circio

Disruptive circRNA technology for genetic medicine

R&D webinar
29 November 2023

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Today's presenters

Dr Erik D Wiklund

Chief Executive Officer

Co-discoverer of circRNA, Pharma consultant at McKinsey & Co and various commercial and R&D roles in biotech, Previously CFO and CBO of Targovax

PhD Cancer epigenetics and RNA biology

Dr Victor Levitsky

Chief Scientific Officer

Deeply experienced tumor immunology scientist from academia and industry, incl Karolinska Institute, John´s Hopkins, Roche and Molecular Partners

MD, PhD Virology and tumor biology

Dr Thomas B Hansen

VP & Head of Research

World-leading pioneer and co-discoverer of circular RNA; 10 years as group leader at Aarhus University in RNA biology and bioinformatics

PhD Molecular and RNA biology

Dr Alexander Wesselhoeft

Dir of RNA Therapeutics, Mass General Brigham

Circular RNA pioneer and founder of ORNA Therapeutics

Optimized circular RNA for in vivo protein expression

PhD Molecular and RNA biology

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Agenda

1	Introduction Dr Erik Digman Wiklund - CEO	15 min

2	Circular RNA Technology - Advances and Challenges Dr R Alexander Wesselhoeft – Dir of RNA Therapeutics, Mass General Brigham	30 min

3	circVec technology overview Dr Thomas B Hansen – VP & Head of Research	20 min

4	Development Strategy Dr Victor Levitsky- CSO	15 min

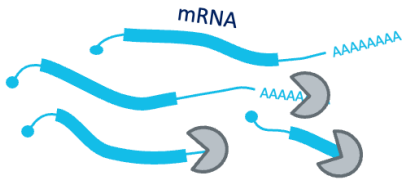
1

Introduction

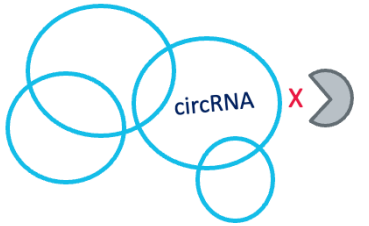
Dr. Erik Digman Wiklund, CEO

circRNA will disrupt gene therapy and vaccines by improving potency and adding novel functionality

Extended RNA durability



Enhanced protein expression

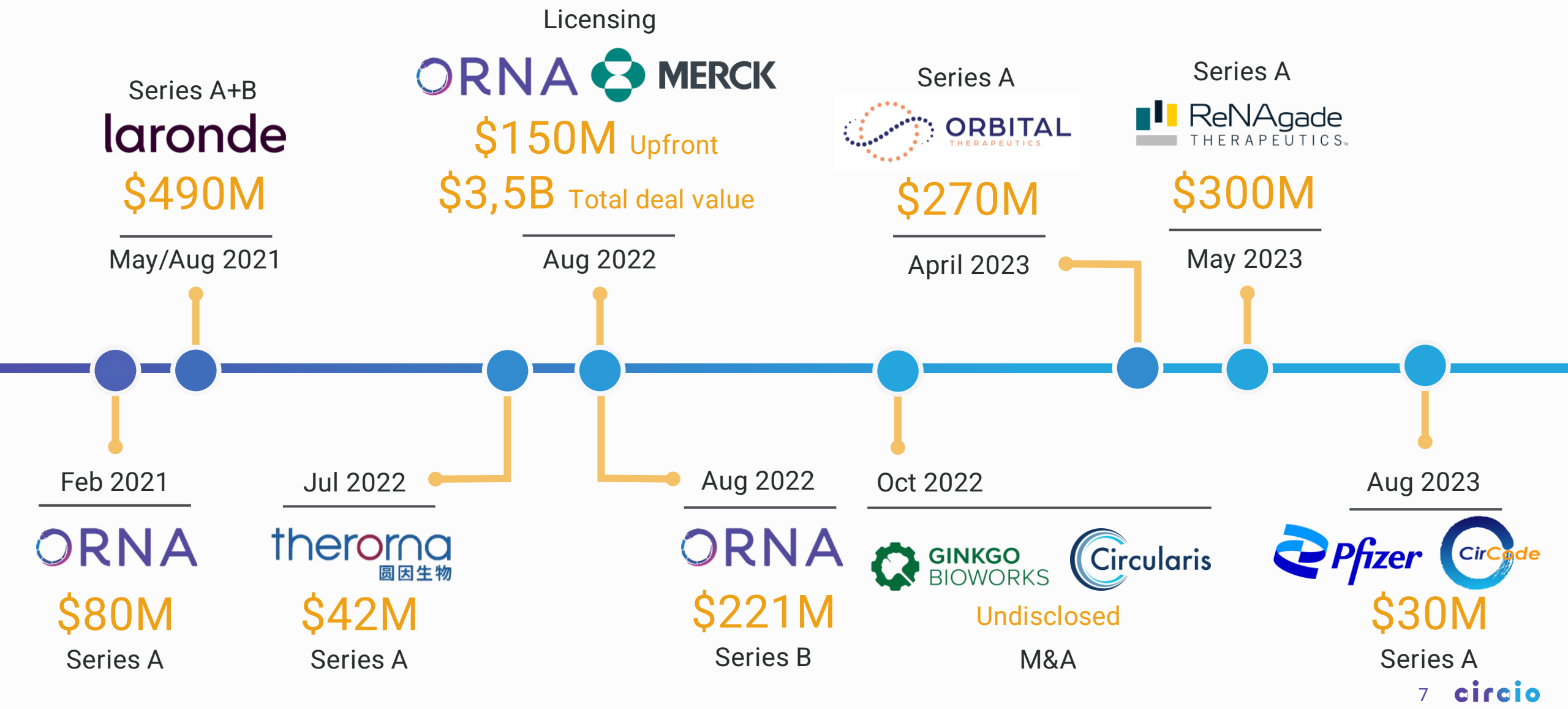


circRNA is resistant to exonuclease degradation, leading to significantly prolonged half-life vs. mRNA within cells

microRNA sponging

Regulatory functionality

circRNA is gaining momentum as a superior mRNA platform



The discoverers of circRNA work for Circio

Dr Thomas B Hansen

Dr Erik D Wiklund

nature — 6,373 citations

Published: 27 February 2013

Natural RNA circles function as efficient microRNA sponges

Thomas B. Hansen, Trine I. Jensen, Bettina H. Clausen, Jesper B. Bramsen, Bente Finsen, Christian K. Damgaard & Jørgen Kjems

THE EMBO JOURNAL — EMBOpress — 30 September 2011 — 922 citations

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miRNA-dependent gene silencing involving Ago2-mediated cleavage of a circular antisense RNA

Thomas B Hansen, Erik D Wiklund, Jesper B Bramsen, Sune B Villadsen, Aaron L Statham, Susan J Clark, Jørgen Kjems

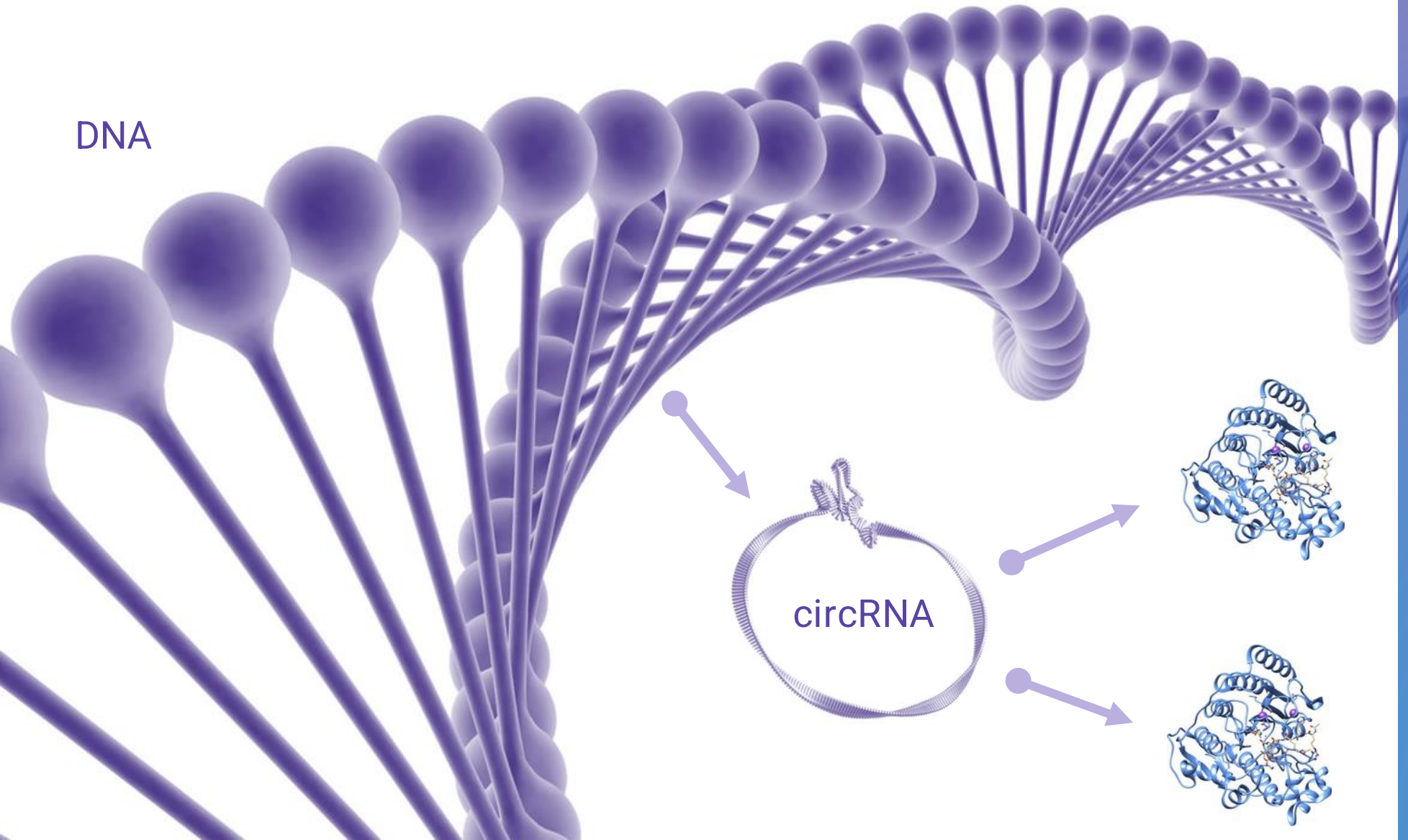
nature reviews genetics — 2,291 citations

Review Article | Published: 08 August 2019

The biogenesis, biology and characterization of circular RNAs

Lasse S. Kristensen, Maria S. Andersen, Lotte V. W. Stagsted, Karoline K. Ebbesen, Thomas B. Hansen & Jørgen Kjems

circVec – Circio`s proprietary vector system for intra-cellular protein expression



circVec
DNA or viral vector

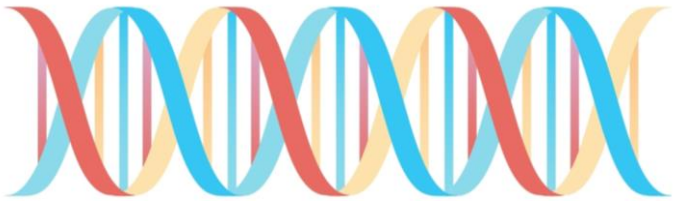
Inject

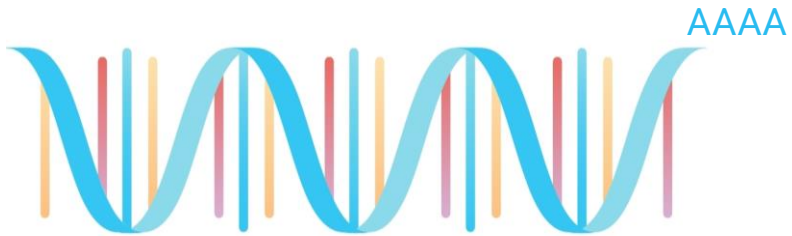
circRNA biogenesis

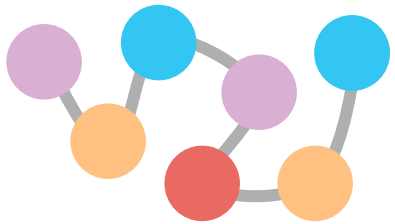
Intra-cellular protein expression

The central dogma of molecular biology

DNA → TRANSCRIPTION → mRNA → TRANSLATION → PROTEIN







- Genetic Code
- Recipe for Proteins
- Durable (“eternal”) in the cell nucleus

- Instruction to generate protein
- Transient: 6-24h in cell cytoplasm

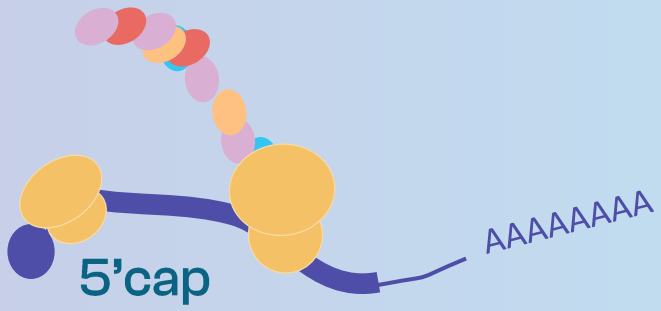
- Performing specific function
- Can be structural or chemical

With new technology, mRNA can be made circular

mRNA

Circular RNA

With new technology, mRNA can be made circular



mRNA

- 5'cap-dependent initiation of translation
  - Rate-limiting step
- Short half-life
  - Hours to days
- 5`cap & 3'poly-A tail – accessible for exonuclease degradation

Circular RNA

- IRES-mediated initiation (Internal Ribosome Entry Site)
  - More efficient than 5' cap-dependent initiation
- Extended half-life
  - Days to weeks
- No 3' or 5' end → exonuclease resistance

2

KOL presentation

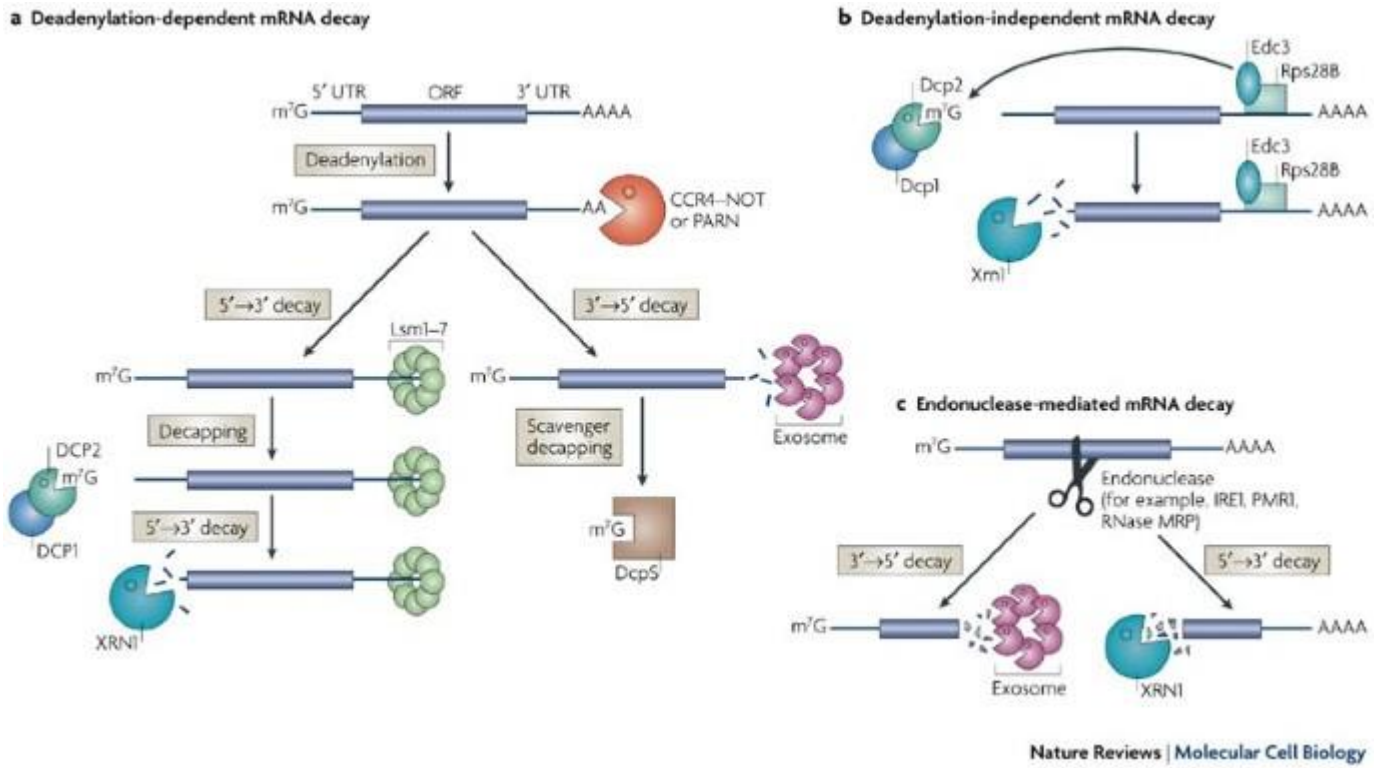
Dr. Alexander Wesselhoeft

Circular RNA Technology: Advances and Challenges

R. Alexander Wesselhoeft

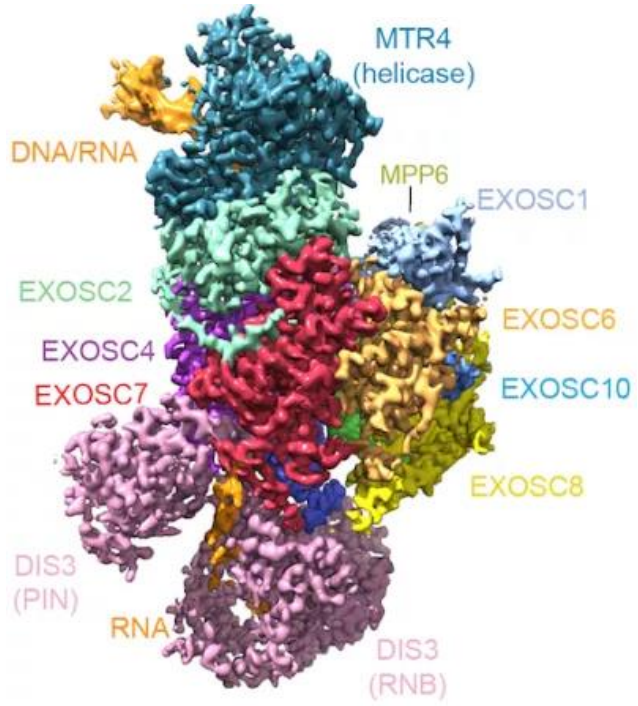
Director of RNA Therapeutics, Gene and Cell Therapy Institute, MGB

Why circularize RNA?



