for vector engineering

AAV Vector Manufacturing – Baculo Advantages and Limitations

	Scalability	Serum Free	Product Yield
Baculo 	Stated as 2000L	Yes	Good on per cell basis, very good on per batch basis

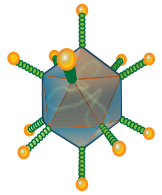
- Comparability to other mammalian platforms a question
- Key features/bugs – high yield & serum free, high cell density/requires 2 or 3 rBac viruses as supply chain, non-optimal stability of rBac (genetic & storage), complex molecular engineering required to support fidelity of AAV life cycle
- EMA Glybera Assessment highlights several gaps in CMC
- Alternate bioreactor format would be required to implement at Woburn; different format for vector engineering

AAV Vector Manufacturing – HEK293 Advantages and Limitations

		Scalability	Serum Free	Product Yield
Plasmid Adherent		Poor	No	Good on per cell basis, low on per batch basis
Plasmid Suspension		Data to 200L, probably could go higher	Yes	Good on per cell basis, good on per batch basis

- Comparability to adherent 293 high
- Key features/bugs – HEK293 platform used for majority of AAV gene therapy trials/plasmid represents high GMP raw material burden
- Readily implemented at Woburn; leverage our core AAV science, support basic PD, de-risk CMO tech transfer

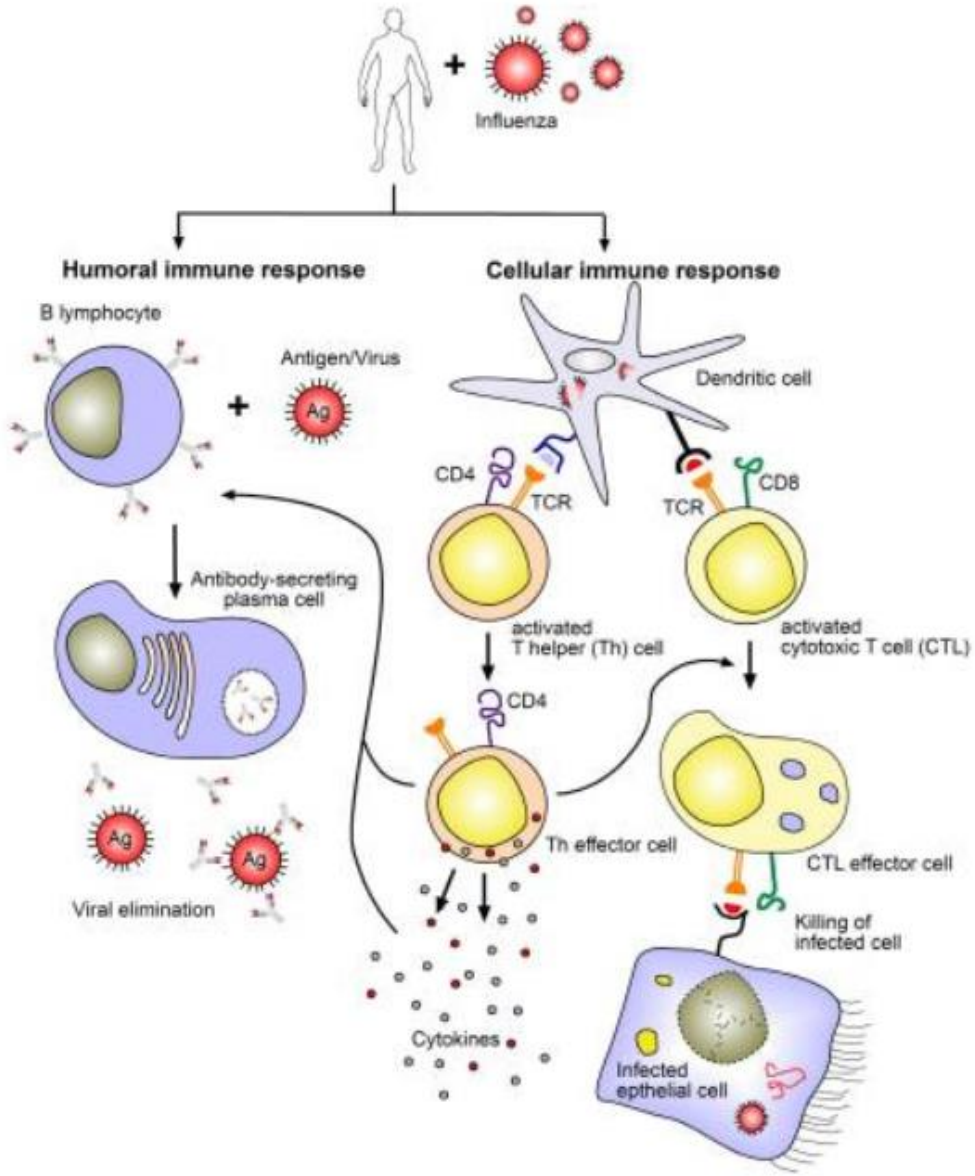
AAV Vector Manufacturing – HeLa Advantages and Limitations

