Technical Journal Club

Marco Losa

21.01.2020

Regulating and controlling gene expression *in vivo* and *in vitro*

Novel tools may open up the next era of gene therapies in medicine

Paper 1

A reversible RNA on-switch that controls gene expression of AAV-delivered therapeutics in vivo

Guocai Zhong[1,3,4,5*], Haimin Wang[1,4,5], Wenhui He[1], Yujun Li[2], Huihui Mou[1], Zachary J. Tickner[1], Mai H. Tran[1], Tianling Ou[1], Yiming Yin[1], Huitian Diao[1] and Michael Farzan[1*]

Nature Biotechnology, 2019

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Paper 2

Dose-dependent activation of gene expression is achieved using CRISPR and small molecules that recruit endogenous chromatin machinery

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Nature Biotechnology, 2019

→ **Harnessing antisense oligonucleotides (Paper 1) and small molecules (Paper 2) to control and regulate (trans)gene expression**

Content

1.) General introduction

2.) Papers

3.) Conclusions

1.) General introduction

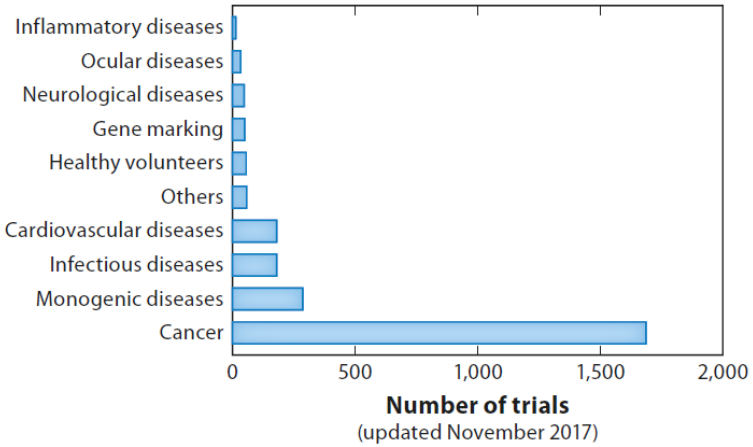
Classification of gene therapies:
a) Class of disease (genetic vs. complex acquired disorder)
b) Route of action
c) Gene delivery vehicle (integrating vs. nonintegrating)
d) Administration route (in vivo or ex vivo)

Anguela and High, Annurev med 2018
Cavazzana-Calvo et al., Science 2000

1.) General introduction

a) **Class of disease**: Gene therapy trials in medicine

- >2500 clinical studies with gene therapies since late 1990s (first one, X-linked SCID, Fischer and colleagues)
- Monogenic diseases, infectious diseases, complex neurodegenerative disorders and cancers



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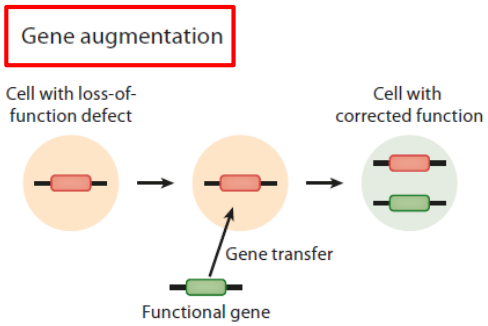
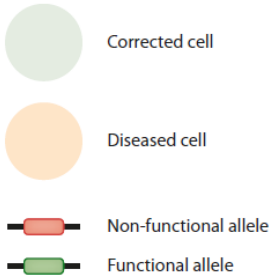
1.) General introduction

b) **Route of action**



- Restore normal cellular function by providing a functional copy of a gene in trans (without affecting diseased gene iteself)
- E.g. ***in vivo*** Leber congenital amaurosis, hemophilias A and B, SMA
- E.g. ***ex vivo*** SCID



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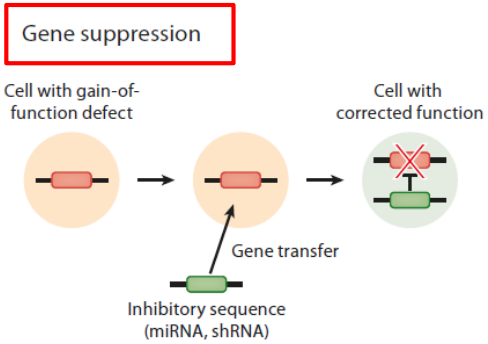
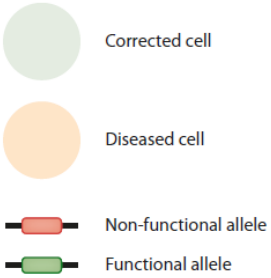


- Gene suppression by reducing expression of the mutated gene via RNA interference
- E.g. Huntington's disease



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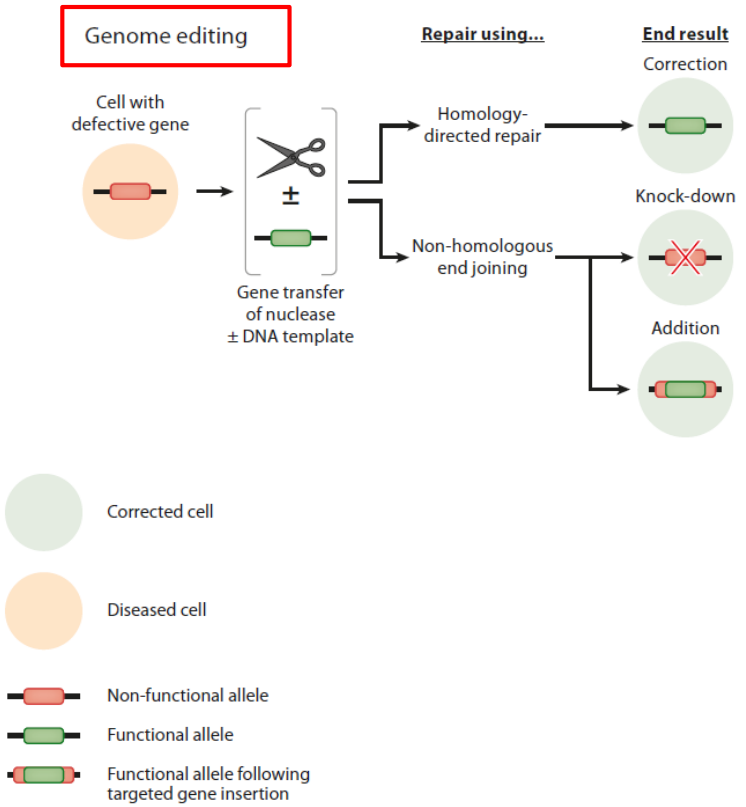

- Gene-specific editing is enhanced by the induction of DNA double-strand breaks at the target site
  → Choice of DNA repair mechanism over another will determine the outcome of genome editing:

- E.g. exogenous template coding for a funcitonal gene, DNA repair may result in a in situ correction of mutated gene via homologous recombination
- E.g. DNA cleavage occurs, break rejoined by non-homologous end joining → knock-down if repair is imperfect
- E.g Insertion of DNA template via non-homologous end joining→ gene addition rather than correction

Anguela and High, Annurev med 2018

1.) General introduction

c) **Gene delivery vehicle**: Viral vectors used

Features	Retroviral	Lentiviral
Viral genome	RNA	RNA
Cell division requirement for target cell	Yes	G1 phase
Packaging limitation	8 kb	8 kb
Immune responses to vector	Few	Few
Genome integration	Yes	Yes
Long-term expression	Yes	Yes
Main advantages	Persistent gene transfer in dividing cells	Persistent gene transfer in transduced tissues

Lentivirus-based systems:

- Belongs to the class of retroviruses (like HIV)
- Carried transgene(s) integrate into genome
- Stable expression in dividing and non-dividing cells (used for stable cell lines)
- Infection of any mammalian cell type possible (VSV-G instead of env gene)