Garneau et al, 2020

Human exosome complex

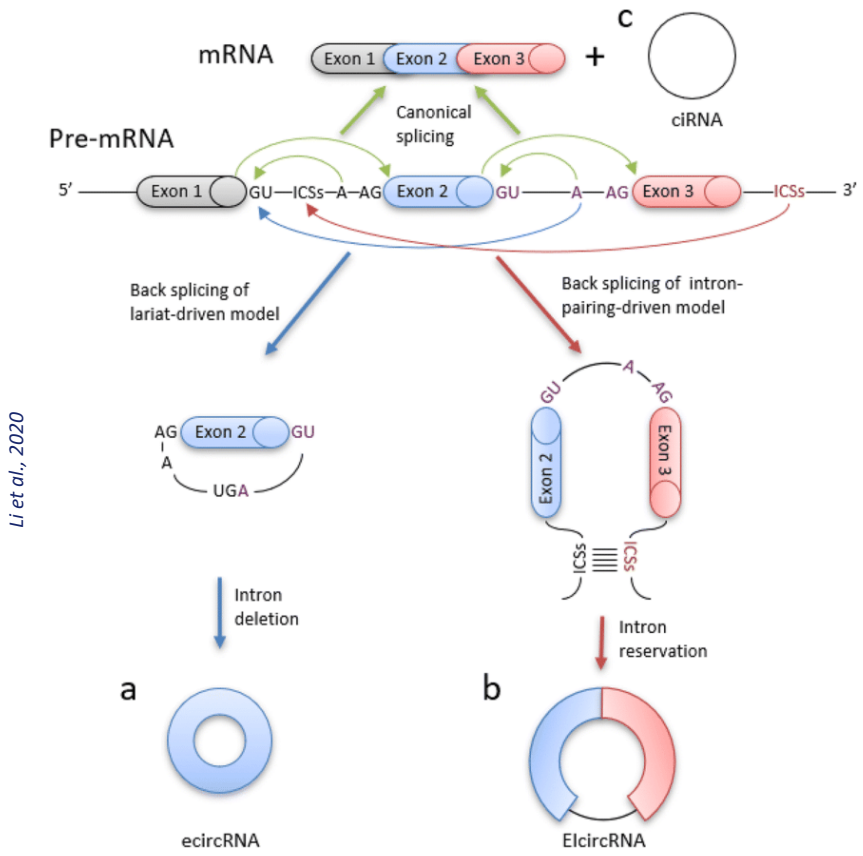


Lima Lab

Circularization → More protein per RNA → Dosing advantage → Better therapeutic index

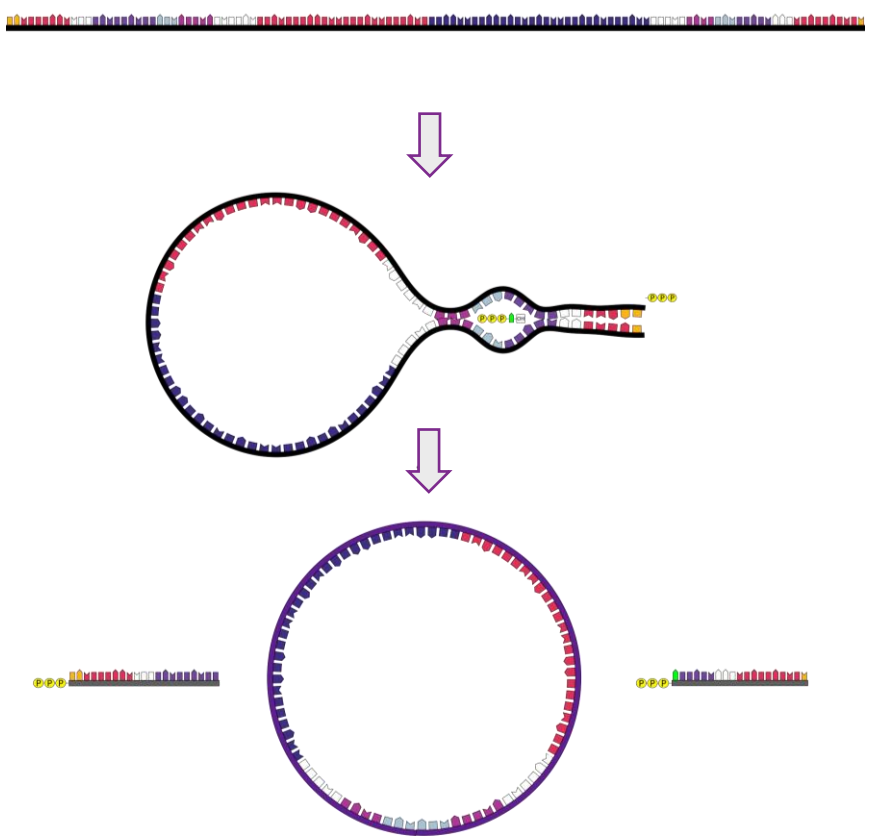
Two types of circles

DNA format Circular RNA



Li et al., 2020

RNA format Circular RNA



Key scientific milestones in the circRNA field

1991

Cell

CellPress

Volume 64, Issue 3, 8 February 1991, Pages 607-613

Article

Scrambled exons

Janice M. Nigro *, Kathleen R. Cho * †, Eric R. Fearon * ‡, Scott E. Kern * †, J.Michael Ruppert * †, Jonathan D. Oliner * ‡, Kenneth W. Kinzler *, Bert Vogelstein * ‡

2013

nature

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nature › letters › article

Published: 27 February 2013

Natural RNA circles function as efficient microRNA sponges

Thomas B. Hansen, Trine I. Jensen, Bettina H. Clausen, Jesper B. Bramsen, Bente Finsen, Christian K. Damgaard & Jørgen Kjems

2018

RNA Circularization Diminishes Immunogenicity and Can Extend Translation Duration *In Vivo*

R. Alexander Wesselhoeft,[1,2] Piotr S. Kowalski,[1,3] Frances C. Parker-Hale,[2,4] Yuxuan Huang,[1] Namita Bisaria,[5] and Daniel G. Anderson[1,3,6,7,8,*]

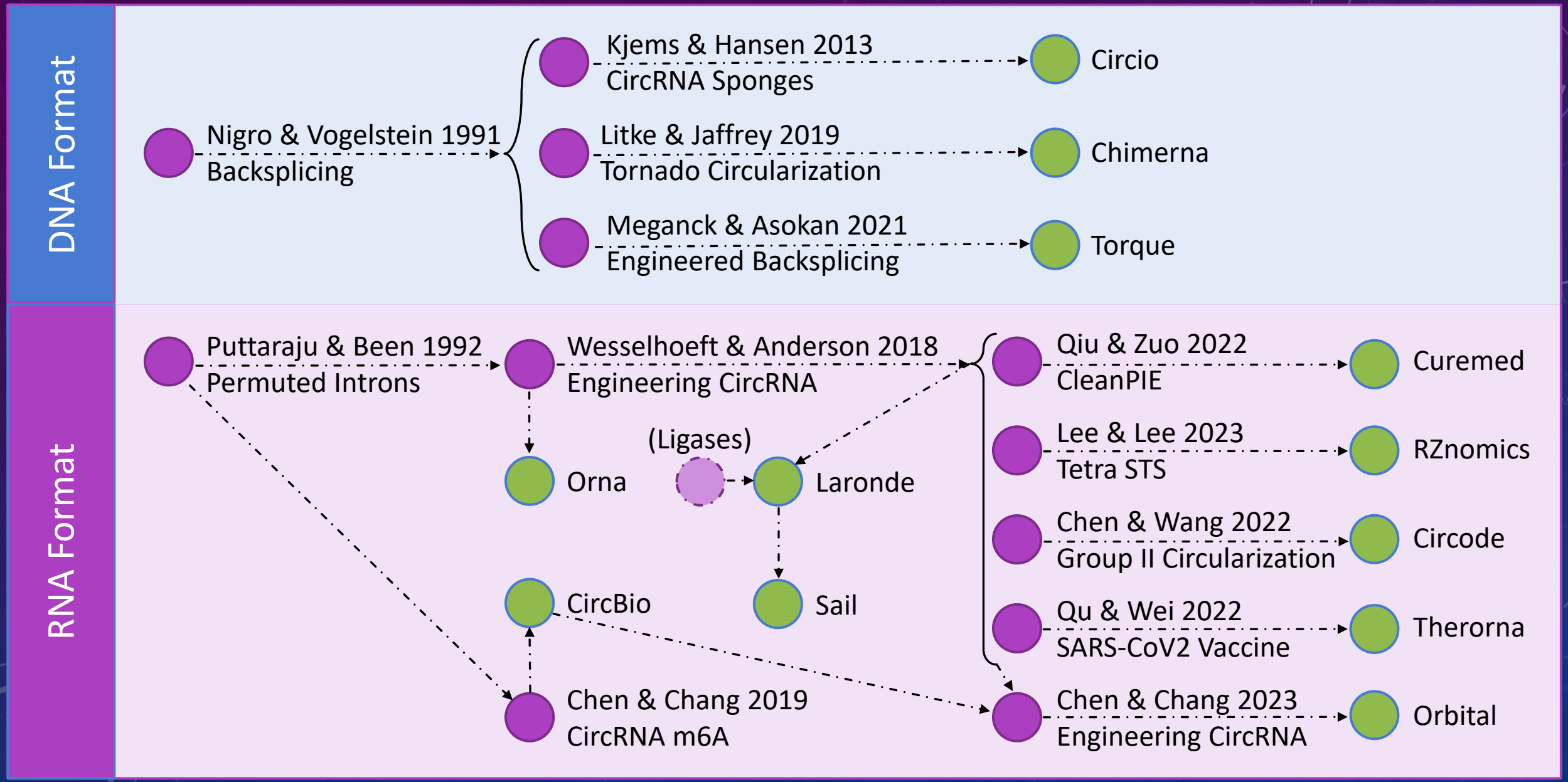
[1]David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02142, USA
[2]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142, USA
[3]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02142, USA
[4]Department of Political Science, Massachusetts Institute of Technology, Cambridge, MA 02142, USA
[5]Whitehead Institute for Biomedical Research, Cambridge, MA 02142, USA
[6]Institute for Medical Engineering and Science, Massachusetts Institute of Technology, Cambridge, MA 02142, USA
[7]Harvard and MIT Division of Health Science and Technology, Massachusetts Institute of Technology, Cambridge, MA 02142, USA
[8]Lead Contact
*Correspondence: dgander@mit.edu
https://doi.org/10.1016/j.molcel.2019.02.015

First natural circular RNA identified

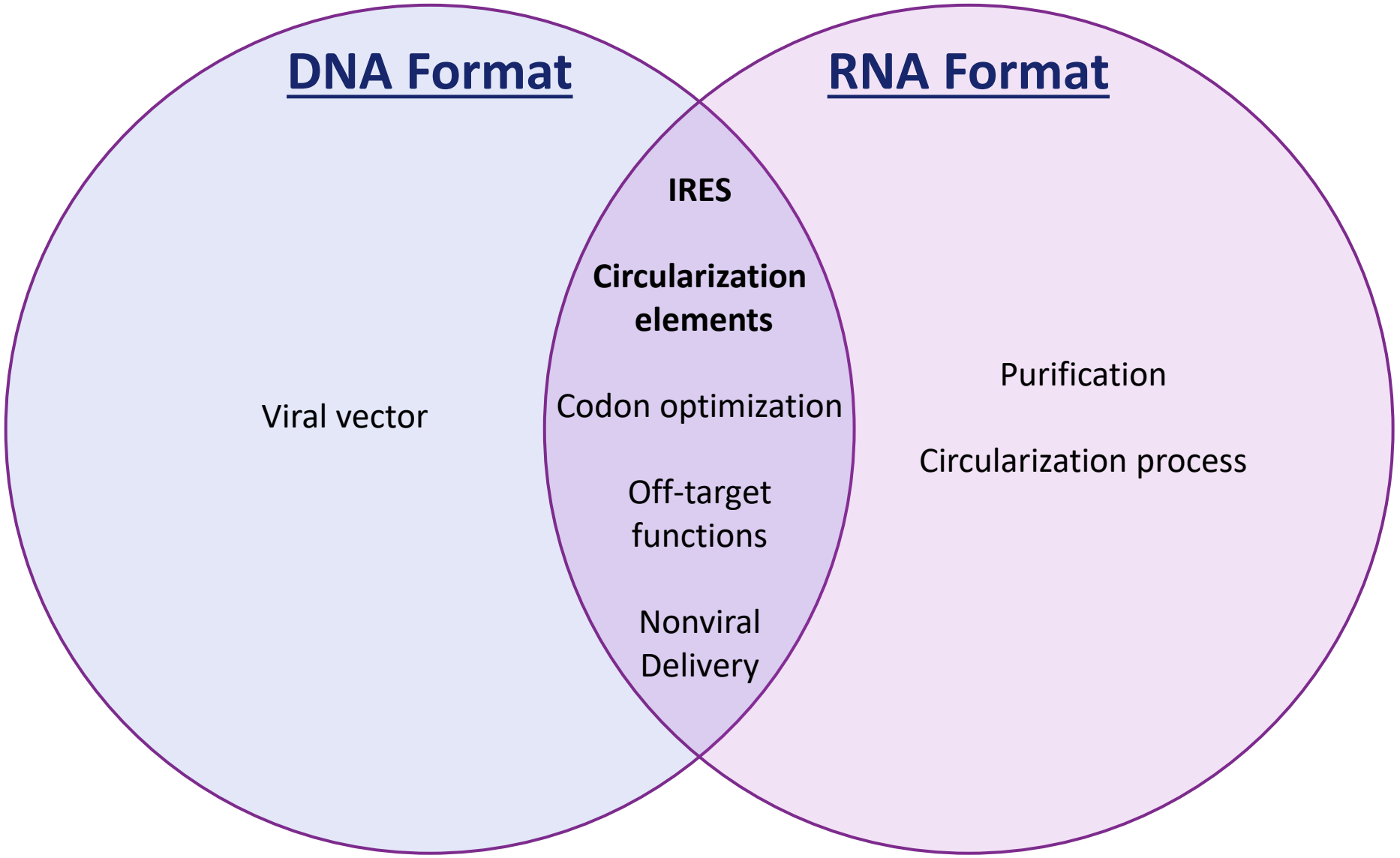
First functional characterization of circRNA