	Scalability	Serum Free	Product Yield
 Ad/HeLa	Proven to 2000L Bioreactor	Yes	Very good on per cell basis, very good on per batch basis

- Comparability to other mammalian platforms good
- Key features/bugs – only clonal system enabling screen for desired features; E/F, rcAAV, other requires high titer Ad helper GMP raw material supply train
- Readily implemented at Woburn; leverage from our core AAV science, support basic PD, de-risk CMO tech transfer

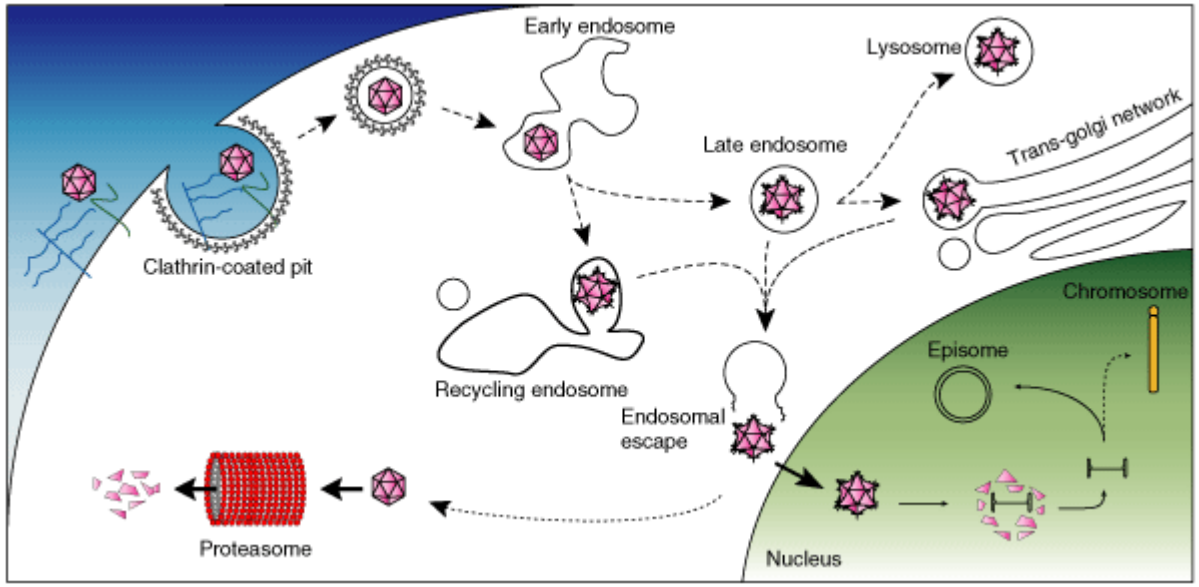
The Immune System – A Primer

- Two pathways – humoral and cellular
- Key signaling includes T-cell, B-cells and NK cells
- AAV vectors retain some viral coding that may be recognized as foreign
  - Capsid
  - Transgene
  - Genome