Anguela and High, Annurev med 2018

1.) General introduction

c) **Gene delivery vehicle**: Viral vectors used

Features	Retroviral	Lentiviral	Adenoviral	AAV
Viral genome	RNA	RNA	DNA	DNA
Cell division requirement for target cell	Yes	G1 phase	No	No
Packaging limitation	8 kb	8 kb	8–30 kb	5 kb
Immune responses to vector	Few	Few	Extensive	Few
Genome integration	Yes	Yes	Poor	Poor
Long-term expression	Yes	Yes	No	Yes
Main advantages	Persistent gene transfer in dividing cells	Persistent gene transfer in transduced tissues	Highly effective in transducing various tissues	Elicits few inflammatory responses, nonpathogenic

Lentivirus-based systems:

- Belongs to the class of retroviruses (like HIV)
- Carried transgene(s) integrate into genome
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- Infection of any mammalian cell type possible (VSV-G instead of env gene)

AAV-based systems:

- Single-stranded DNA genome, infects human and some primates
- Virus lacks of pathogenicity (very mild immune response)
- Naturally occuring AAV poorly integrates into genome but only at AAVS1 locus on Chr. 19 (safe harbor)
- If no integration occures (used vectors), genome persists episomal (extrachromosomal)

Anguela and High, Annurev med 2018

1.) General introduction

AAV serotypes defines tissue specificity

Serotype	Primary target tissue	Description
AAV-1	Muscle	Best for cardiac muscle, skeletal muscle, neuronal and glial tissue.
AAV-2	Muscle, Liver, Retina	Most commonly-used serotype. Best for neurons, muscle, liver, and brain.
AAV-3	Megakaryocytes	Best for megakaryocytes, muscle, liver, lung, and retina.
AAV-4	Retina	Best for neurons, muscle, brain, and retina.
AAV-5	Lung	Best for lung, neurons, synovial joint, retina, and pancreas.
AAV-6	Muscle, Lung	Best for lung, liver, and heart.
AAV-7	Muscle, Retina, Neurons	Best for muscle, neurons, and liver.
AAV-8	Liver	Best for muscle, brain, liver, and retina.
AAV-9	Various	Best for muscle, heart, liver, lung, and brain.
AAV-10	Pleura, CNS	Cloned from Cynomolgus, almost identical with AAVrh10 except for 12 amino acids in VP1. Best for lung, muscle, heart, NCS and liver.
AAV-DJ	Various	A mixture of 8 naturally-occurring serotypes. Efficiently transduces a wide variety of cell types *in vitro*.
AAV-DJ/8	Various	A variant of AAV-DJ with a heparin binding domain (HBD) mutation, which permits infection of liver as well as other tissues *in vivo*.

Table 2. List of widely-used AAV serotypes

https://www.genecopoeia.com/resource/aav-vs-lentivirus-choosing-for-dna-delivery/

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Table 2. List of widely-used AAV serotypes

Some clinical trials using AAV-based vectors

Indication	Gene	Route of administration	Phase	Subject number	Status
Cystic fibrosis	*CFTR*	Lung, via aerosol	I	12	Complete
	CFTR	Lung, via aerosol	II	38	Complete
	CFTR	Lung, via aerosol	II	100	Complete
Hemophilia B	*FIX*	Intramuscular	I	9	Complete
	FIX	Hepatic artery	I	6	Ended
Arthritis	*TNFR:Fc*	Intraarticular	I	1	Ongoing
Hereditary emphysema	*AAT*	Intramuscular	I	12	Ongoing
Leber's congenital amaurosis	*RPE65*	Subretinal	I–II	Multiple	Several ongoing and complete
Age-related macular degeneration	*sFlt-1*	Subretinal	I–II	24	Ongoing
Duchenne muscular dystrophy	*SGCA*	Intramuscular	I	10	Ongoing
Parkinson's disease	*GAD65, GAD67*	Intracranial	I	12	Complete[24]
Canavan disease	*AAC*	Intracranial	I	21	Ongoing
Batten disease	*CLN2*	Intracranial	I	10	Ongoing
Alzheimer's disease	*NGF*	Intracranial	I	6	Ongoing
Spinal muscular atrophy	*SMN1*	Intravenous and Intrathecal	I–III	15	Several ongoing and complete
Congestive heart failure	*SERCA2a*	Intra-coronary	IIb	250	Ongoing

https://www.genecopoeia.com/resource/aav-vs-lentivirus-choosing-for-dna-delivery/
https://www.wikipedia.org

1.) General introduction

Potential complications of clinical gene therapies

Potential complications of gene therapy	Strategies to mitigate risks
Gene silencing—repression of promoter	Use endogenous cellular promoters, avoid viral-derived regulatory sequences
Genotoxicity—complications arising from insertional mutagenesis	Use vectors with safer integration profile (e.g., self-inactivating lentiviral vectors) Sequence-specific integration (i.e., genome editing)
Phenotoxicity—complications arising from overexpression or ectopic expression of the transgene	Control transgene expression spatially (e.g., endogenous, tissue-specific promoters) and temporally (on/off switch)
Immunotoxicity—harmful immune response to either the vector or transgene	Carefully monitor T cell reactivity to the vector and transgene to initiate immune suppression if needed
Risk of horizontal transmission[a]—shedding of infectious vector into the environment	Monitor vector shedding in preclinical models when developing novel vectors
Risk of vertical transmission—germline transmission of donated DNA	Use of barrier contraceptive methods until vector shedding is negative

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Paper 1 and 2

One remaining challenge (out of many):

→ **There is a lack of safe, controllable, small and *in vivo* compatible regulatory mechanisms for gene therapies useful in (human) diseases**

Paper 1

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Nature Biotechnology, 2019

Introduction

- Gene therapies are limited by the lack of small genetic switches with wide dynamic ranges that control transgene expression without the requirement of additional protein components.

- Problem: ***In vivo* regulation and control** of transgene expression

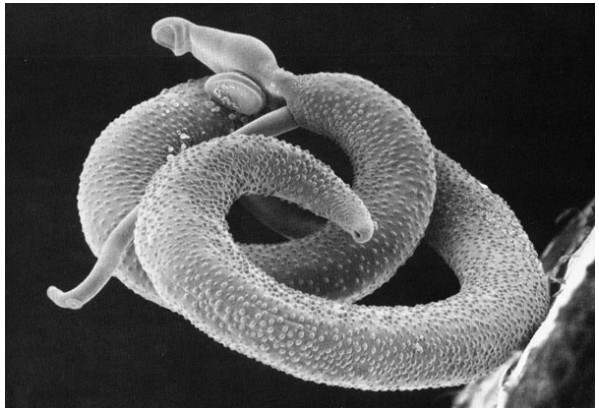
- **Goals:**
  (a) Create a novel ON-switch ***in vivo***
  (b) **Reversible** system which increases safety and reliability
  (c) The switch must **not be a protein** and should be able to administer in humans/mice
  (d) Switch should be **small**
  (e) Switch should be **incorporated into** the gene therapy **vector**
  (f) The introduced system/transgene should **'by default'** be **inactive without leakage**
  (g) **Long-term** and high (trans)gene **expression** should be reliable

Ribozymes

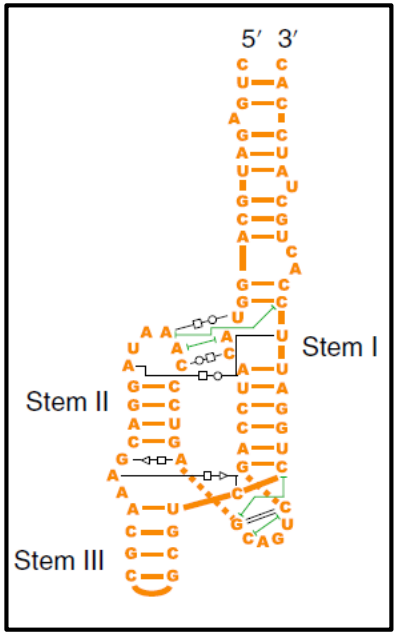
- Ribozymes are RNA molecules with a catalytical ability
- Small in size (<200bp)
- Conserved and present in many species
- Different classes and types of ribozymes:

-E.g. 28S-rRNA synthetizes peptid bond in translation (protein required for stabilization of ribozyme)
-E.g. Spliceosomes, which are ribozymes (protein required for stabilization of ribozyme)

- **Ribozymes without the assistance of a protein: Hammerhead-Ribozymes (HHR)**
- **Some** classes of ribozymes do **have self-cleaving capability** to form their final funcional state



Parasite causing bilharziosis and bladder cancer in humans



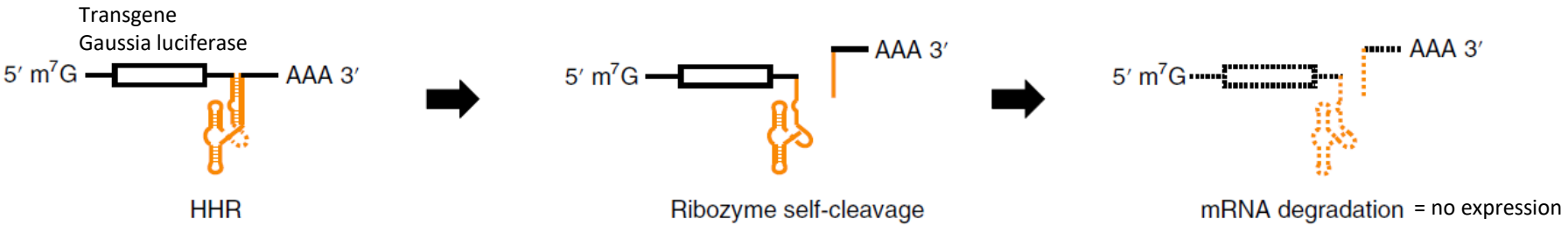
Watson-Crick base-pairing: orange lines
Hydrogen-bonding interactions: black lines
Nonadjacent base stacking: green line

Schistosoma mansoni **type I HHR**

Methods

Rationale/Idea

- Introduction of an AAV delivered transgene system
- Usage of a hammer-headed ribozyme (HHR) sequence 3'- to the transgene
- Engineering of a powerful and reliable self-cleaving HHR
- Upon self –cleavage release of mRNA 3'-UTR that leads to mRNA degradation 'by default'



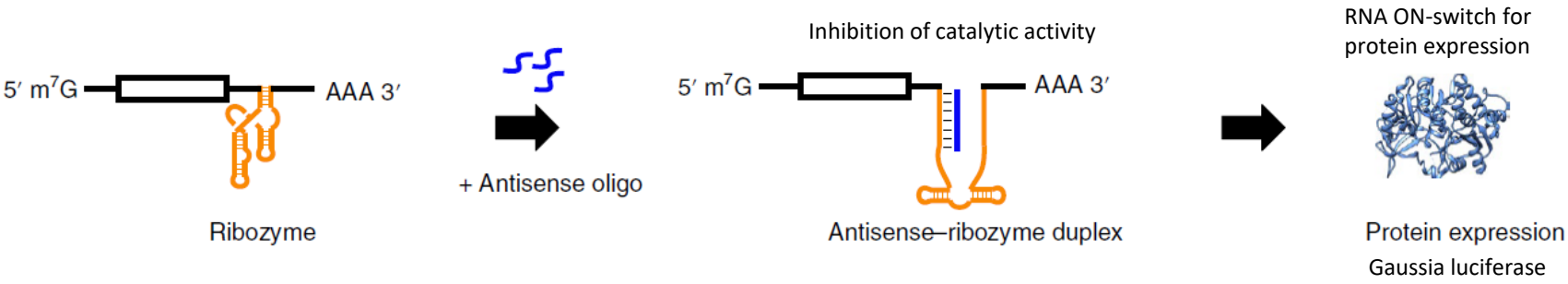
- **Cell culture (293T cells) based reporter inhibition assay** to test ribozymal catalytic activity:

→ Ribozyme sequence introduced 3'-UTR of Gaussia luciferase (Gluc) gene

→ Read out: fold inhibition of Gluc expression relative to expression observed with a corresponding inactive mutant)

Methods

Rationale/Idea



→ Antisense oligonucleotide (Morpholino) administration leads to cutting deficiency of HHR and ‘switches’ protein expression on

Methods

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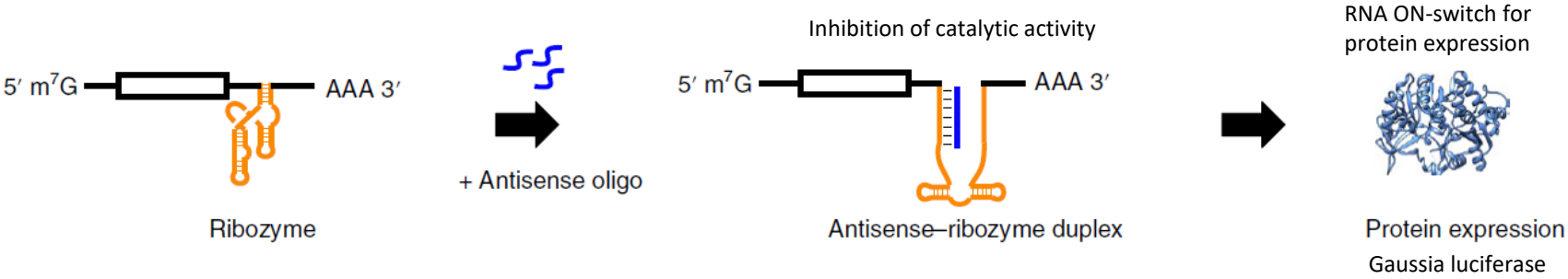

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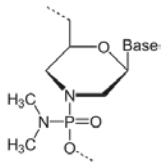
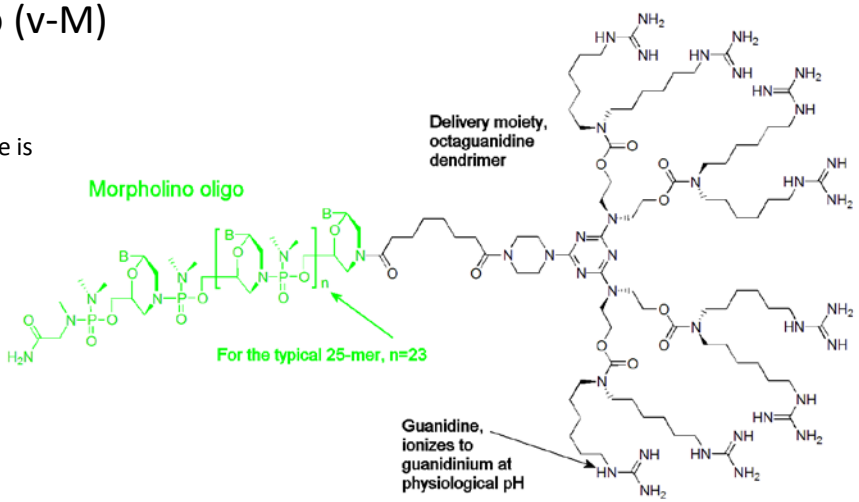
Vivo Morpholino (v-M)