First *in vivo* protein expression from engineered circRNA

Circular RNA translation to industry

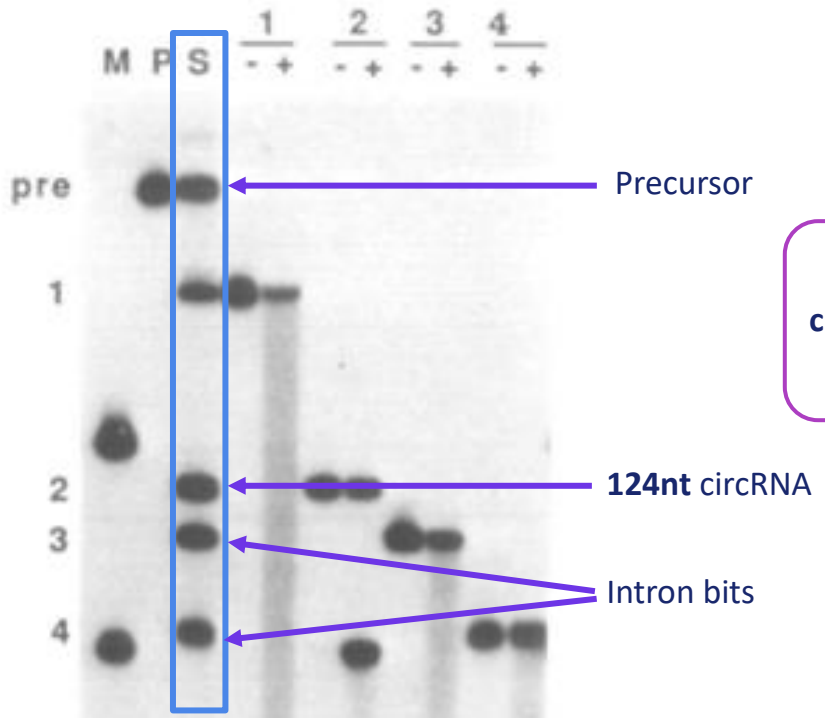


Different platforms, similar challenges



Challenge 1: Circularization

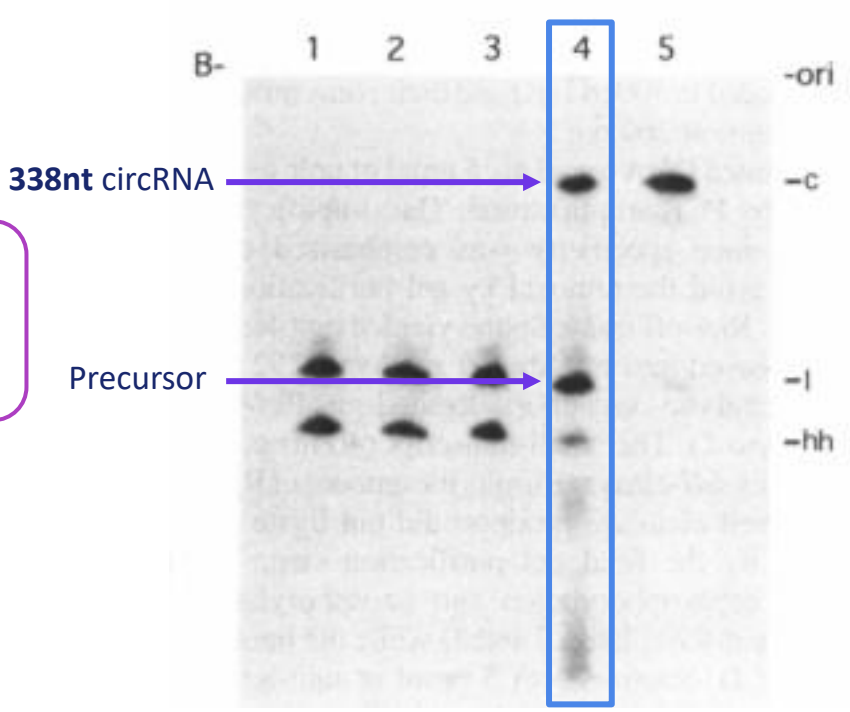
Permuted intron



Puttaraju and Been, 1992

~50% circularization efficiency

Enzymatic ligation

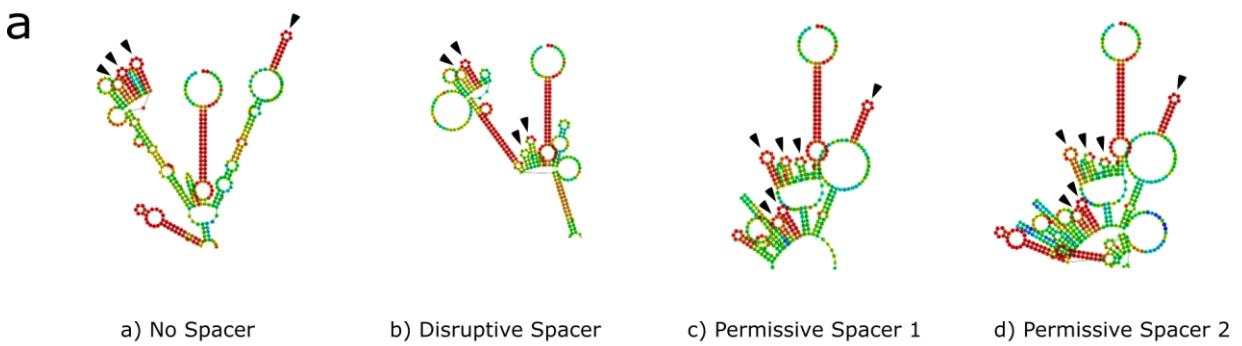


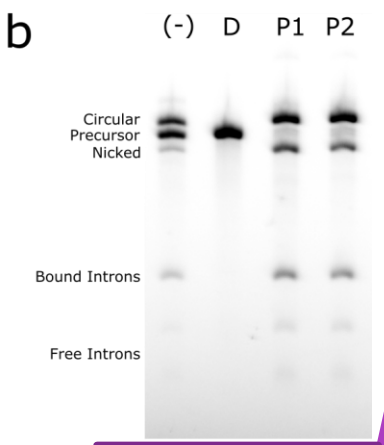
Beaudry and Perreault, 1995

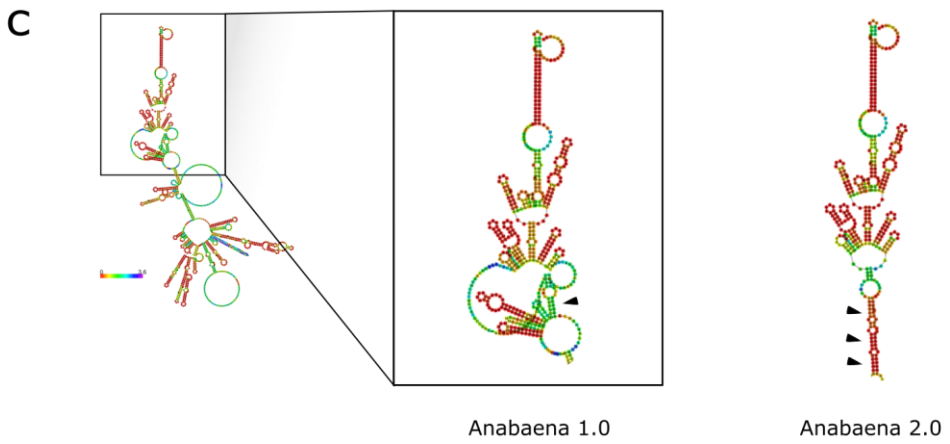
- RNA circularization is not a new idea
- There were multiple attempts at circularizing RNA in the 1990s, with a particularly interesting permuted intron strategy introduced by Puttaraju and Been in 1992
- Efficiencies were low even for small RNAs; mRNA circularization was largely unattainable

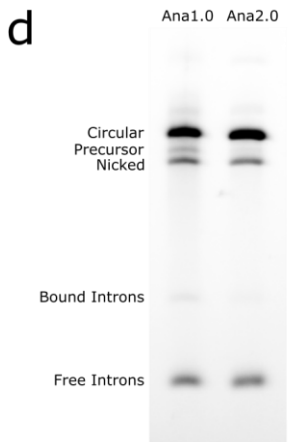
Additional design strategies improve circularization

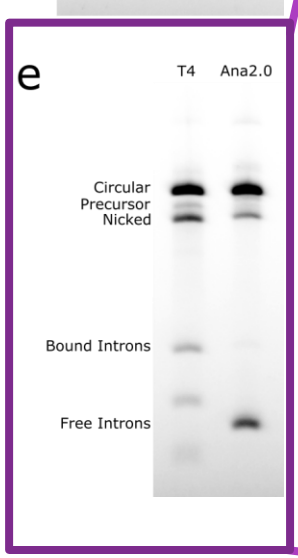
Spacers can dramatically facilitate correct intron folding

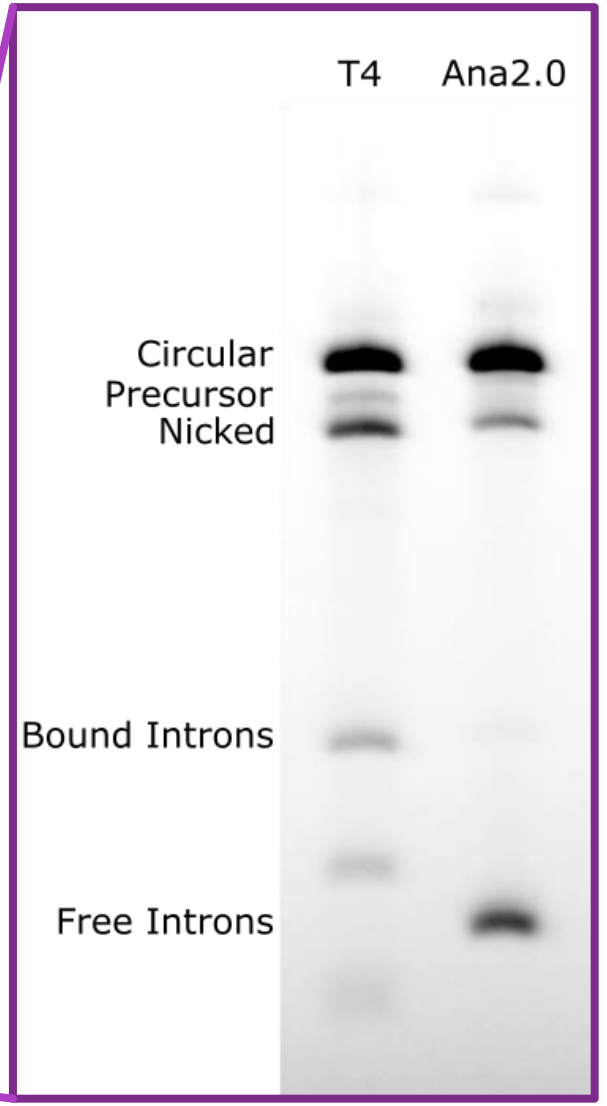










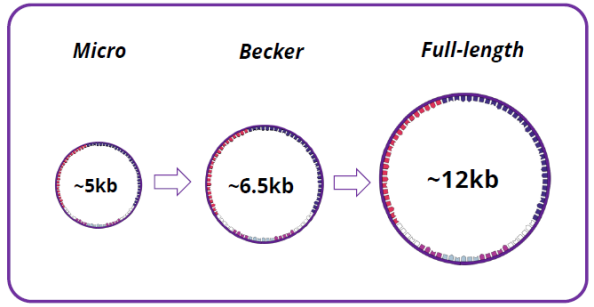


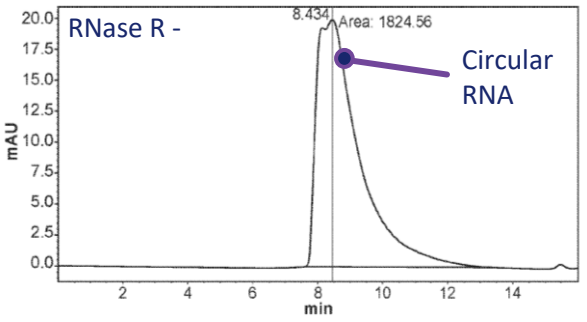
Alternative introns can be more efficient

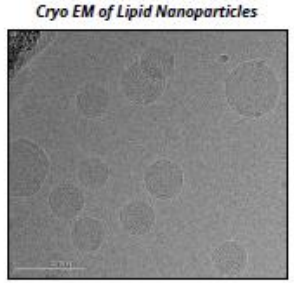
MIT

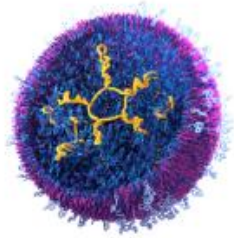
Very large circRNAs have been successfully made

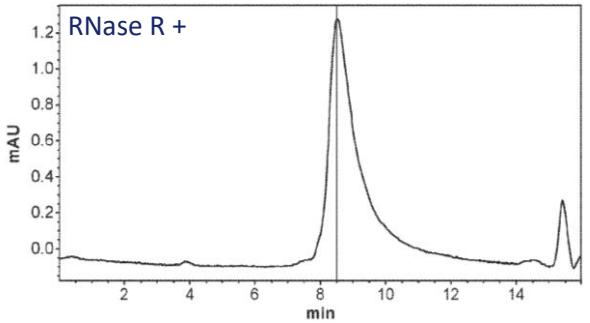
- Generating circular RNA encoding full-length dystrophin and smaller variants of dystrophin is a major challenge





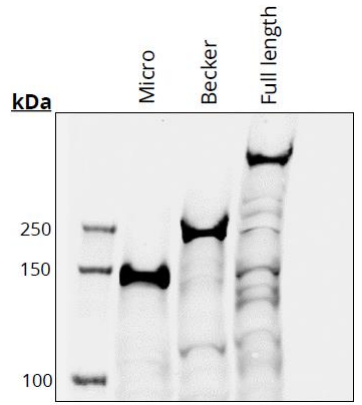




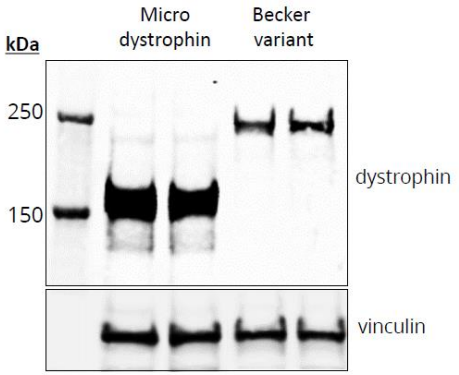


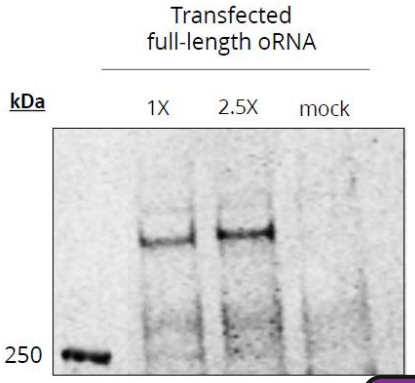
- Through sequence engineering and process optimization, intact ~12kb circRNAs can be successfully generated

- A cell-free translation assay shows products of the correct size for all three constructs



- These circRNAs can also be translated in primary human myotubes!

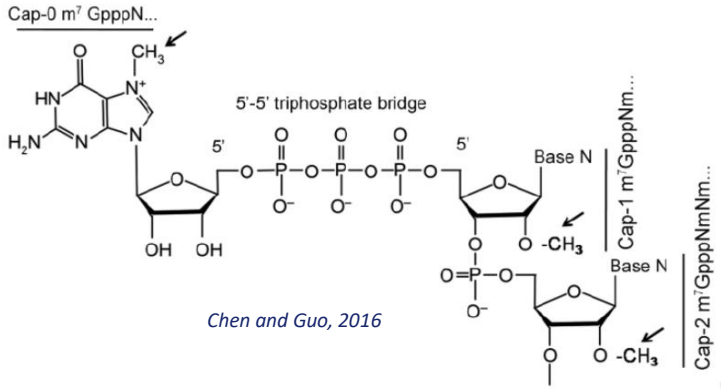




Orna

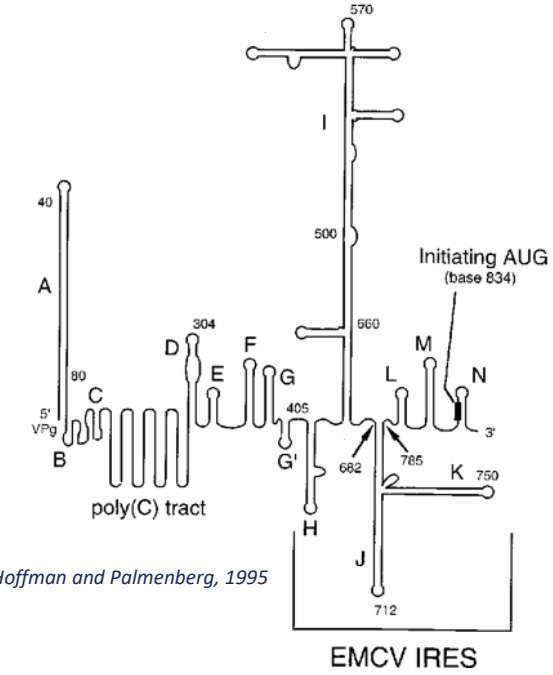
Challenge 2: Translation

- mRNA has a cap structure that initiates ribosome scanning
- Circular RNA doesn't have a cap
- **How can we get cap-independent translation within big synthetic circular RNAs?**

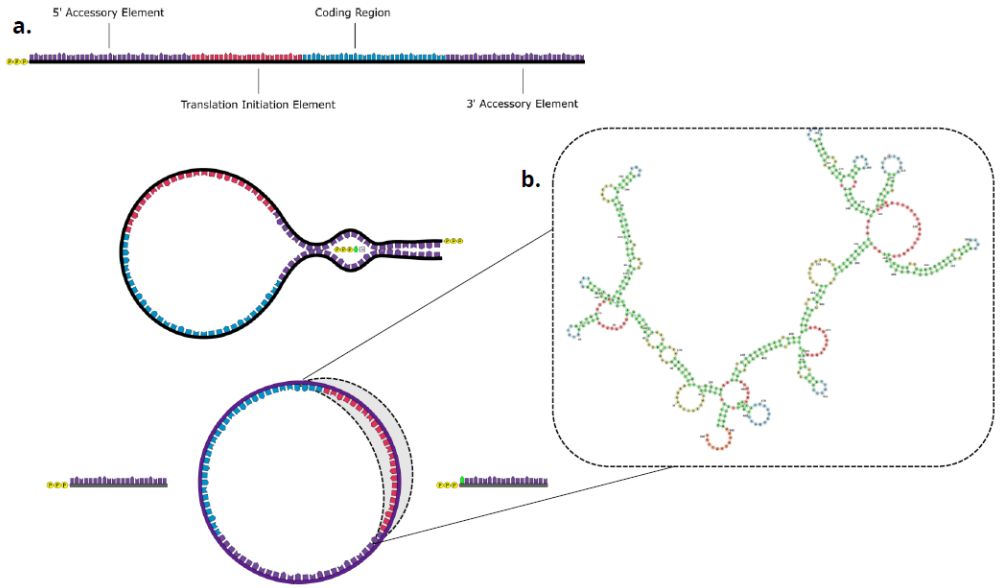


Chen and Guo, 2016

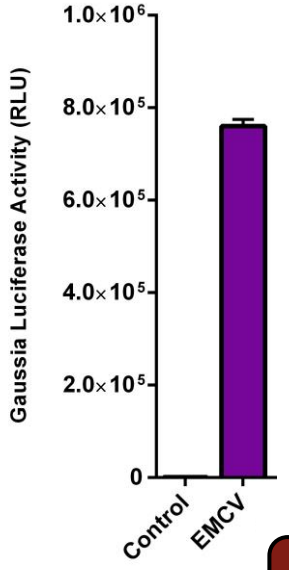
Viruses have a solution: The Internal Ribosome Entry Site (IRES)



Hoffman and Palmenberg, 1995



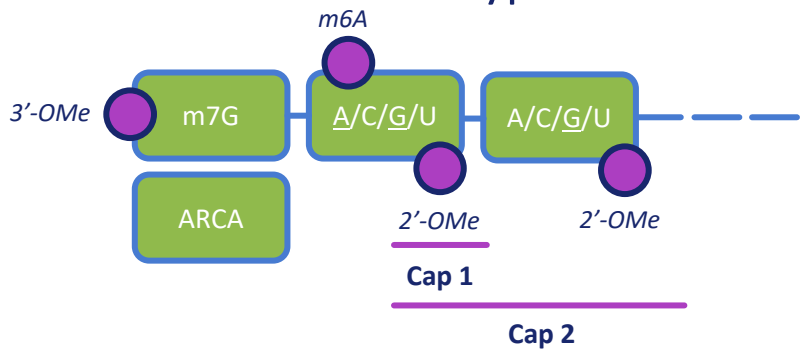
And get protein!