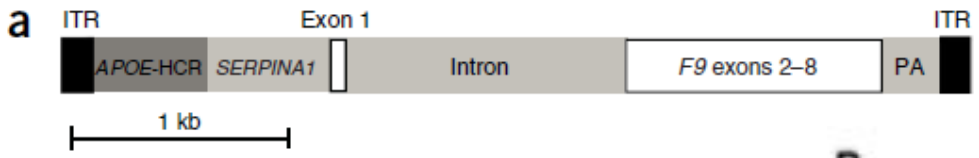


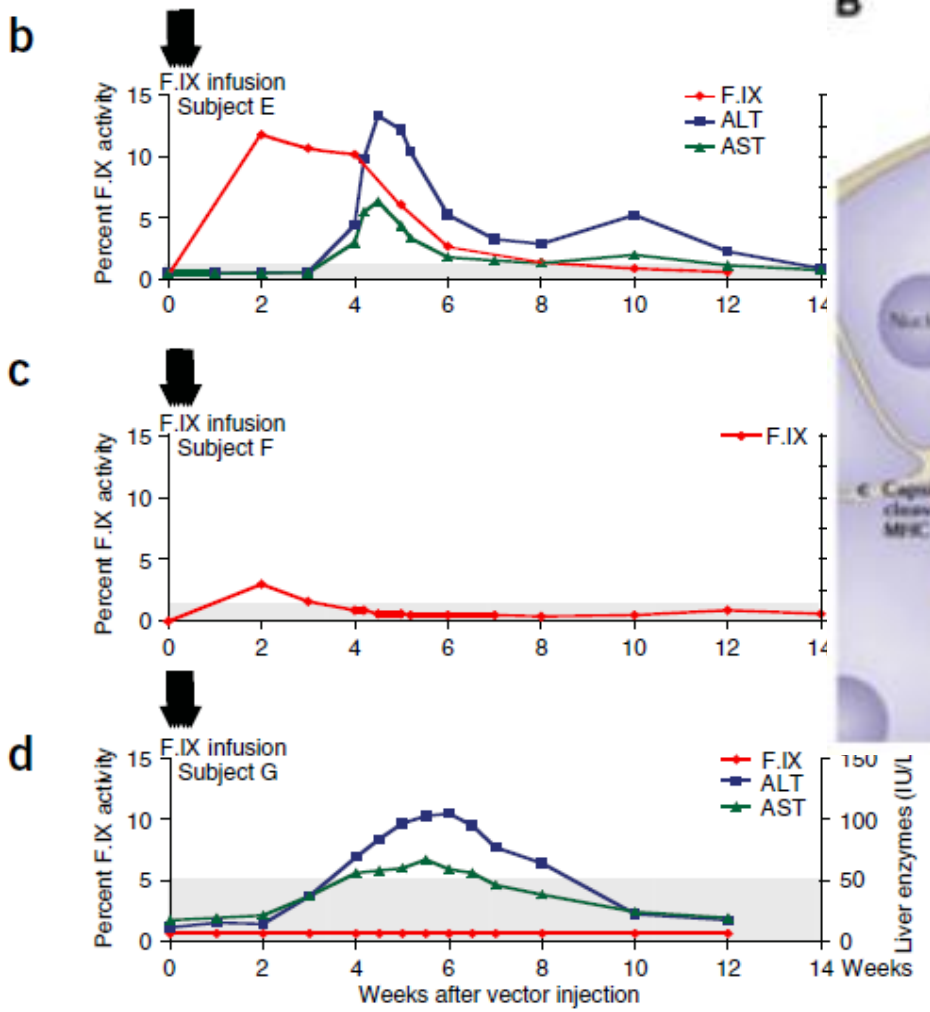
Potential Goals of immune intervention in AAV GT

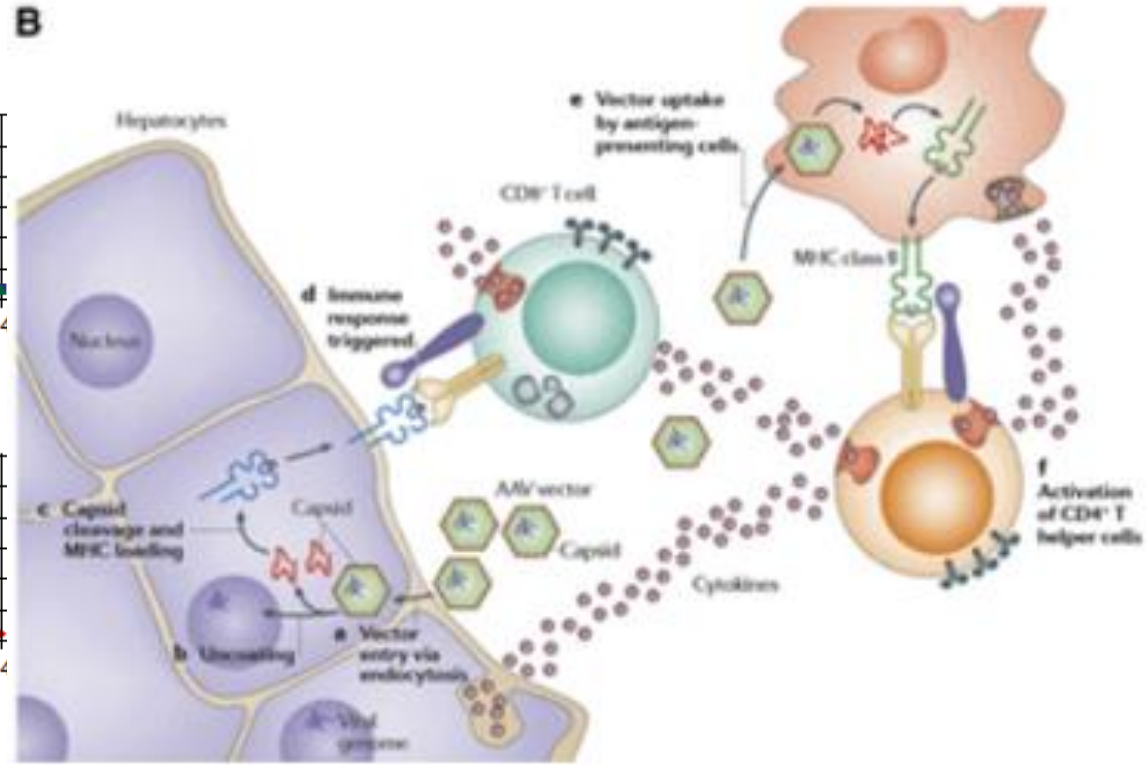
- T cell responses to capsid antigens?
- T cell responses to therapeutic protein?
- B cell responses to capsid antigens?
- B cell responses to therapeutic protein?



First Observation of a Reduction in AAV Gene Therapy – First Liver Delivered Hemophilia Trial







First Introduction of Steroids to Address Reduction in Efficacy – Nathwani 2011

- Steroids broadly suppress the immune system
- Nathwani's use of steroid was likely serendipitous
- Outcome was favorable and patients have sustained activity beyond 6 years