Morpholino oligo: backbone is methylene-morpholine



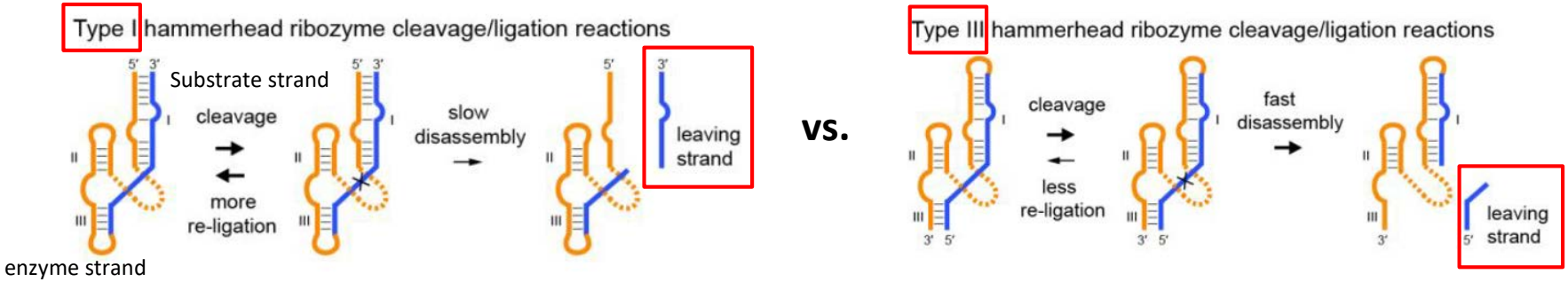


Morpholino, similar to Evans et al. 2019, Nature Comm.

Results

Engineering a class of highly efficient hammerhead ribozymes

Hypothesis



→ Idea: Less leakage due to faster disassembly and less re-ligation

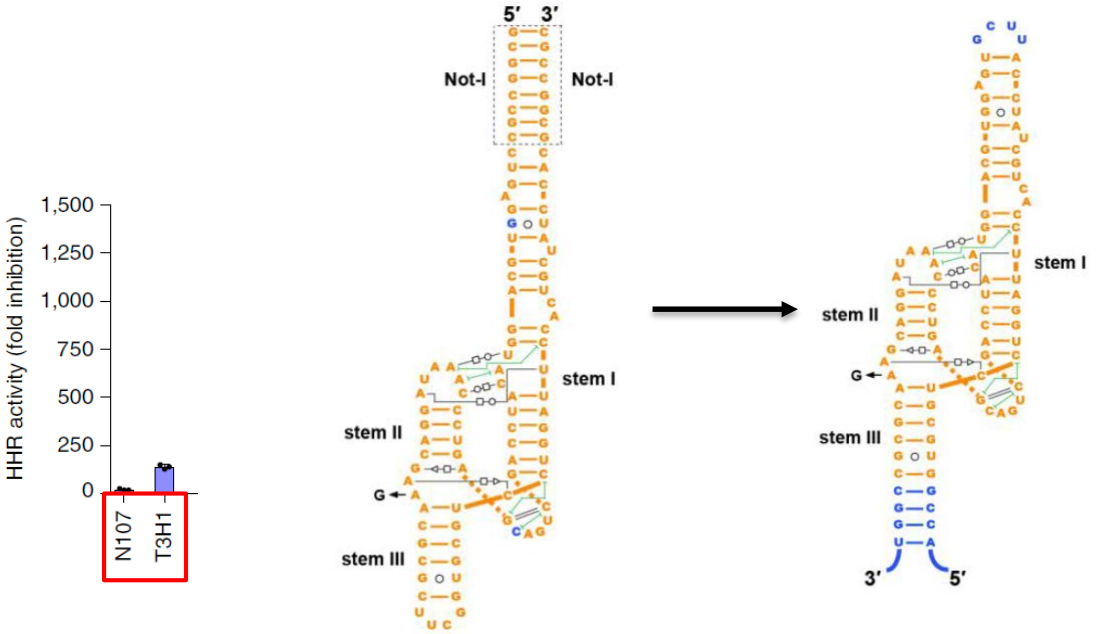
→ Type III: Faster disassembly and **less re-ligation** due to shorter leaving strand and less energy needed

→ Shorter leaving strand may has fewer tertiary interactions

Results

Engineering a class of highly efficient hammerhead ribozymes

Evolution of N107 ribozyme in this study



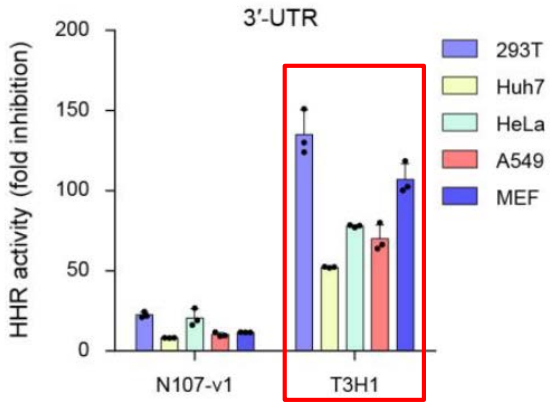
N107
Type I HHR
18-fold inhibition

T3H1
Type III HHR
130-fold inhibition

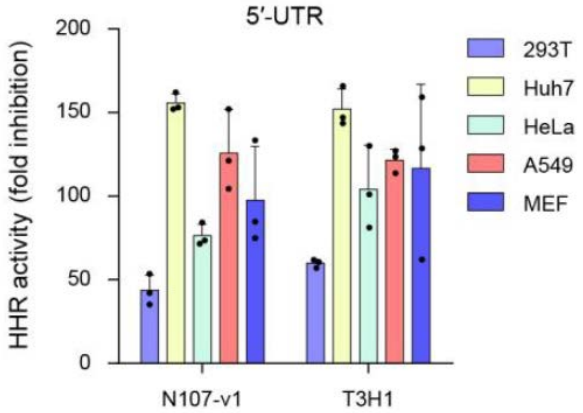
→ **Converting type I HHR to type III significantly improved ribozyme activity (from 18-to 134-fold)**

Results

Ribozyme insertion on 3’-UTR improves inhibition



- **T3H1 outperformed N107 in all cell lines when insertd at 3’-UTR**



- Insertion on 5’-UTR not much of a difference

→ Maybe cellular helicases promote disassembly of both ribozymes

→ Not reasonable to put the switch on 5’-UTR

Results

Engineering a class of highly efficient hammerhead ribozymes

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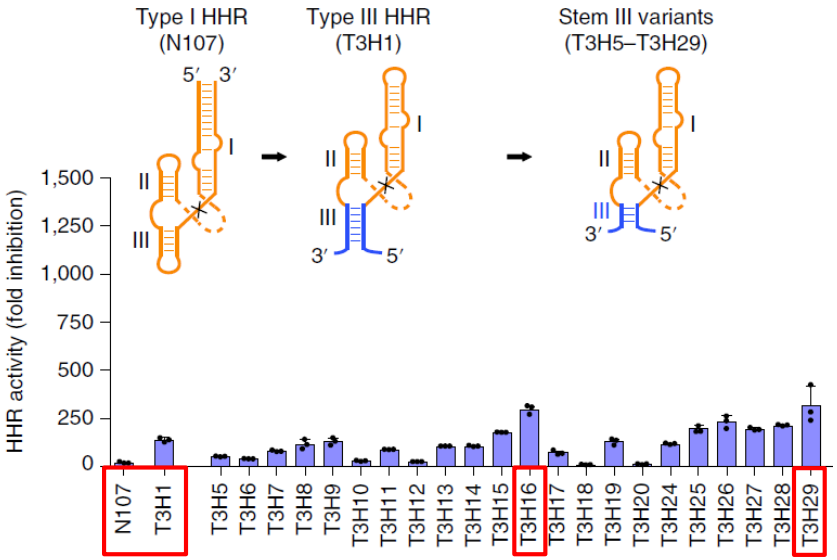
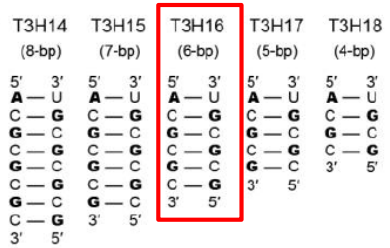
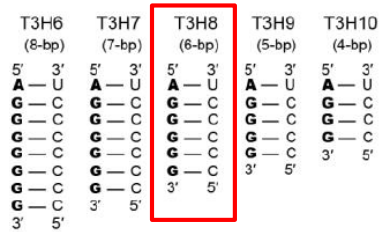
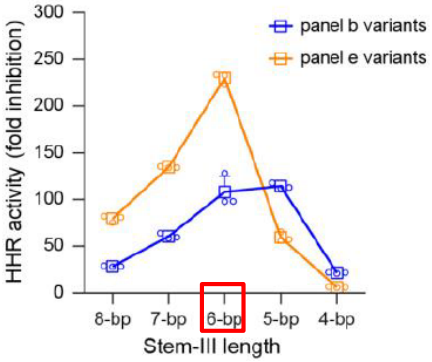