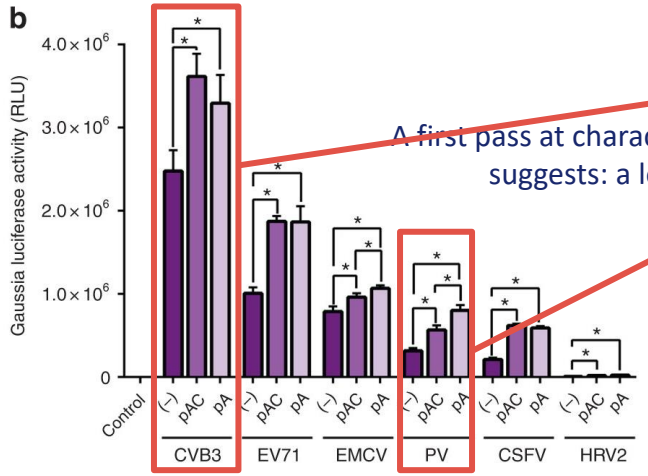
MIT

What is the IRES?

- There are a few different types of mRNA caps:

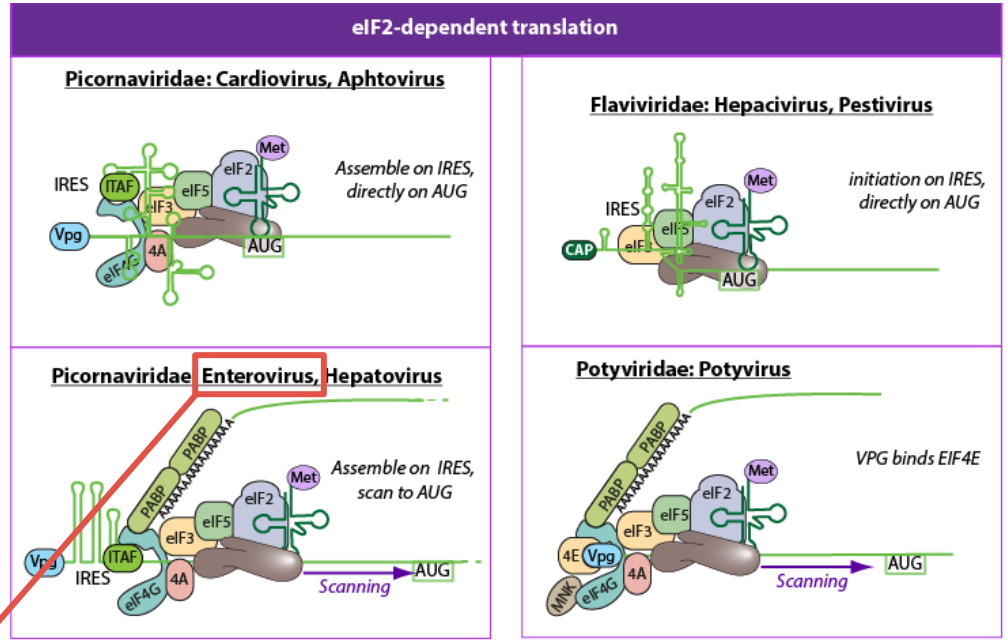


- With key decisions being first nucleoside and methylation pattern
- **What kind of chemical and functional diversity can we expect from IRESs?**



A first pass at characterization suggests: a lot!

- Like caps, there are a few types of IRESs:

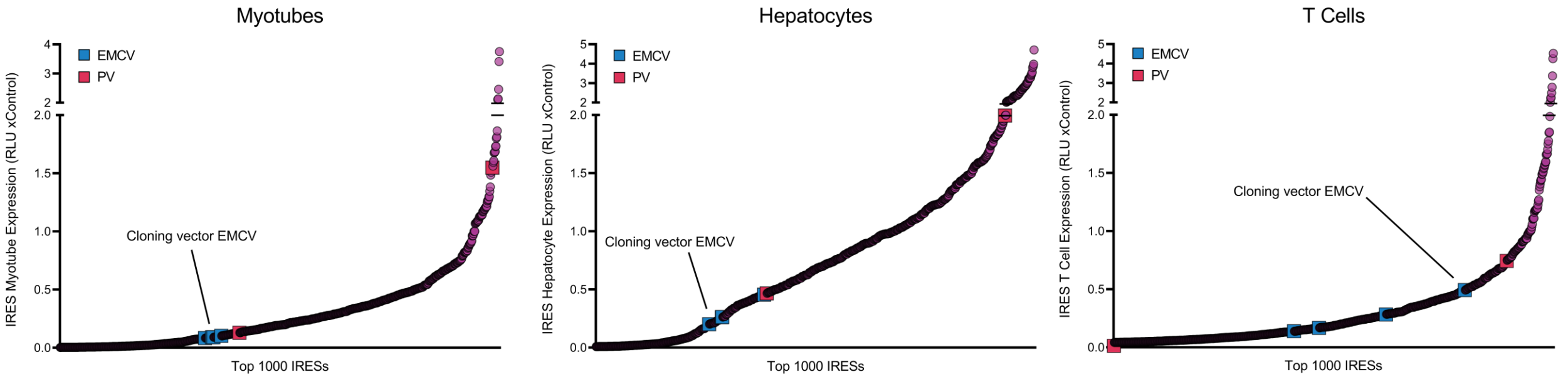


Viralzone

- But within IRES types, there appears to be a high level of sequence and functional diversity
- **What is the best way to discover potent IRESs?**

MIT

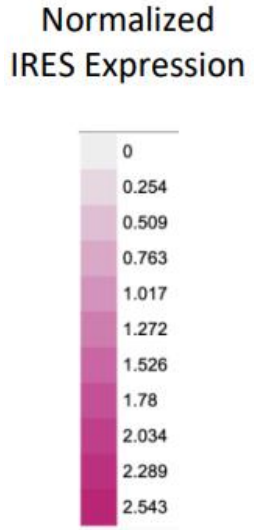
Unlocking a new world of IRES elements

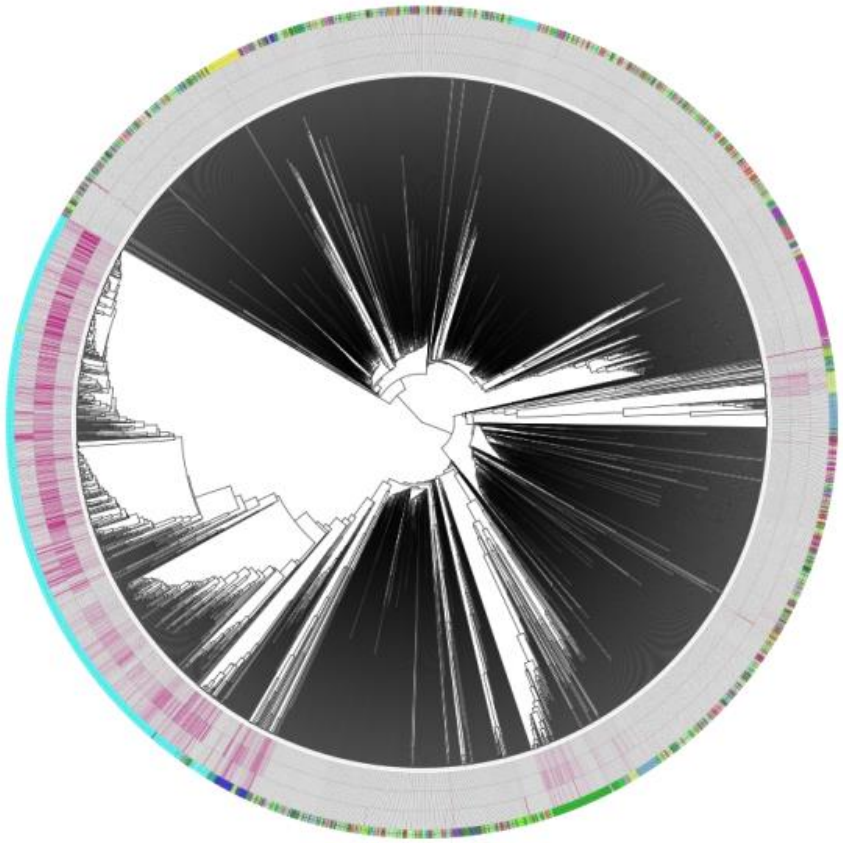


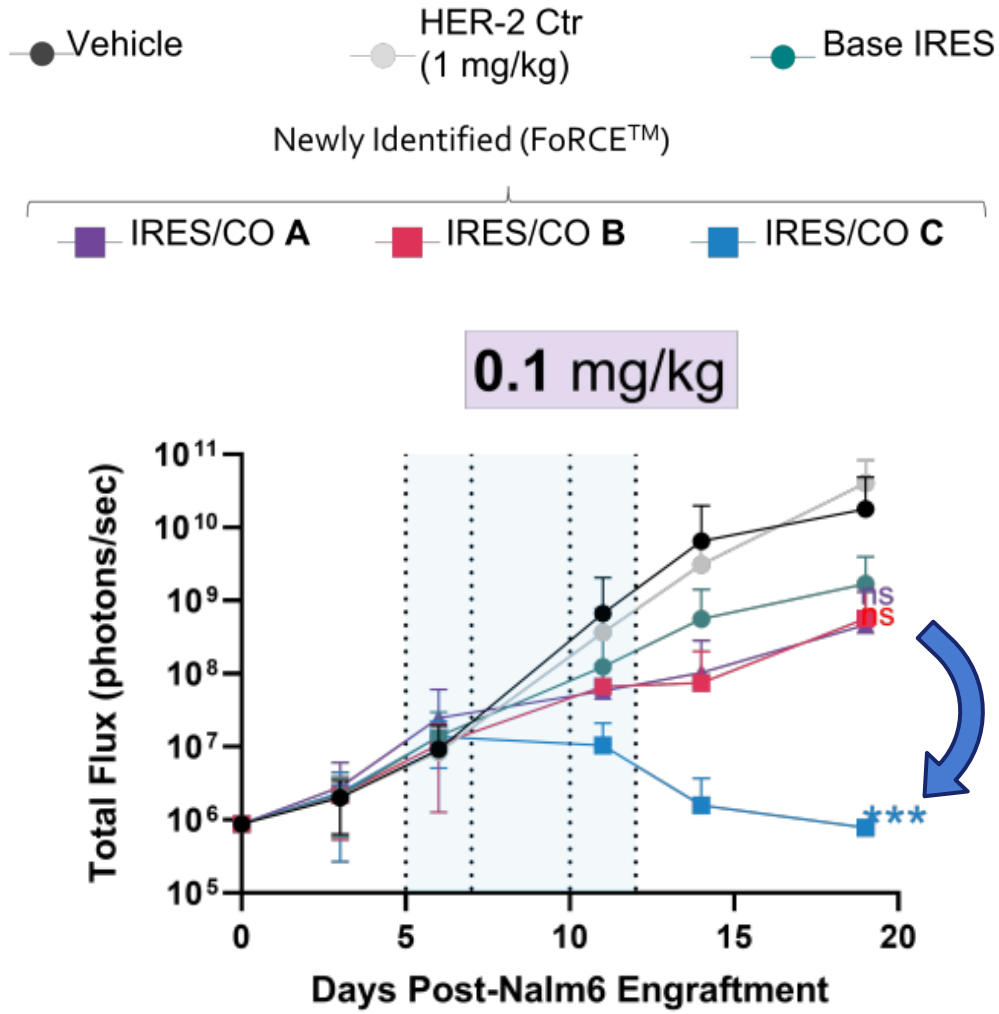
- Orna identified hundreds of new IRES elements highly active in oRNA
- Some IRESs are 10-40x stronger than the commonly used EMCV-type IRESs
- There can be significant differences even between IRESs from highly similar viruses, showing the importance of empirical approaches

Orna

Technology optimization leads to improved preclinical signal



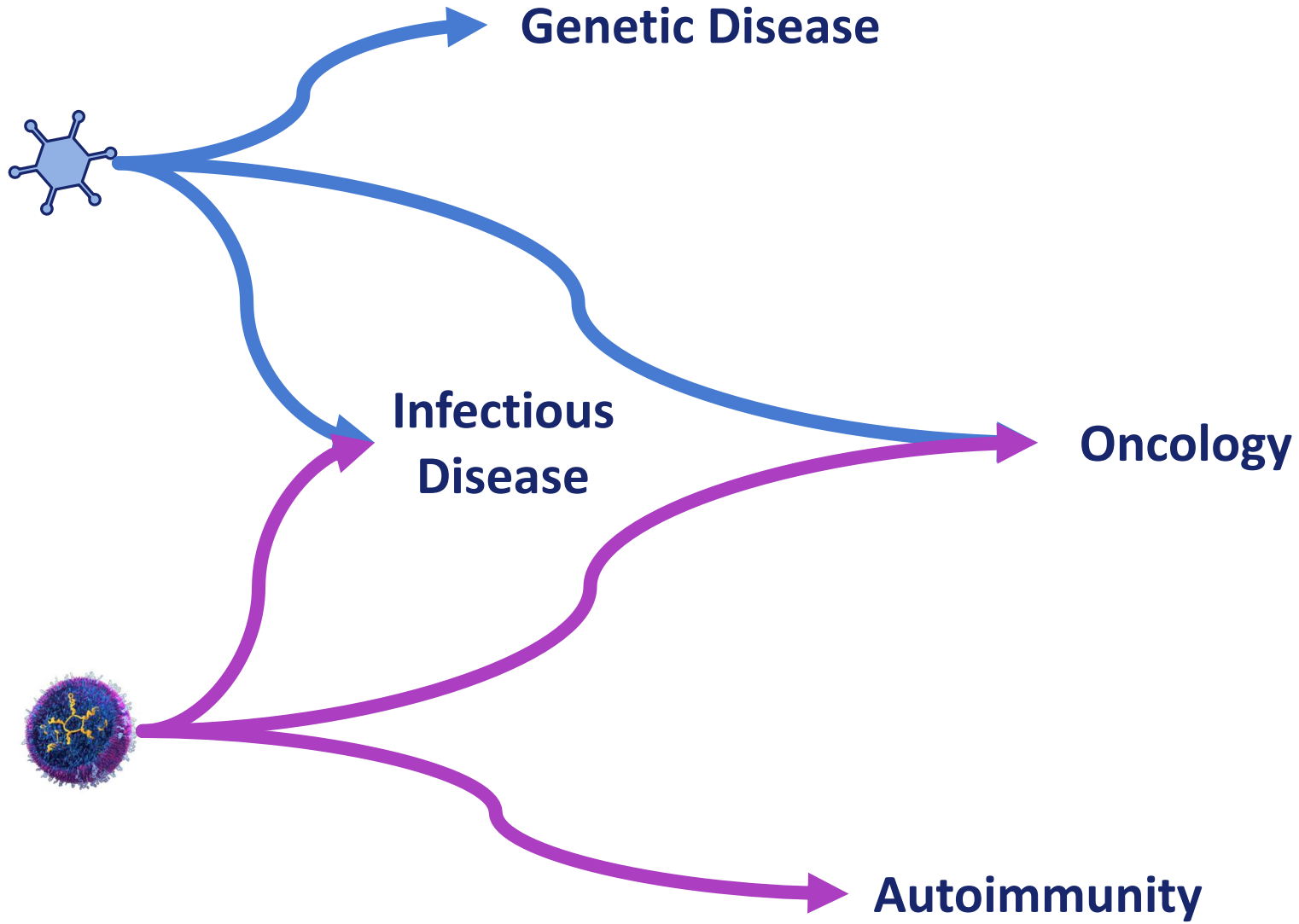




Orna

CircRNA applications

- DNA and RNA format circRNA technologies share several broad application areas, but delivery and duration of expression bias use towards specific areas
- Improving translation potency through circularization reduces dosing requirements across all potential applications, resulting in a wider therapeutic index



Perspectives on the future of circRNA

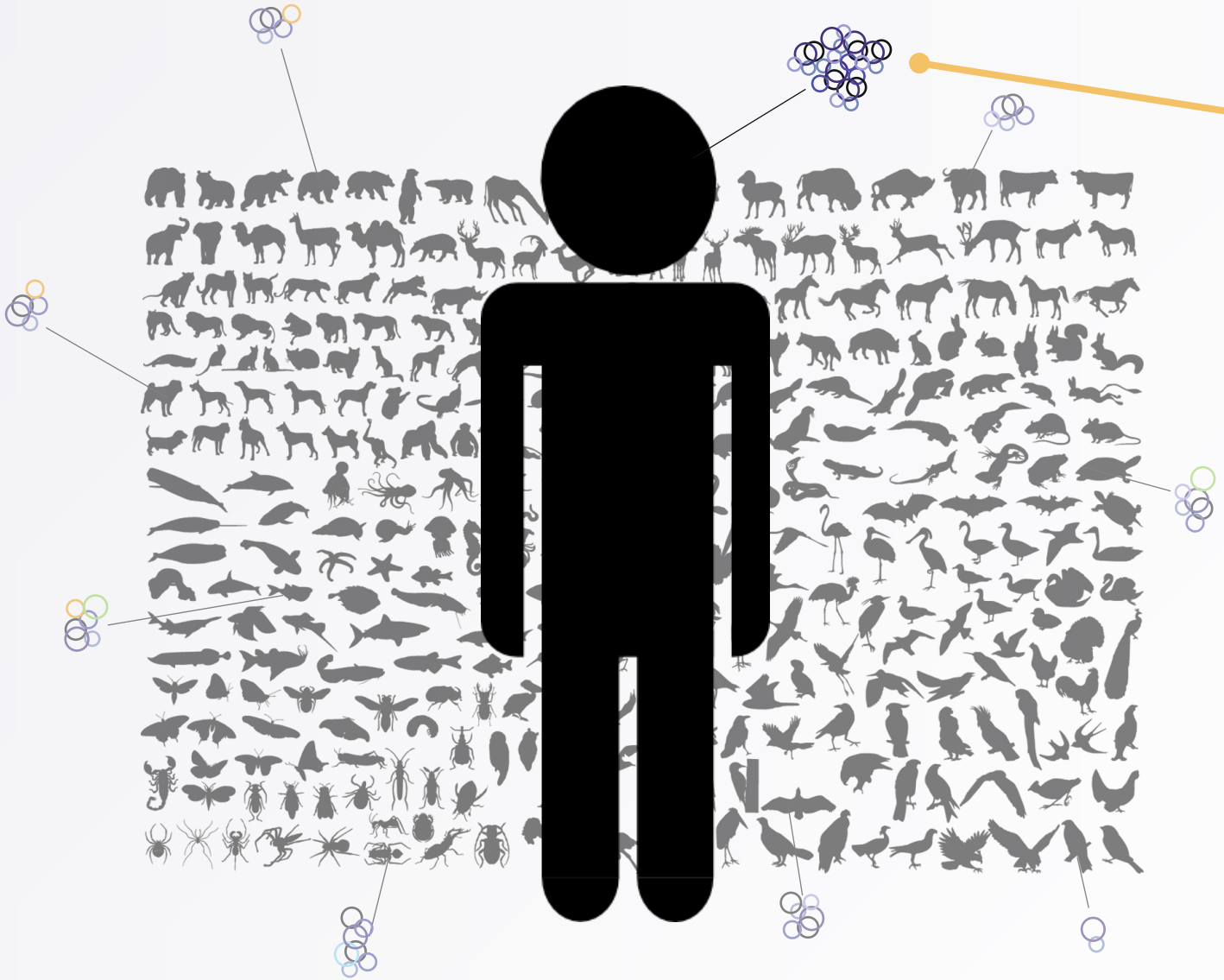
- CircRNA has RNA format and DNA format applications, wherein the core advantage is **increased potency** through a combination of enhanced stability and expression
- **Extensive optimization is required** to realize the full potential of circRNA
- CircRNA already compares favorably to linear RNA, and may see **another ~10x potency improvement** in both formats as development continues over the next few years
- **Circularization is solved** but most circRNA companies differentiate themselves by circularization method
- There is room for innovation in RNA format circRNA manufacturing, particularly with respect to **purification**
- **Targeted delivery is not a fully solved problem** for both formats and nucleic acid therapeutics at large

3

circVec overview

Dr. Thomas Birkballe Hansen
VP & Head of Research

Circular RNA – a natural design



High natural prevalence of circular RNA in all eukaryotes, particularly in humans

Why use circRNA?