Panel e



Panel b





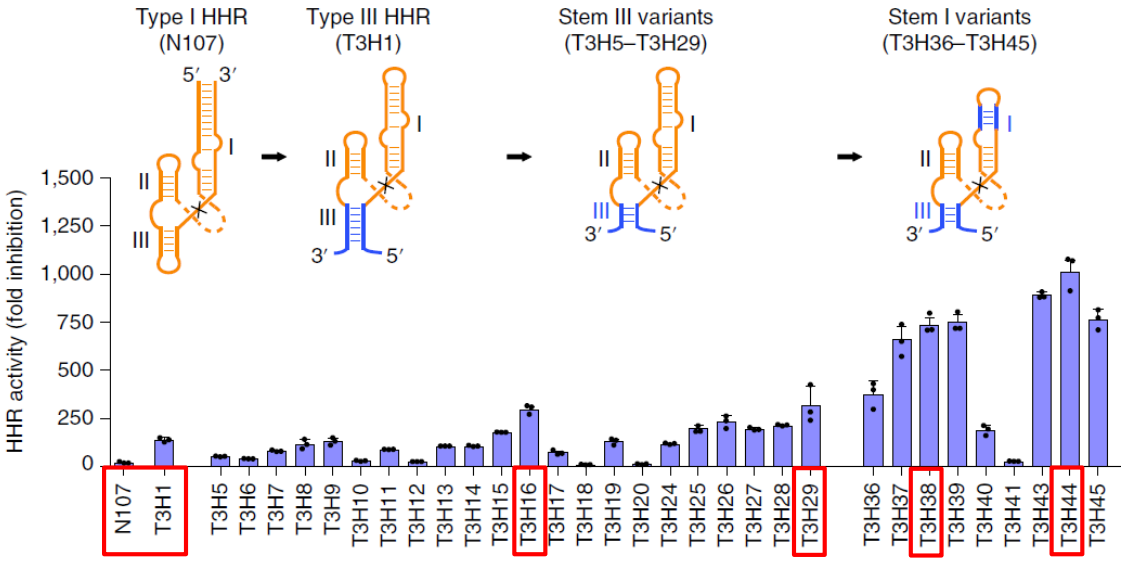
→ **Modification of HHR stem III significantly improved ribozyme activity (from 134-fold to approx. 300-fold)**

→ **Enzymatic activity seems to be optimal with 6-bp stem III**

Results

Engineering a class of highly efficient hammerhead ribozymes

Evolution of N107 ribozyme in this study



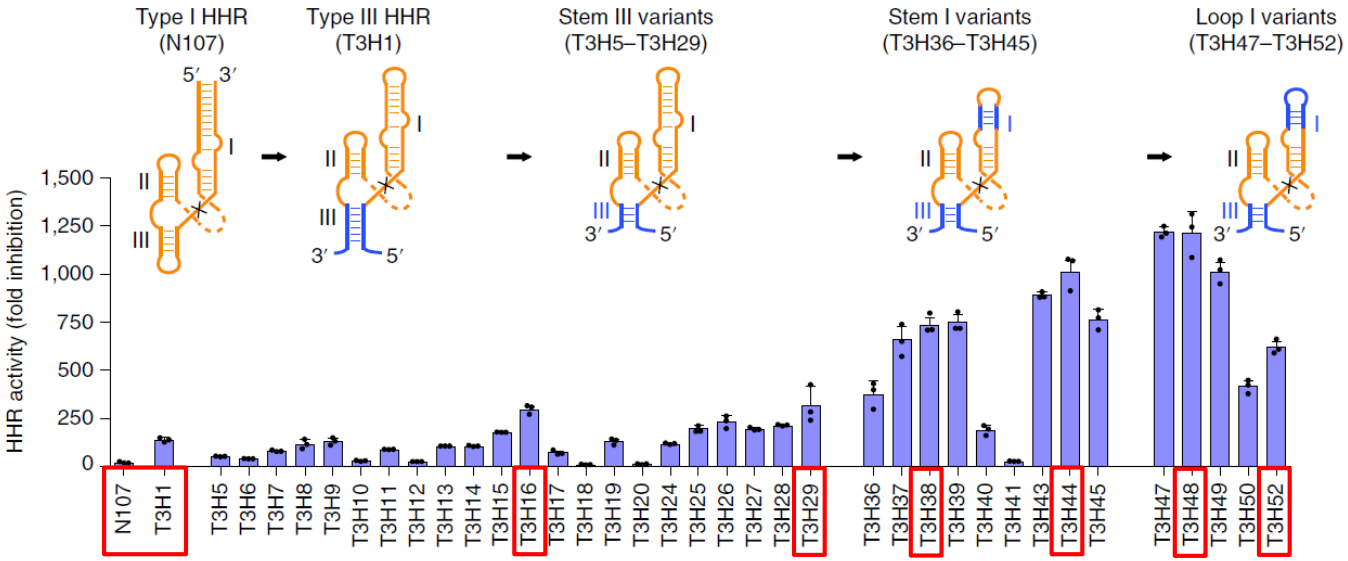
→ **Engineer type I HHR to III HHR,**

→ **Modification of stem III and I significantly improved ribozyme activity (from approx. 300-fold to 730-fold)**

Results

Engineering a class of highly efficient hammerhead ribozymes

Evolution of N107 ribozyme in this study



→ **Engineer type I HHR to III HHR, stem III, stem I on loop I significantly improved ribozyme activity**

→ **Total increase from 18-fold to 1200-fold inhibition (increased catalytical activity→ less re-ligation→ less GLuc)**