Growth-decay model:

$$X(t) = Ce^{-Dt} + \frac{P}{D}$$

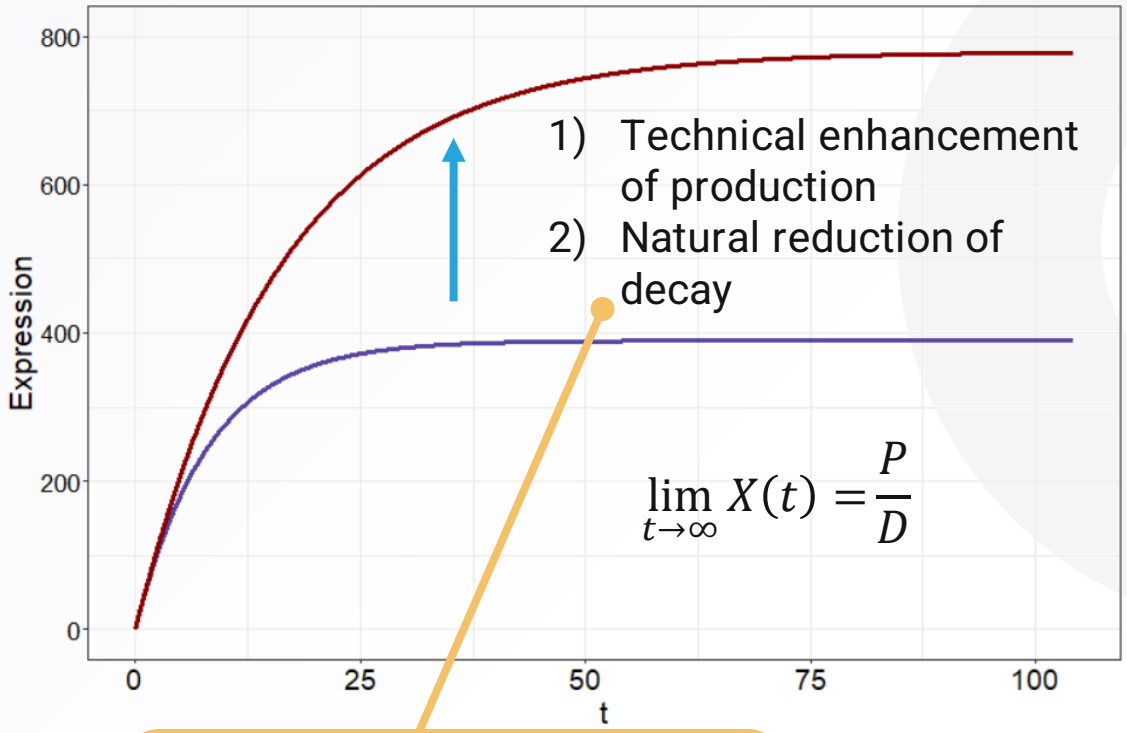
Expression

$P = production\ rate$

$D = decay\ rate = \frac{\log_e 2}{T½}$

Gene expression is determined by production rate and decay rate.

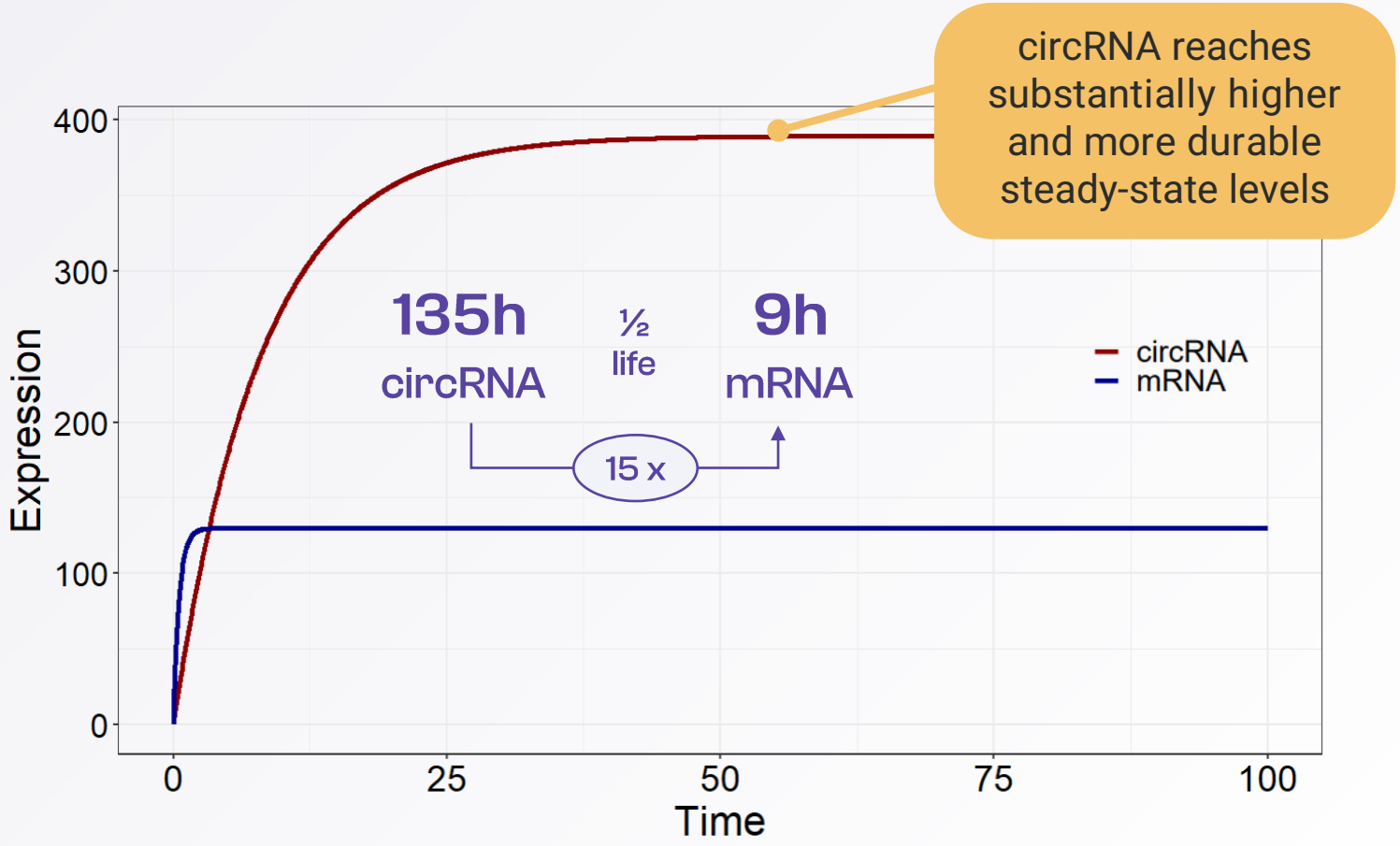
Two ways to increase expression



circRNAs are significantly more stable than linear mRNA

Bioinformatic simulation demonstrating advantage of vector-expressed circRNA vs. mRNA

Temporal vector-based RNA expression dynamics; circRNA vs. mRNA



Input assumptions for simulation:

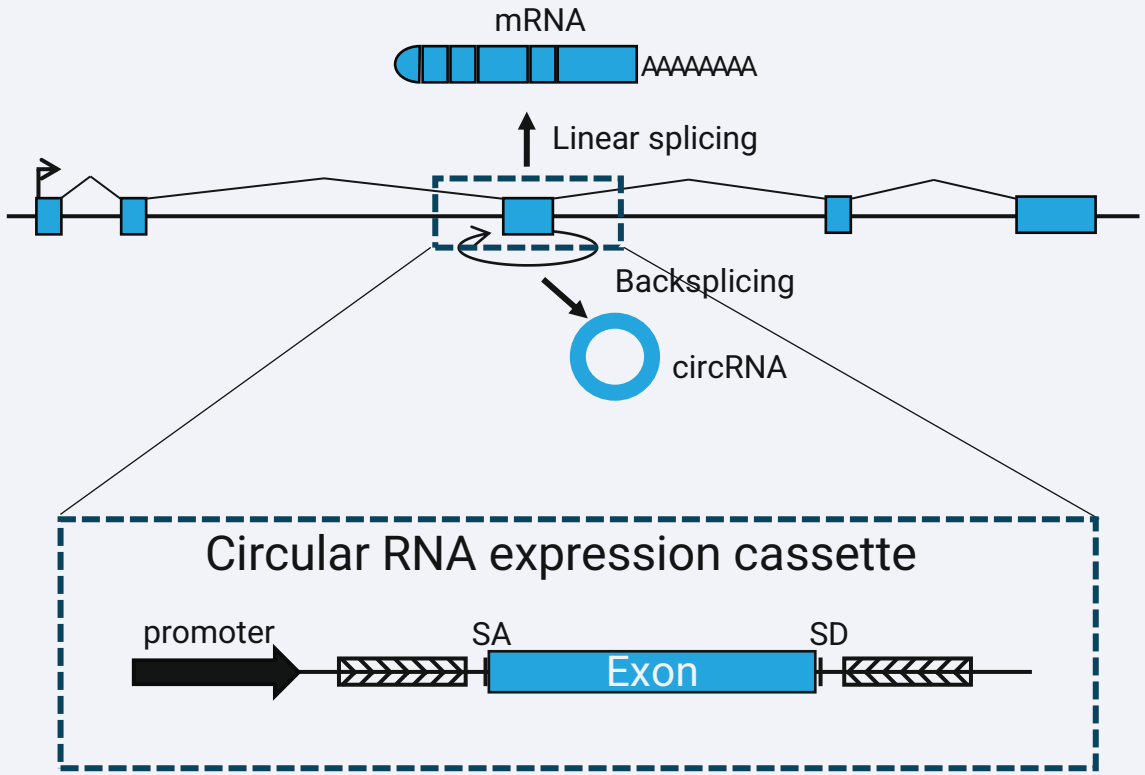
Non-dividing target cells

mRNA production:	10 molecules / hr
mRNA half-life:	9 hrs *
circRNA production:	2 molecules / hr *20% of mRNA rate*
circRNA half-life:	135 hrs * *15x mRNA ½-life*

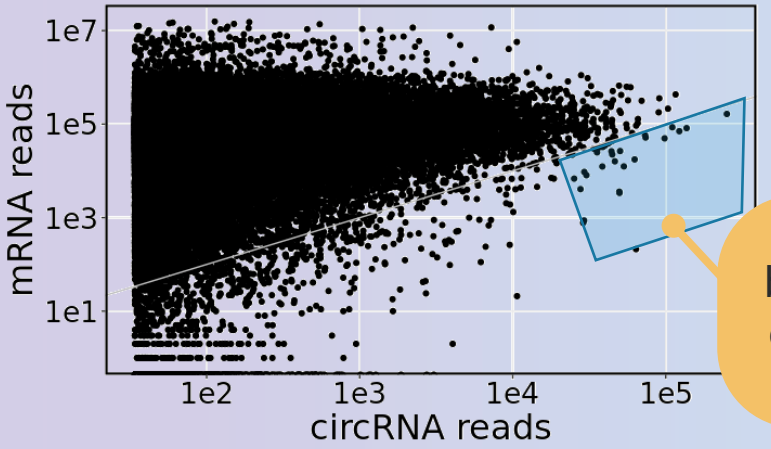
→ circRNA translation 5x mRNA rate gives >25x peak protein expression*

* Based on circVec experimental data

circVec is based on nature´s best design

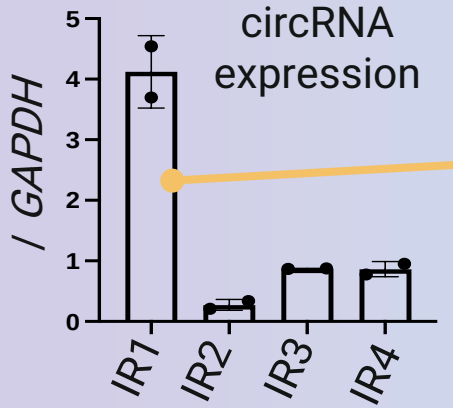


Expression of human endogenous circRNA
NGS analysis of 300+ RNAseq datasets



Maximum biogenesis-rate of endogenous circRNA

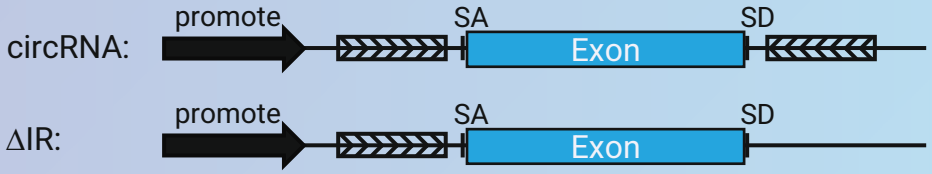
Screening of most effective natural sequences

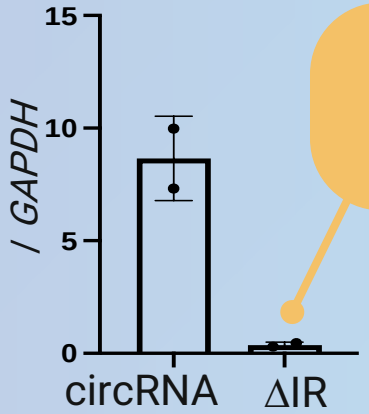


IR1 cassette design shows best circRNA biogenesis

Validation of circRNA biogenesis

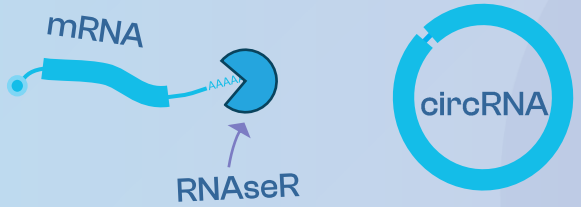
IR-dependent signal

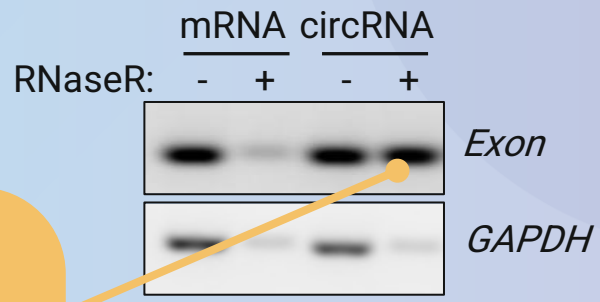




No production without IR element

RNAseR resistant

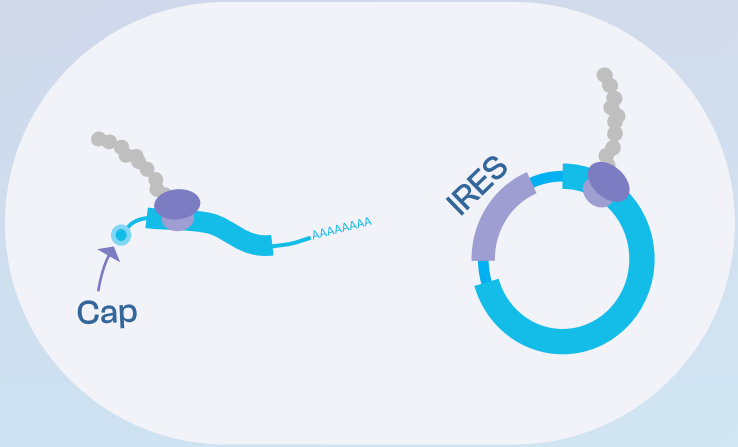




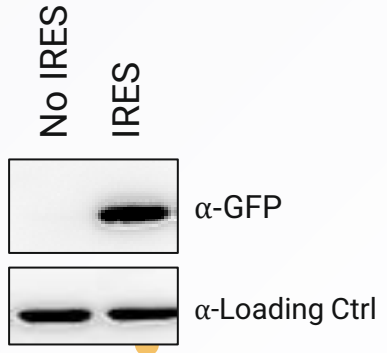
Resistance towards exonucleolytic degradation

IRES: enabling cap-independent translation from circular RNA

circRNAs are translated in a cap-independent manner

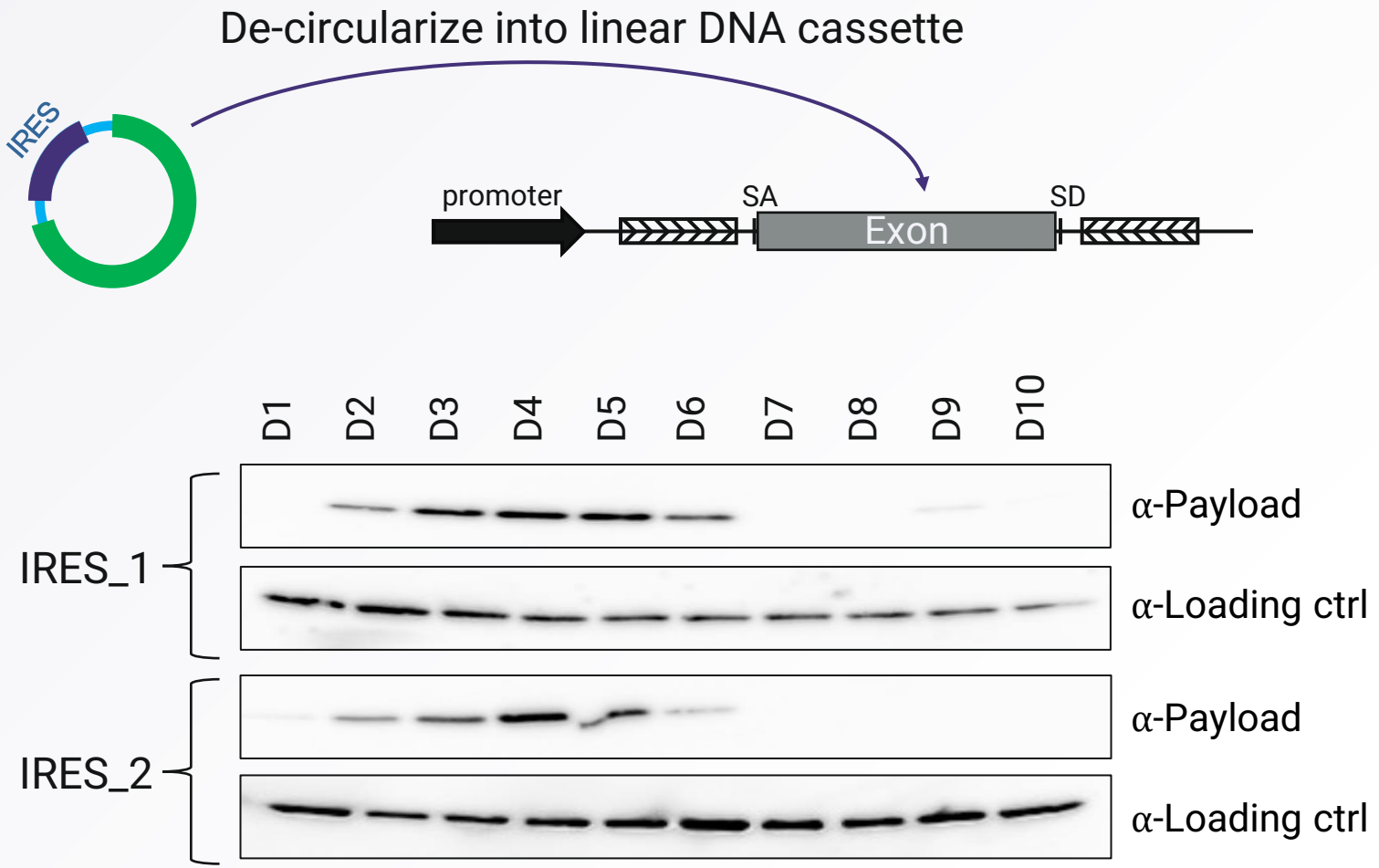


Protein expression, Western blot

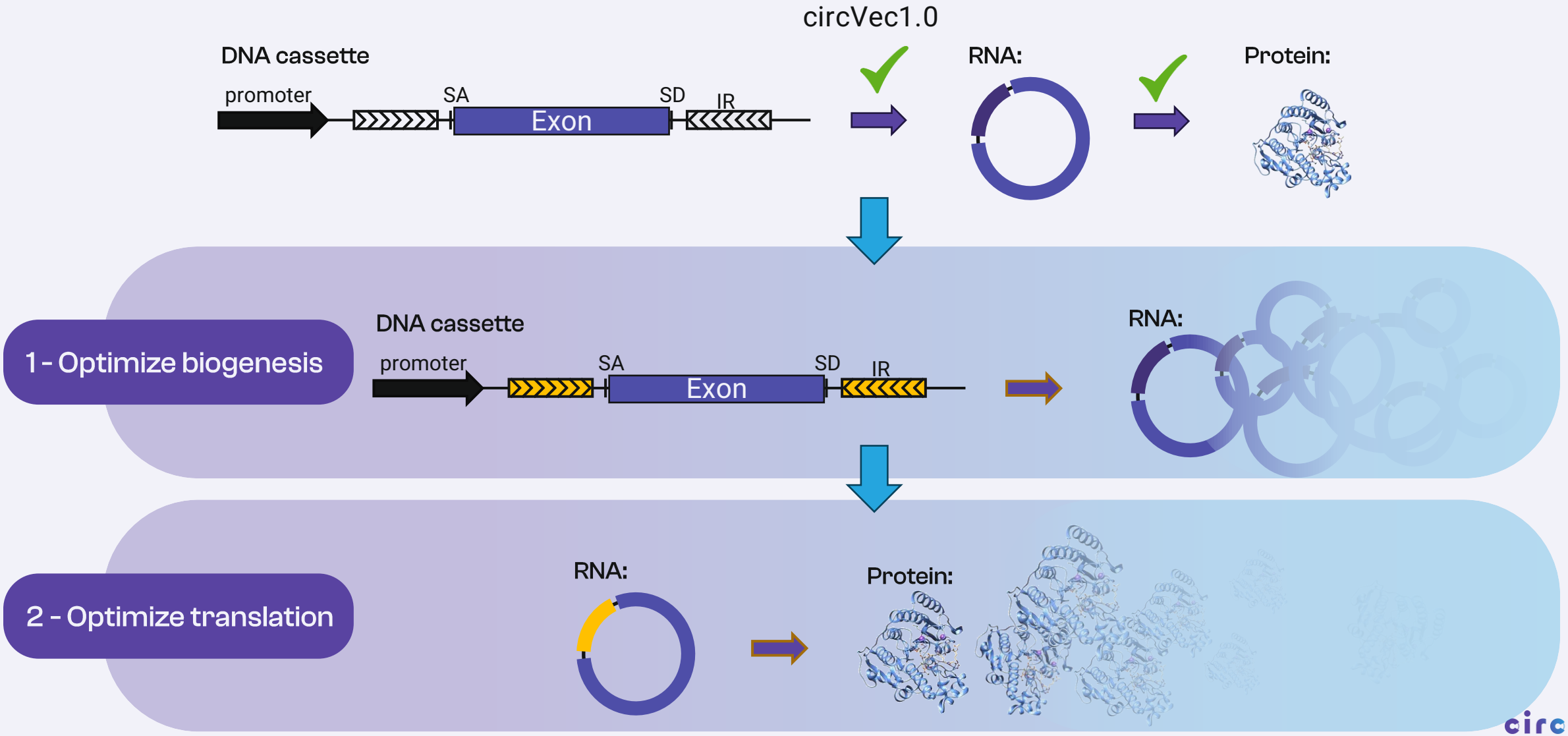


IRES element is required for circRNA translation

Design rules: Cassette composition is critical



Optimization scheme

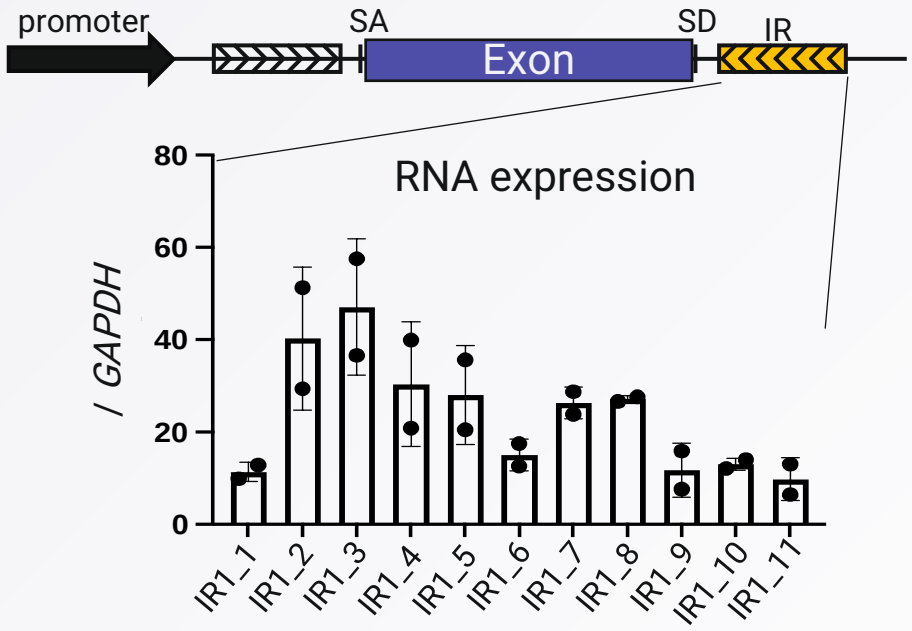


circio

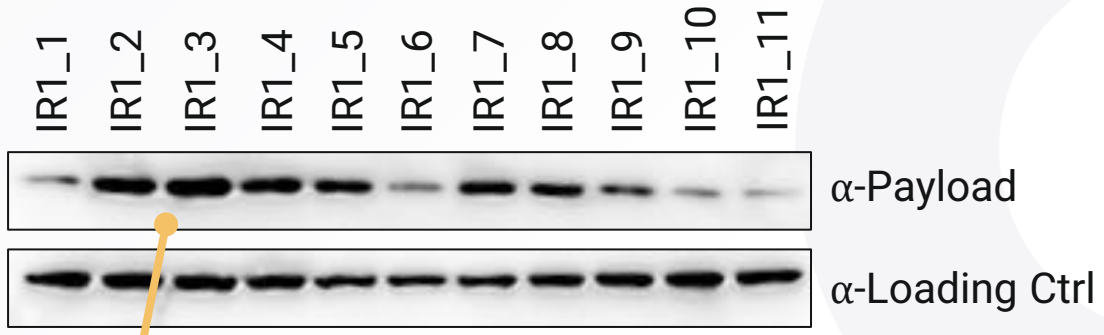
Optimizing flanking IR improves circRNA biogenesis

1 - Optimize biogenesis

circRNA expression, RT-qPCR



eGFP expression from circRNA, western blot

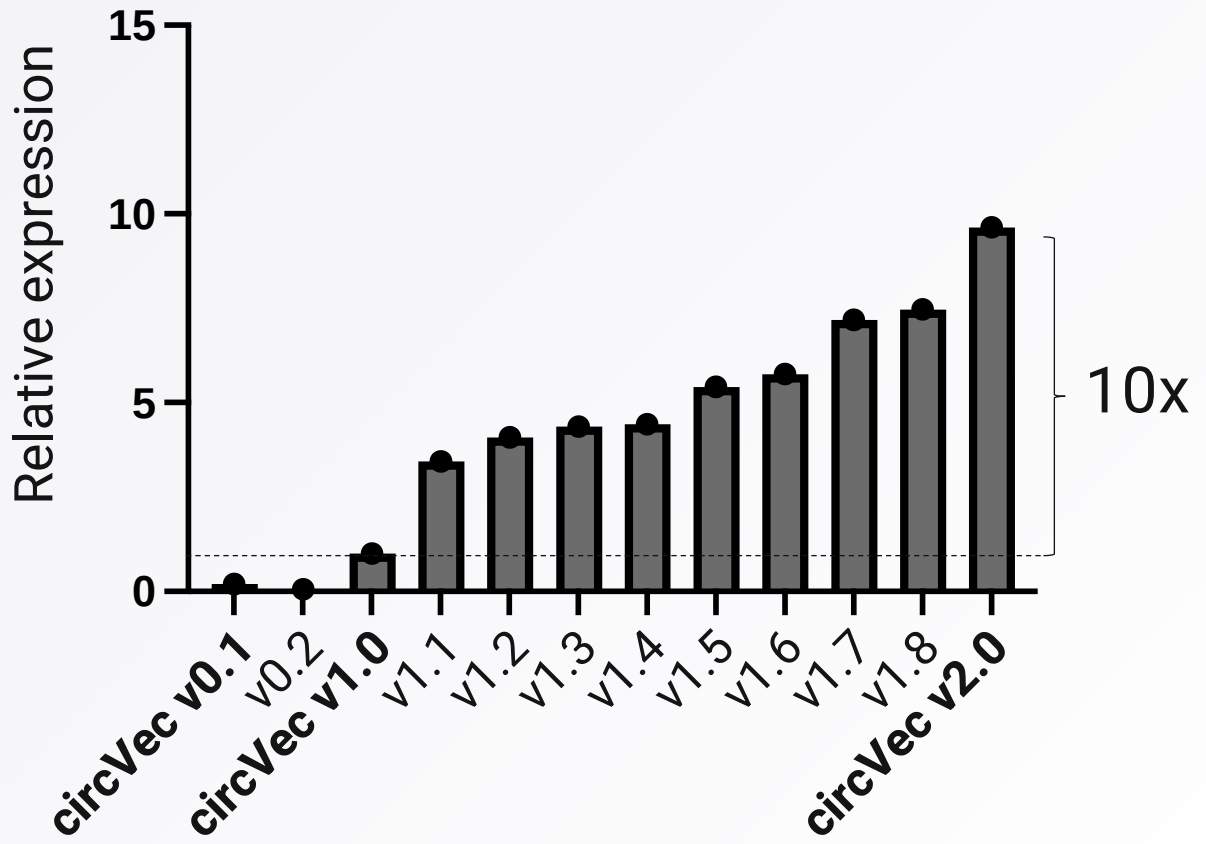


Modification of downstream inverted repeat (IR) element enhances both circRNA biogenesis and protein expression

IRES optimization results in ~10x higher protein expression

2 - Optimize translation

circVec design optimization, protein expression level @48h post-transfection



circVec v2.0 achieves up to 10x[1] higher protein yield than v1.0 design

1 Level of improvement in range of 3-10x depending on cell type

circVec substantially outperforms the expression level and durability of mRNA-based systems

Increased expression level

Prolonged durability

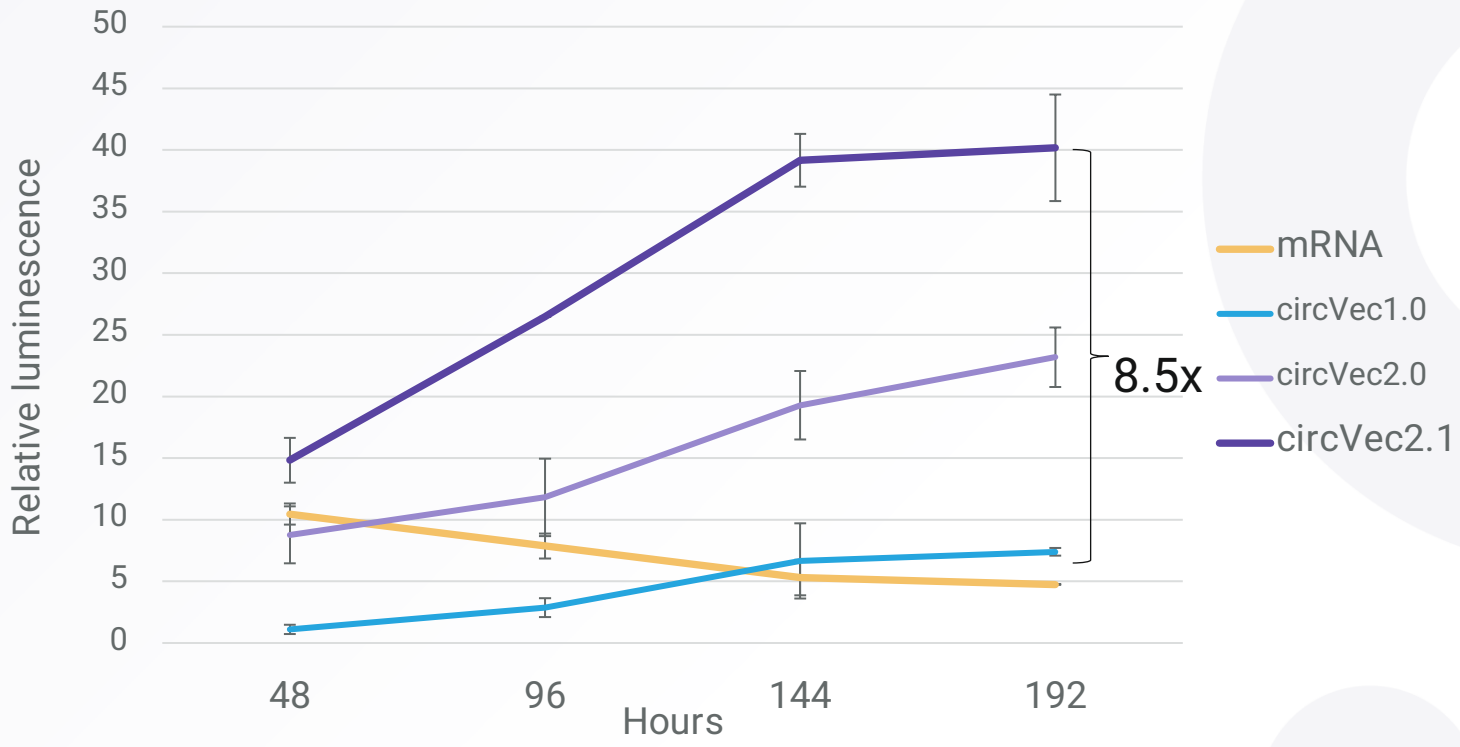
Enhanced therapeutic potency

"Due to its significant advantages, circRNA systems can be expected to replace mRNA-based expression for DNA format therapeutics in the future – just as synthetic circRNA can be expected to replace current mRNA formats"