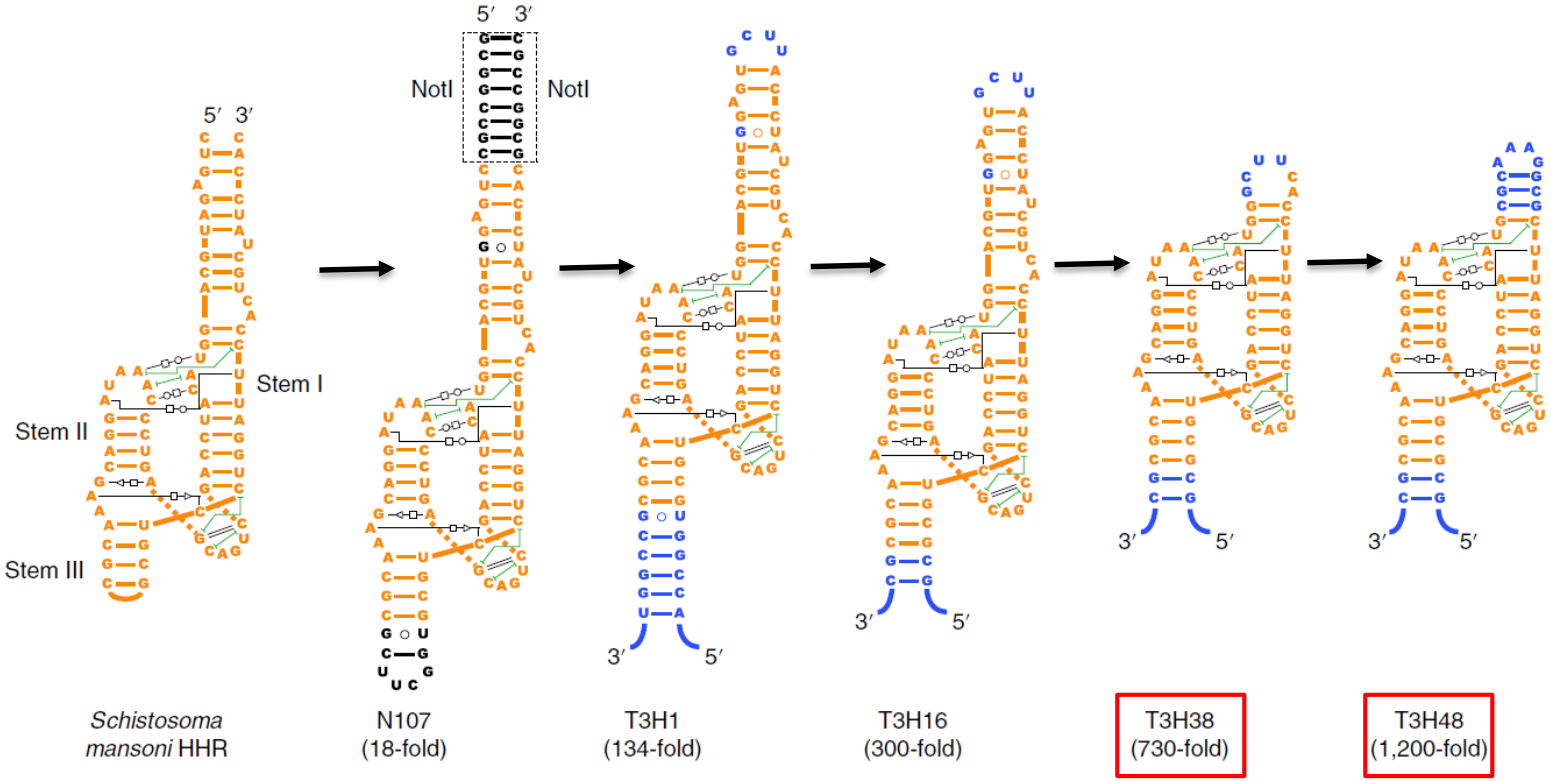
→ **No 'leakage'**

→ **T3H38, T3H48, T3H52 were taken for further experiments**

Results

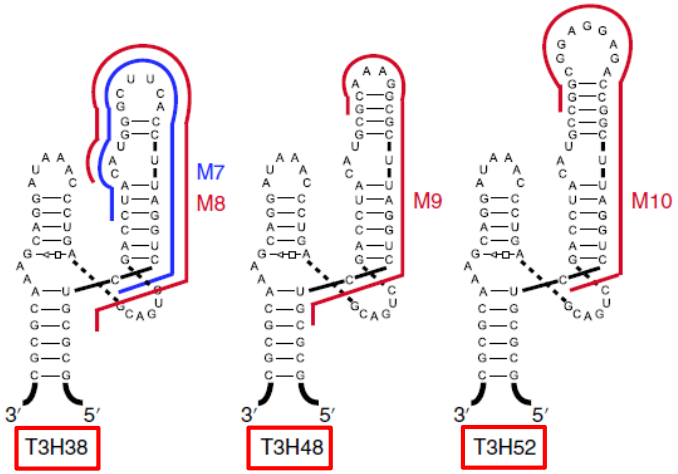
Engineering a class of highly efficient hammerhead ribozymes

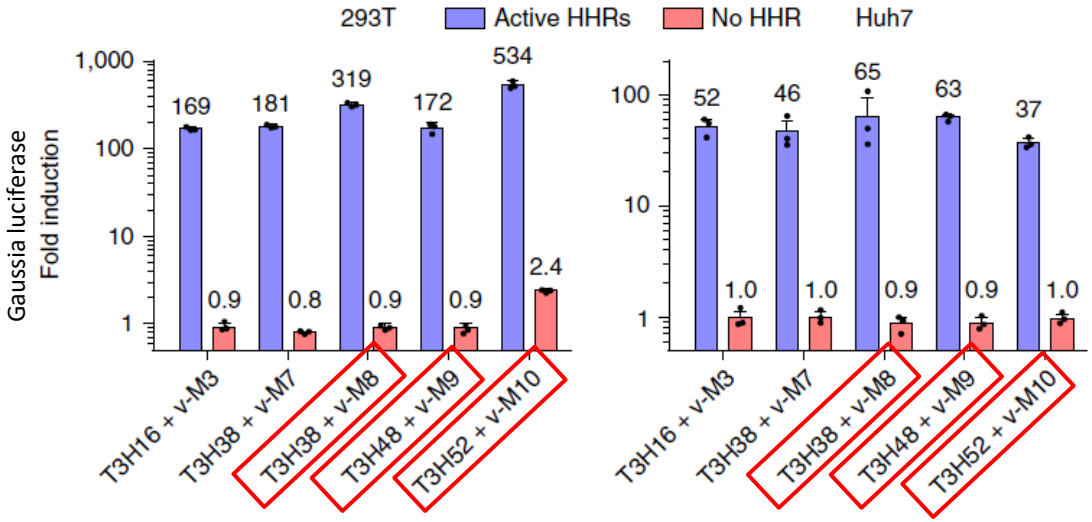
Summary of evolution of N107 ribozyme in this study



Results

Efficient regulation of gene expression using optimized type III HHR ribozymes





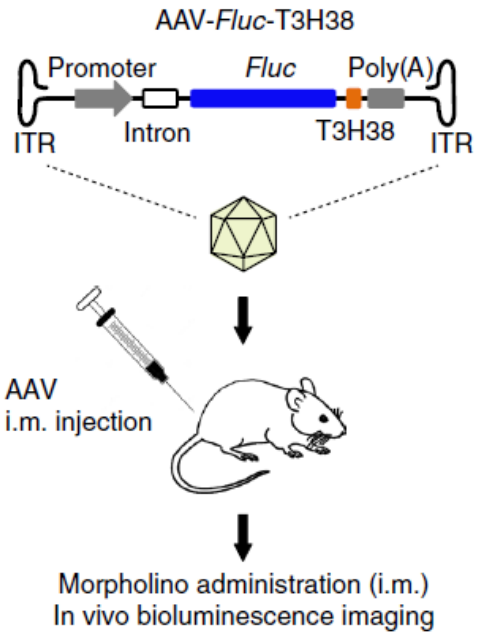
→ **Cell type dependent activities of ribozyme variants and v-M8 Morpholino**

→ **They proceeded with T3H38+v-M8**

Results

In vivo induction of an AAV-delivered reporter transgene (fire fly luciferase (Fluc))