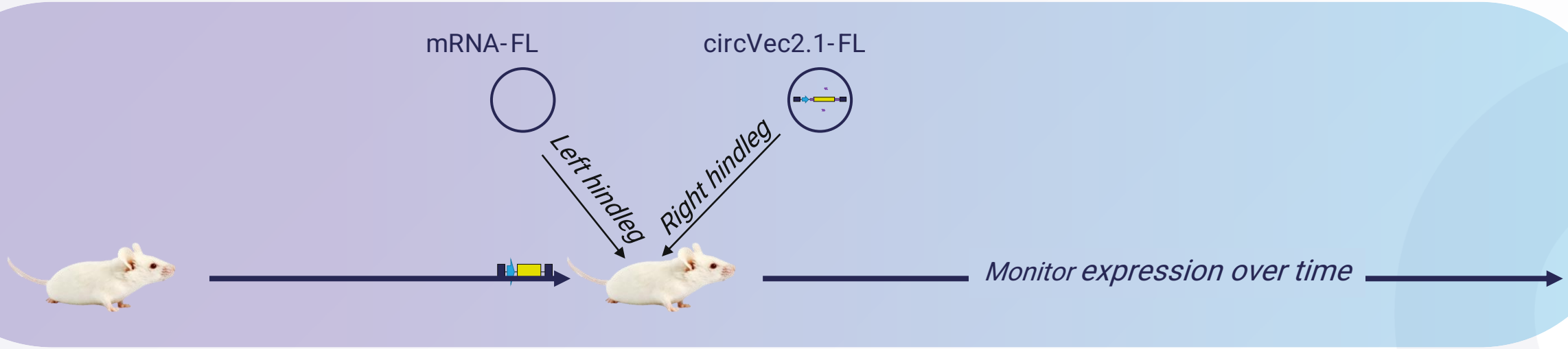
Dr. Alex Wesselhoeft

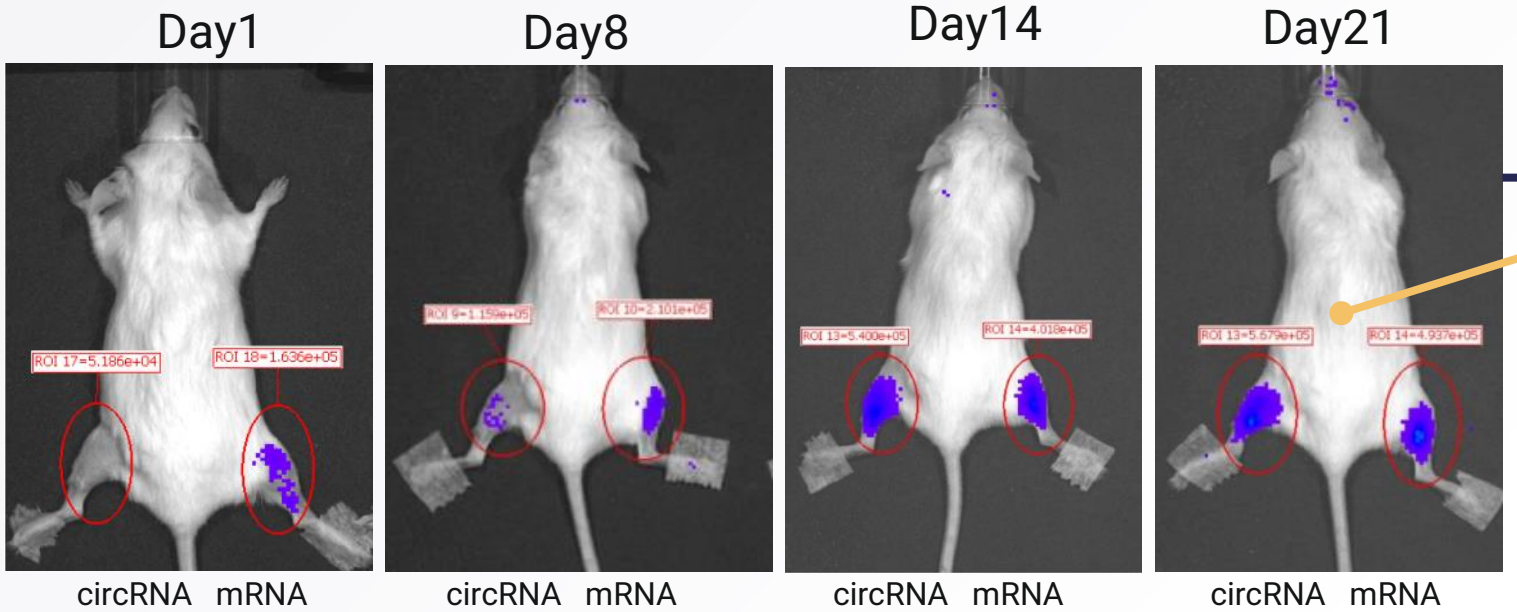
Scientific founder
oRNA Therapeutics

circVec vs. mRNA luciferase reporter expression; time course



Characterizing circVec v2.1 performance in vivo

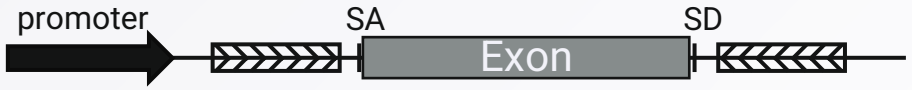




Realtime monitoring ongoing

Efficient circRNA expression established in mouse model demonstrating progressive accumulation

Adding more functionalities to circVec



circVec

Expression of missing protein in loss-of-function scenarios!

Removal of protein in gain-of-function scenarios?

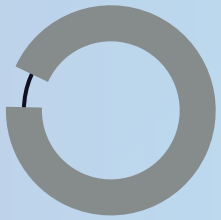
Remove-and-Replace design!

Remove-and-Replace concept enables expression of missing protein while depleting aberrant transcripts

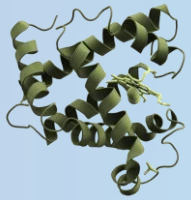
circVec-*remover*

Expression of missing protein in loss-of-function scenarios!

circRNA

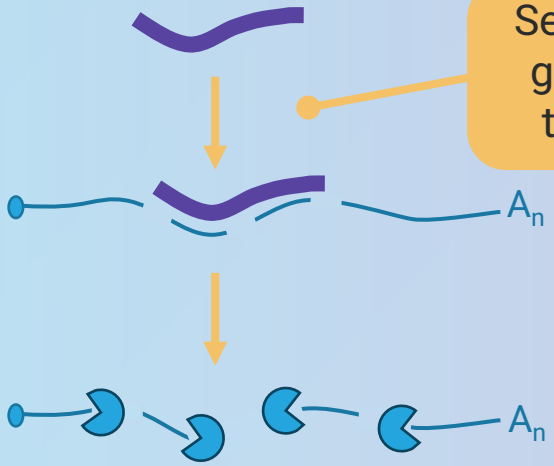


Loss-of-function protein expression



Removal of protein in gain-of-function scenarios!

Remover



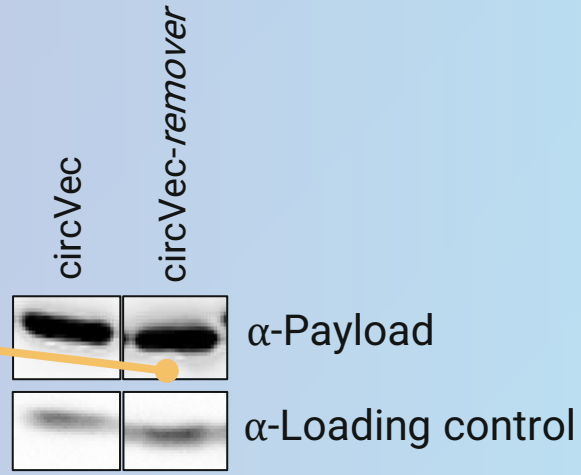
Sequence-specific gain-of-function target cleavage

Remove-and-Replace concept enables expression of missing protein while depleting aberrant transcripts

circVec-*remover*

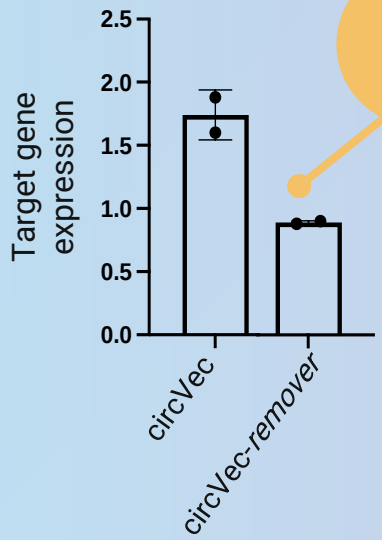
Expression of missing protein in loss-of-function scenarios!

CircRNA expression unaffected by remover modality



Removal of protein in gain-of-function scenarios!

Effective depletion of target gene by *remover*



Technical development status

- ✓ Substantially optimized circRNA biogenesis and protein expression
- ✓ Multifunctional *Remove-and-Replace* concept established
- ✓ In-vivo validation ongoing in multiple settings
- ✓ Now testing circVec 2.1 in therapeutically relevant applications

4

R&D Strategy

Dr. Victor Levitsky - CSO

Major opportunities identified for the circVec platform in gene therapy and vaccines

Current focus areas

Rare disease

"Remove-and-replace" concept with durability and safety advantages

-

Major long-term potential

Vaccines

Enhanced potency, single dose vaccine concept with simplified administration

-

Early partnering option

Cancer gene therapy

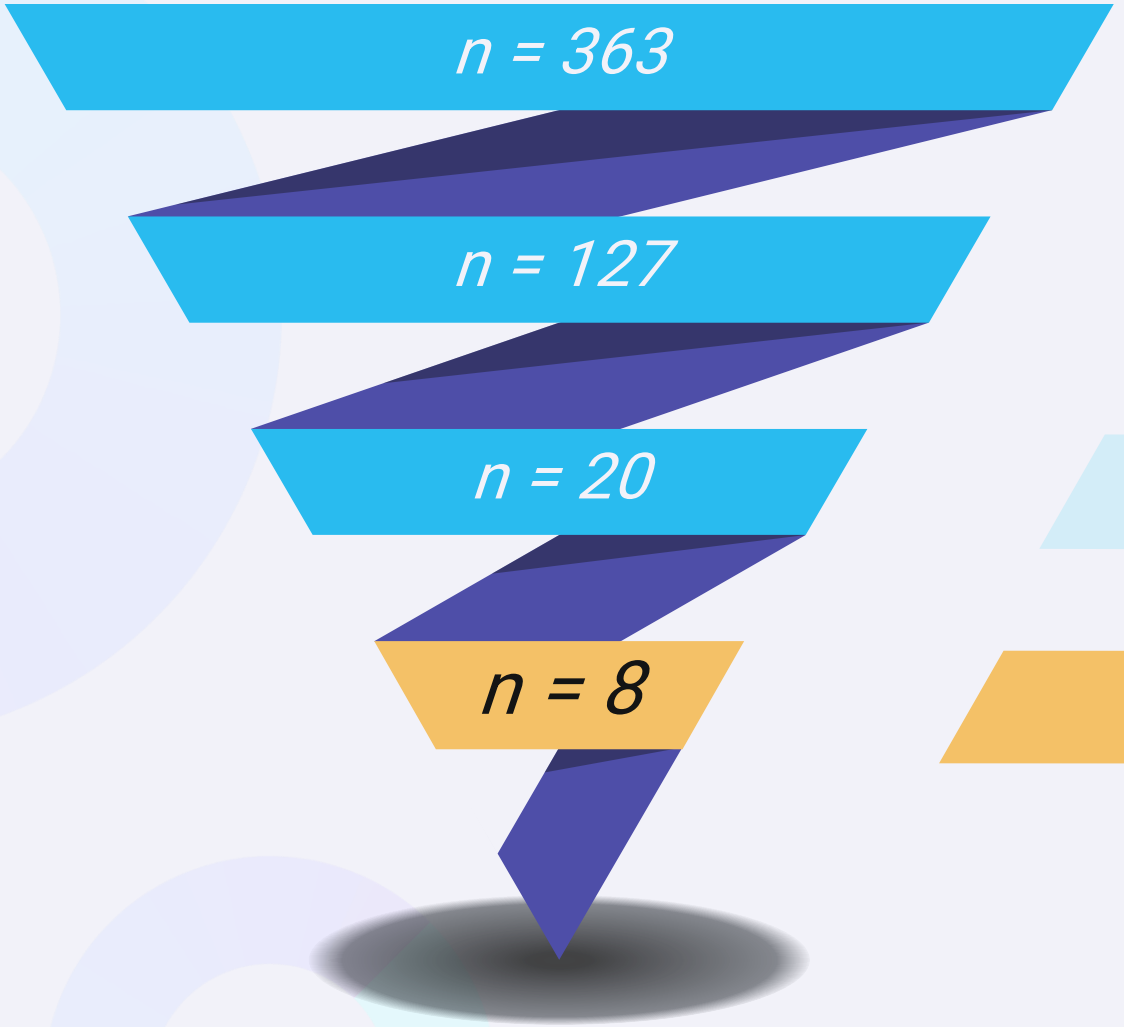
Efficient and durable expression of therapeutic proteins in solid tumors

-

Unique oncology concept

Designed for intra-cellular circRNA supply, durable protein expression and targeted regulatory functionality

Broad analysis performed to identify target rare diseases suitable for circVec approach



Initial Screening

- Therapeutic Focus: Rare genetic disorders
- Development Stage: Clinical or Market validation achieved

1st Prioritization Criteria

- Etiology: monogenic disorders only (exclude polygenic disorders)
- In vivo validation: availability of suitable in vivo models

2nd Prioritization Criteria

- Epidemiology: From Moderate (1/50,000) to High (<1/2,000)
- Gene size: ORF ≤ 4,500 Kb

Indication Prioritization Final Step

Identified short-list of eight monogenic disorders with strong fit for circVec

Short-list of 8 rare monogenic disorders particularly suitable for circVec approach

Lead Indication

Alpha-1 Antitrypsin Deficiency (AATD)

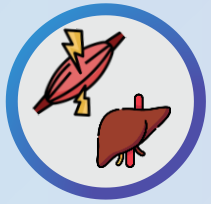
Second priority

Ornithine Transcarbamylase Deficiency (OTCD)

Citrullinemia Type I (CTLN1)

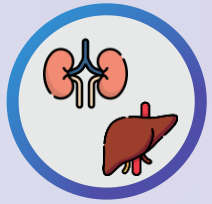
Argininosuccinate Synthetase Lyase Deficiency (ASLD)

Third priority

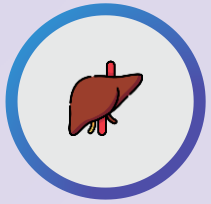


Pompe Disease

Wilson Disease



Glycogen Storage Disease 1A

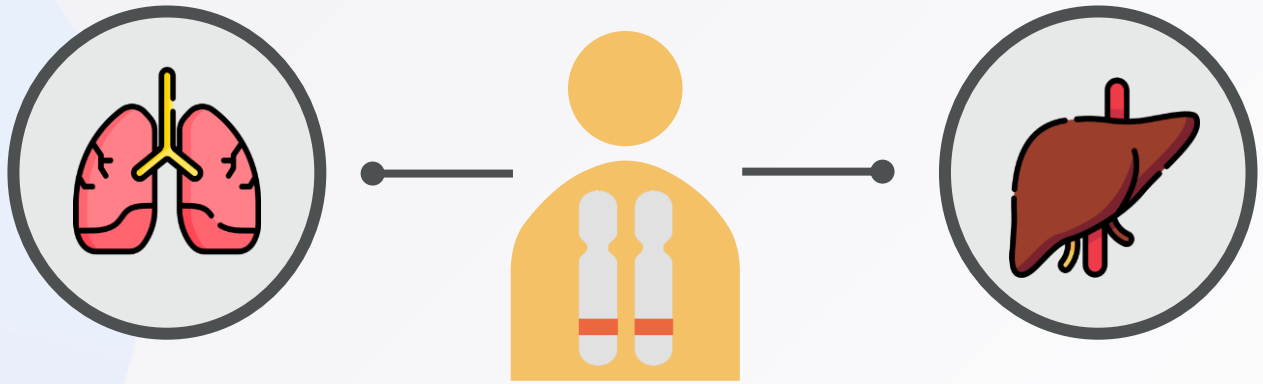


Tyrosinemia Type I

Commercial and regulatory assessment ongoing

Lead indication: Alpha-1 antitrypsin deficiency (AATD)

AATD is a major unmet medical need manifested in liver and lung



- Lack of functional AAT protein
- Emphysema and/or chronic bronchitis

- Accumulation of toxic mutant protein
- Cirrhosis

Patients with moderate to severe AATD

120K in EU

75K in US

Lung-associated AATD

- Replacement therapy with an alpha-1 proteinase inhibitors
- Weekly IV infusions
- Bronchodilators and inhaled steroids used for mild symptoms

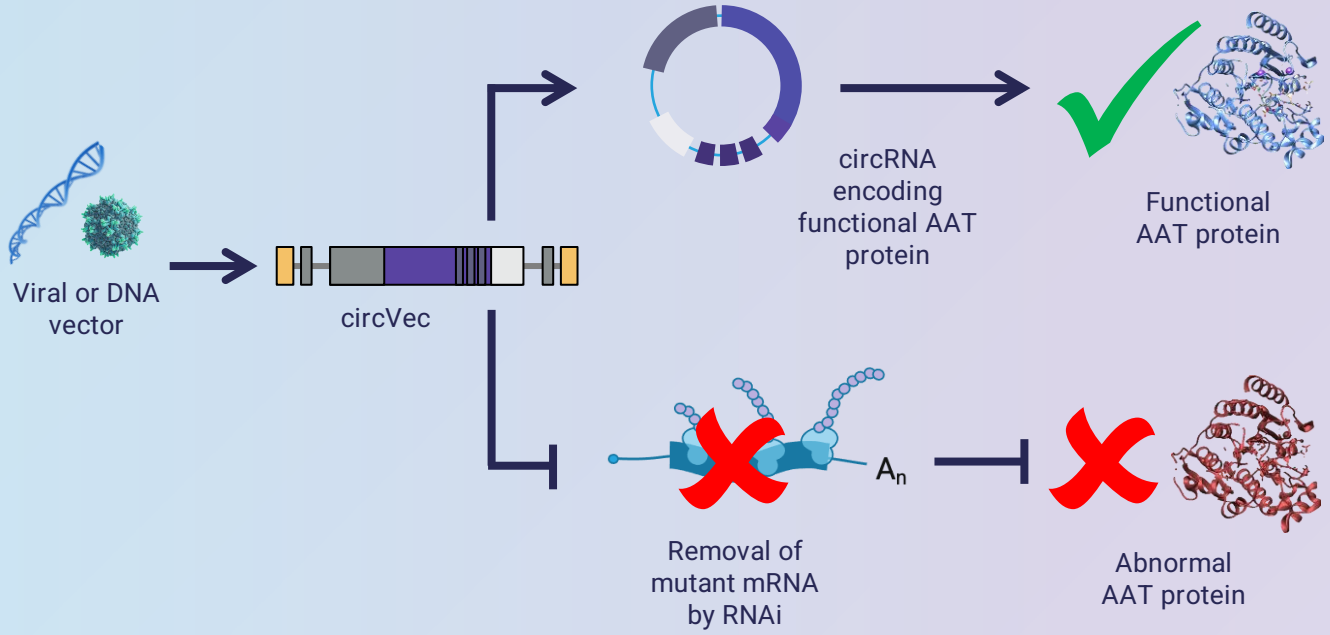
Liver-associated AATD

- No approved therapeutics
- Liver transplantation is the only treatment alternative in severe cases

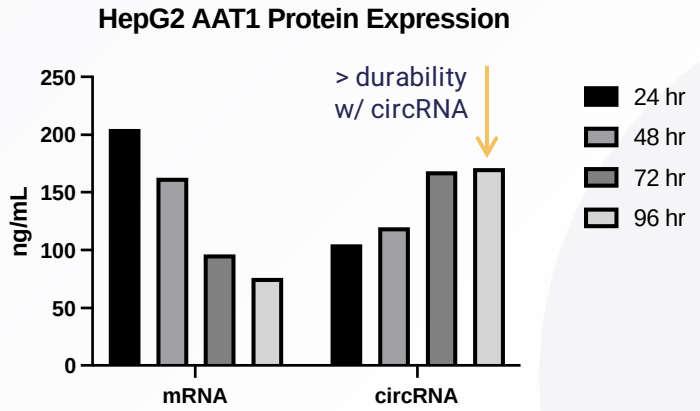
circVec "Remove-and-Replace" concept for AATD

Depleting mutant form and replenishing functional protein by circVec

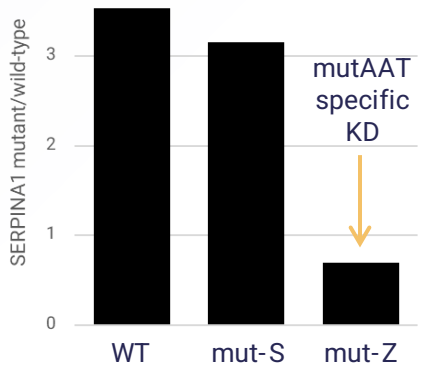
- *reverses toxic protein accumulation in liver and restores normal function in lung*



circVec v1.0 AAT expression in liver cells



circVec mutAAT knock-down

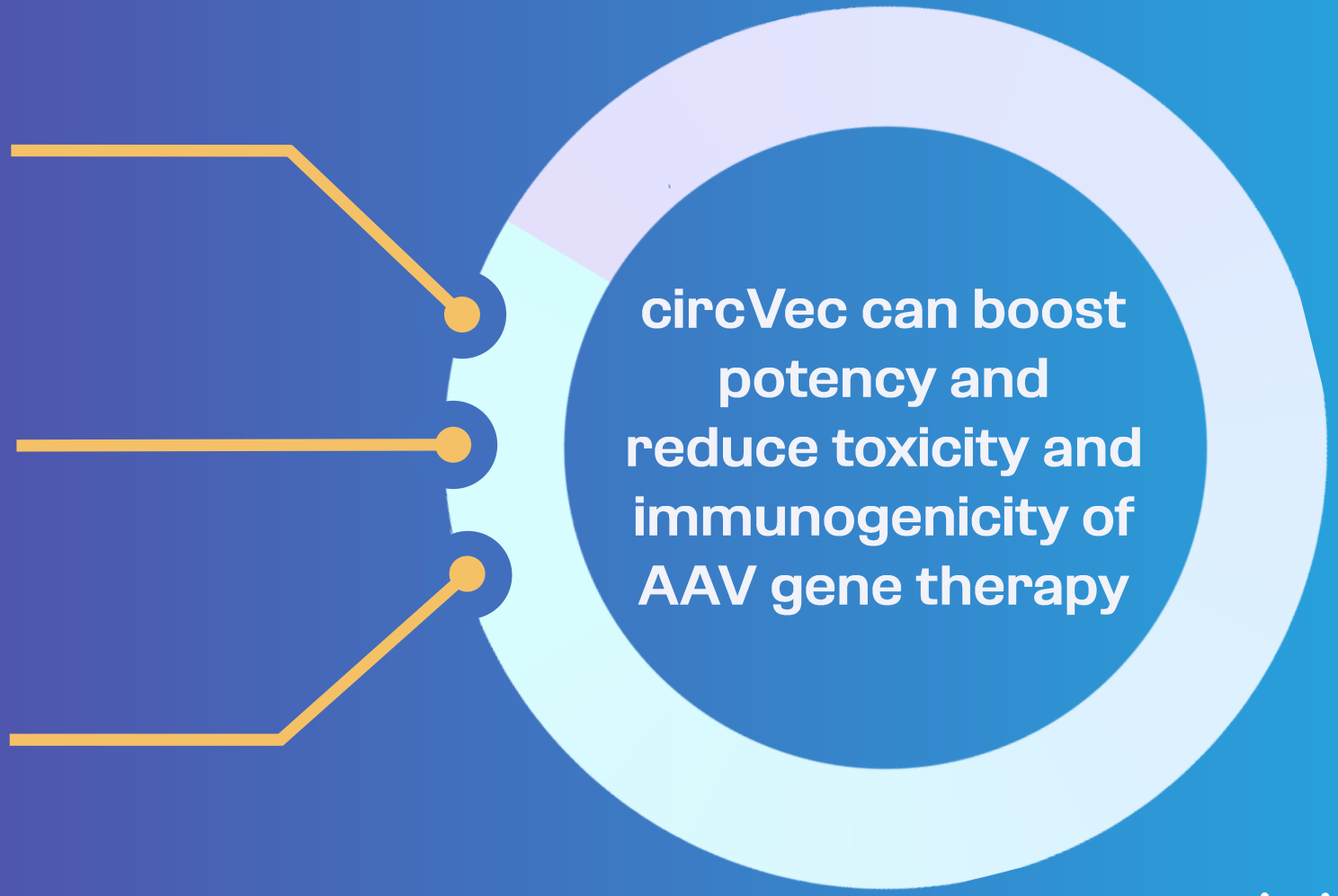


High dosing requirement is a substantial shortcoming for AAV-based gene therapy

Safety issues
Liver toxicity, innate immunity

High dose = high immunogenicity
No repeat dosing

Manufacturing cost
10^{14} – 10^{15} VPs per dose



AAV: circVec proof-of-concept for gene therapy

vector

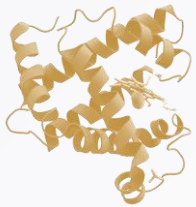
Standard mRNA approach

circVec circRNA approach

RNA

Shorter durability

15x durability
5x translation rate



Protein

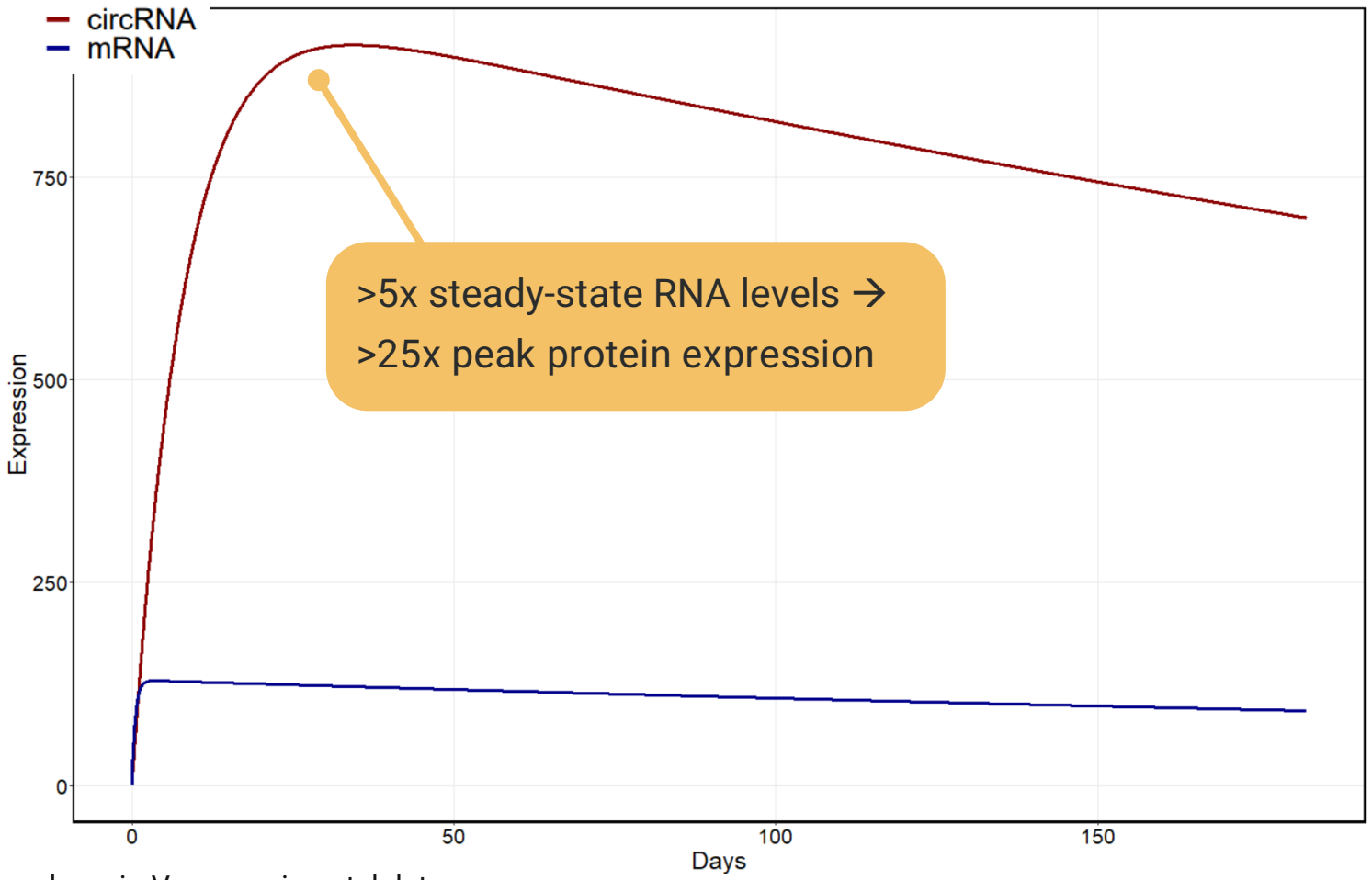
Lower steady-state protein levels

>25x maximum protein levels

circVec has the potential to substantially boost the potency of AAV-based gene therapy

circVec-based AAV therapy can translate into major improvement in long-term expression dynamics

Temporal AAV-based RNA expression dynamics; circRNA vs. mRNA



* Based on circVec experimental data

Input assumptions for simulation:

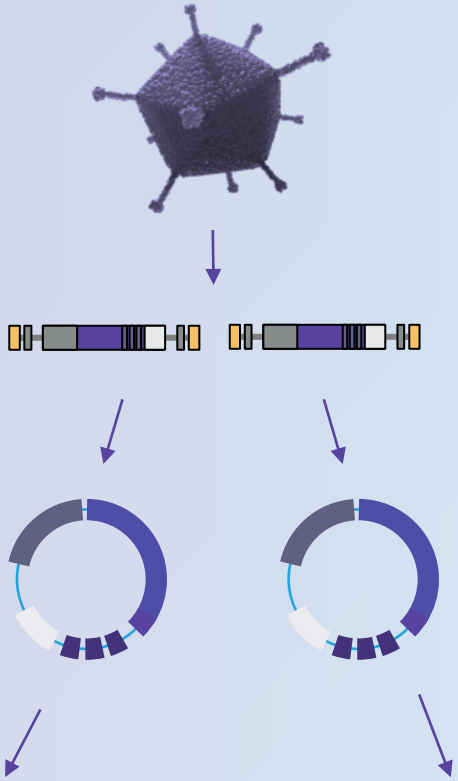
Non-dividing target cells

AAV half-life:	365 days
mRNA production:	10 molecules / hr
mRNA half-life:	9 hrs *
circRNA production:	5 molecules / hr
circRNA half-life:	135 hrs *
	15x mRNA ½-life

→ circRNA translation 5x mRNA rate gives >25x peak protein expression*

circVac: AdV circVec system for potent vaccination

Replication-deficient
AdV vector *35kb genome*



circVec inserts
>7kb size feasible

One or more circRNAs
2-6kb size per circRNA

Durable protein or
antigen expression

+RNAi

Additional booster,
miRNA sponging

Non-replicating AdV advantages

- Large cargo capacity 7+ kb, 50% more than AAV vectors
- Opportunity to express multiple circRNAs from one vector
- Potent and well-described for vaccine applications
- Established delivery to muscle and lung (intra-nasal/inhaled)

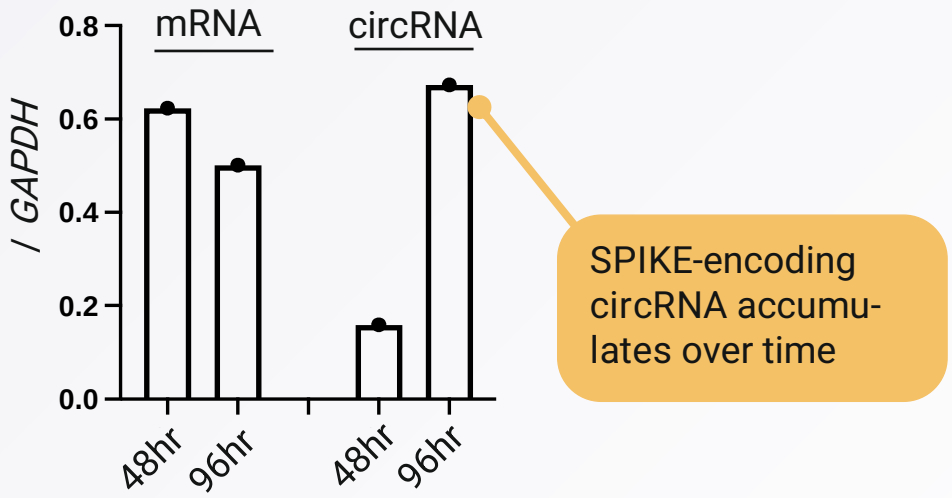
Upcoming milestones

4Q´23:	circVac v1.0 Spike vaccine *in vivo* data
1Q´24:	circVac v2.0 Flu intra-nasal *in vivo* data
1H´24:	circVac v2.0 Spike vaccine *in vivo* data

Durable expression of COVID Spike protein demonstrated for circVac 1.0

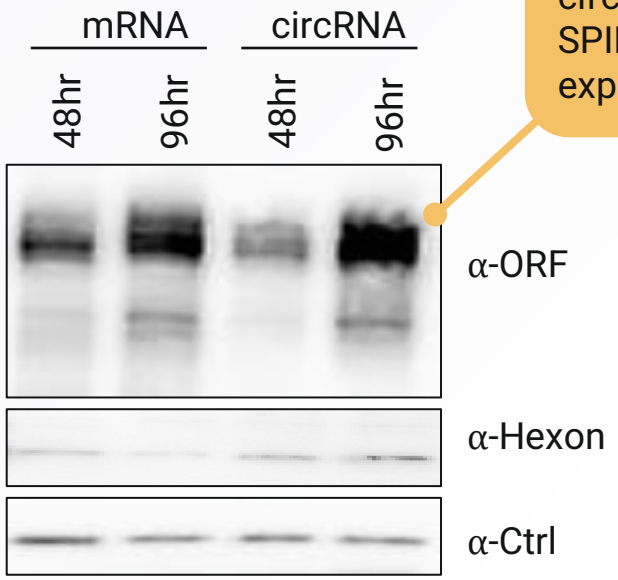
circVac v1.0 COVID Spike expression, RNA and protein level

RNA expression



SPIKE-encoding circRNA accumulates over time

Protein expression



circRNA > mRNA SPIKE protein expression @ 96hr

circVac v1.0 Spike protein *in vivo* experiment ongoing, circVac v2.0 Spike in production

Circio has a unique position in the circRNA field

- Circio is the only significant player in the DNA-format circRNA space

- Enhanced durability and protein expression from circRNA is expected to translate into lower dosing of DNA-format applications, which may solve both potency, toxicity and cost challenges facing current ”gold-standard” gene therapy

- Vector-expressed circRNA has the potential to become the preferred format for any DNA-based therapeutic in the future
  - *Just as synthetic circRNA is expected to become the preferred format for long RNA-based therapeutics in the future*