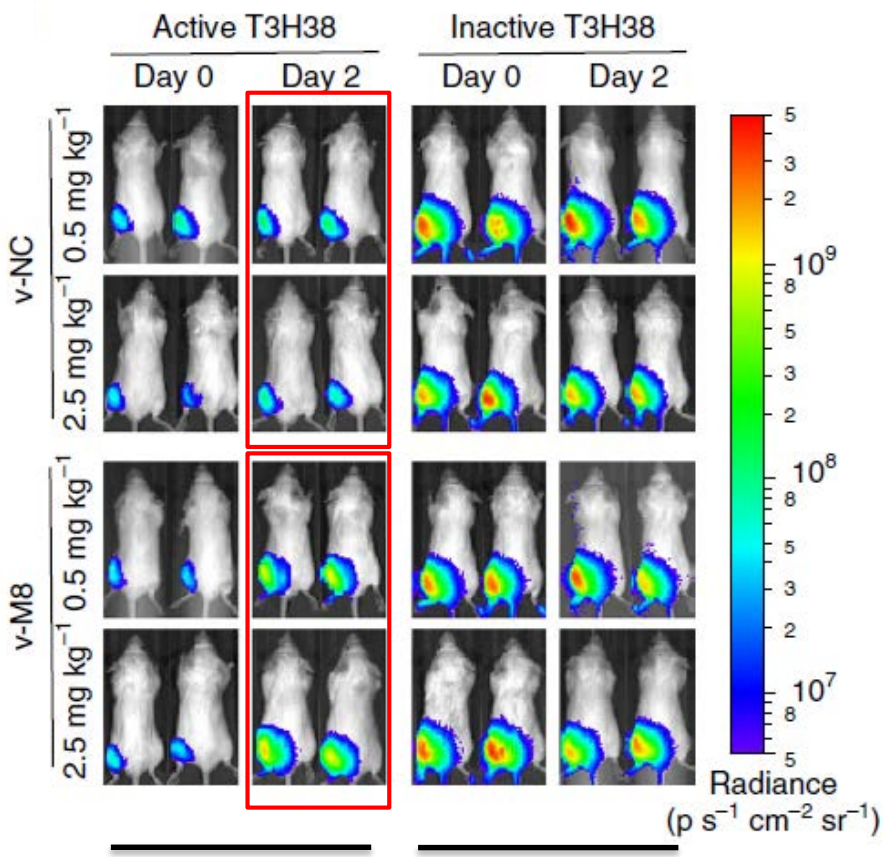
In vivo schematic



AAV1 is muscle specific

Read-out

No targeting Morpholino



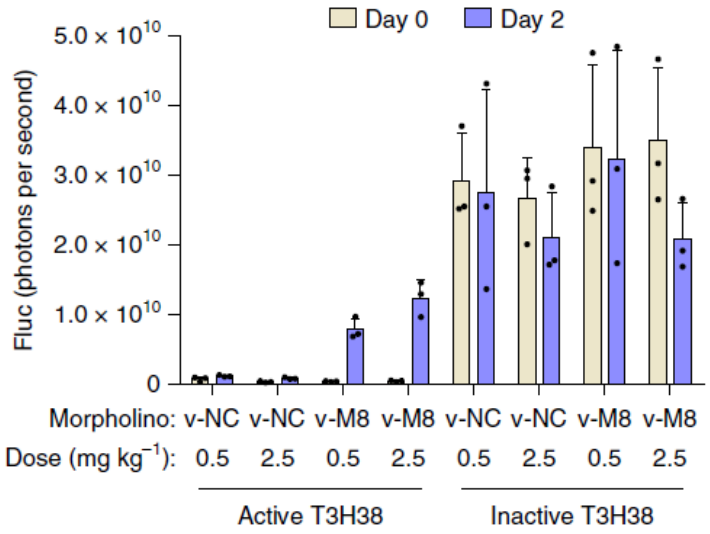
No Fluc Expression (catalytic cleavage) v-M8 induced expr. *in vivo*

Fluc Expression (no catalytic cleavage)

- ITRs in cis (close to transgene) and form hairpins (self priming) and allow primase-independent synthesis of second DNA strand
- Can anneal and form concatemers
- Important for encapsidation of virus
- Rep and cap proteins in trans

Results

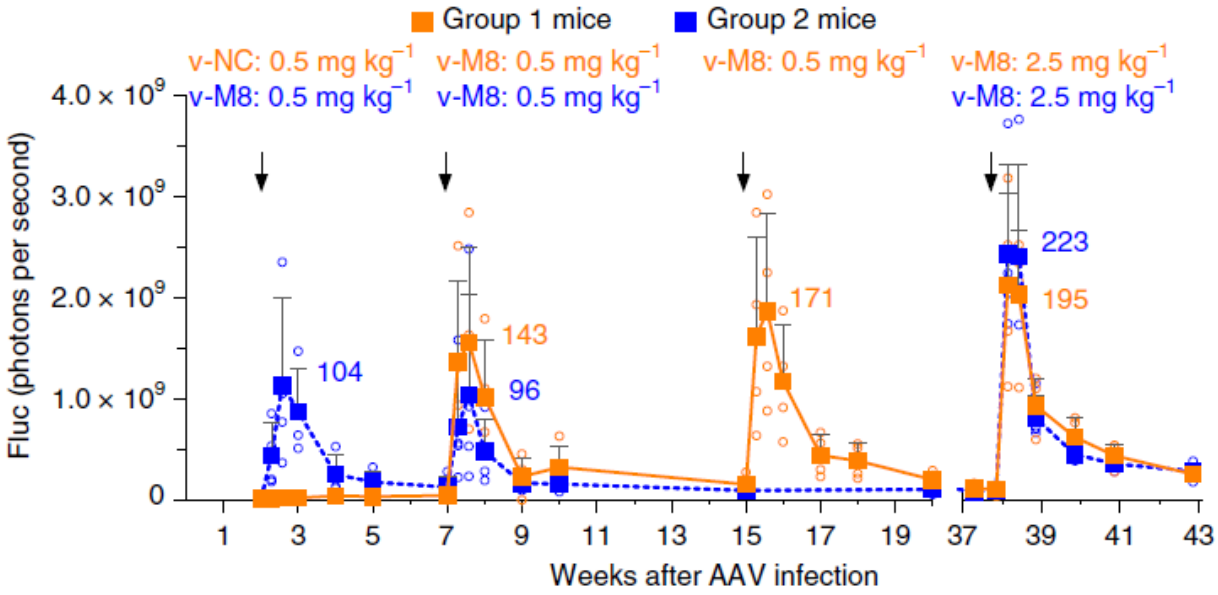
In vivo induction of an AAV-delivered reporter transgene (fire fly luciferase (Fluc))



→ **Dose-dependent induction of luciferase expression**

Results

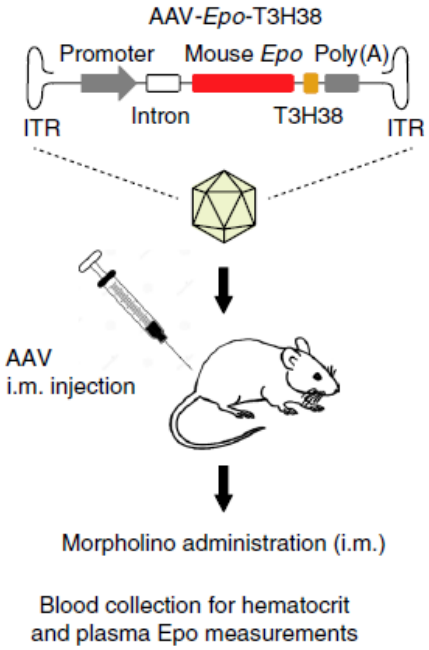
In vivo induction of an AAV-delivered reporter transgene (fire fly luciferase (Fluc))

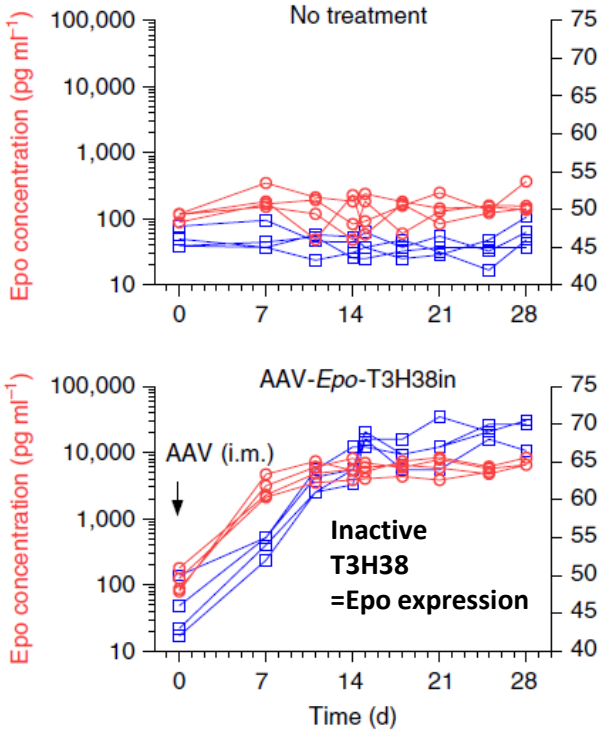


→ **T3H38+ v-M8 system induces Fluc for several weeks (long-term gene expression)**

Results

Reliable *in vivo induction of* Erythropoietin (Epo) using the engineered type III HHR system



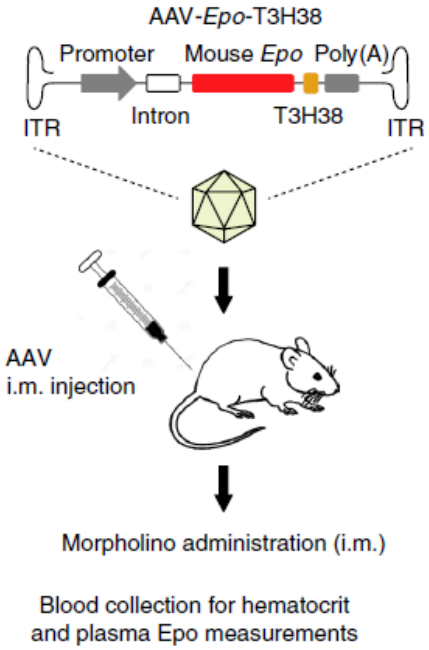


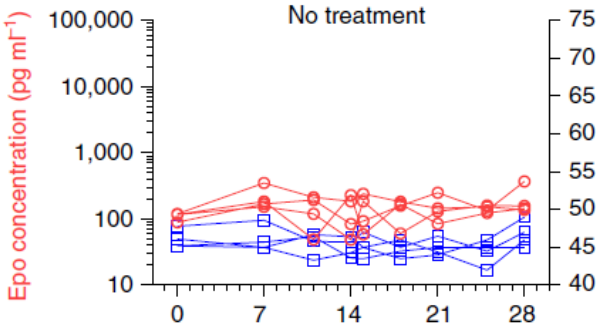
→ **T3H38+ v-M8 system induces reliably Epo expression**

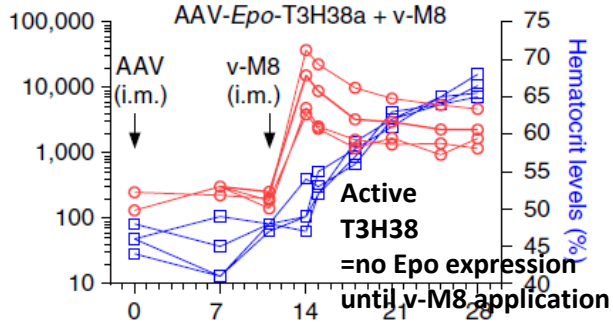
→ **Initial high hematocrit levels**

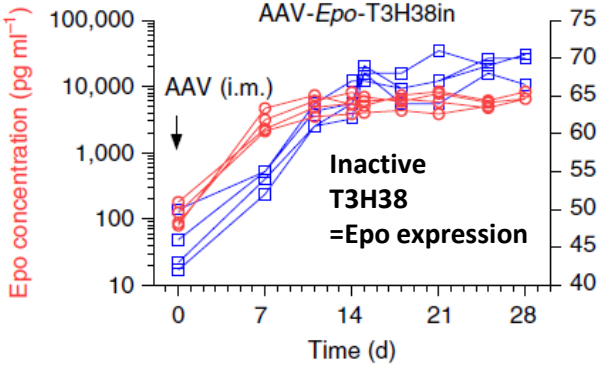
Results

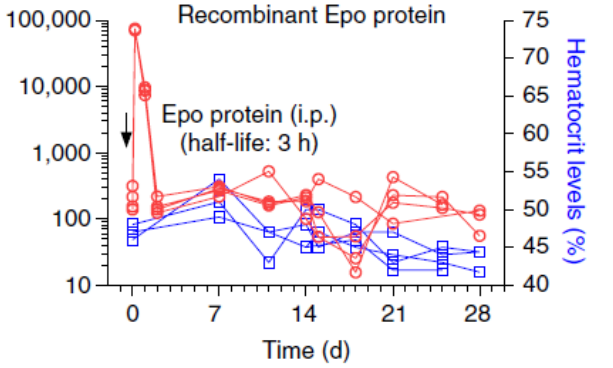
Reliable *in vivo induction of* Erythropoietin (Epo) using the engineered type III HHR system









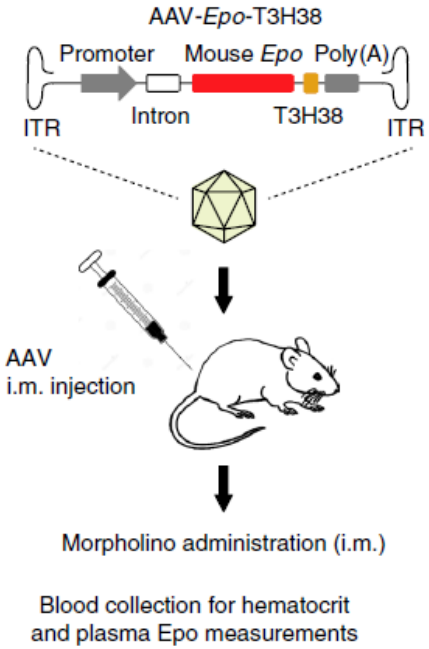


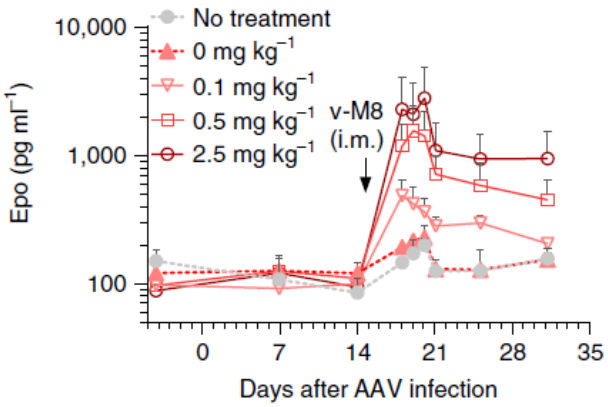
→ **T3H38+ v-M8 system induces reliably Epo expression**

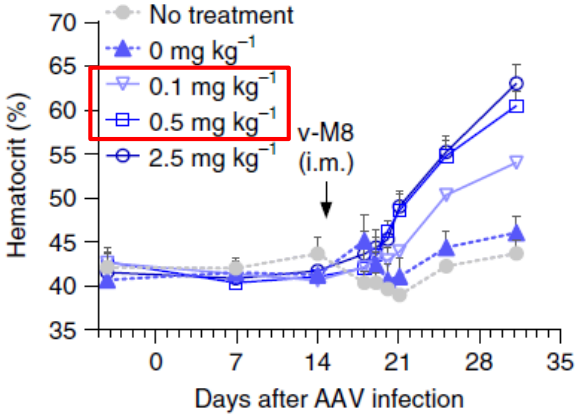
→ **Initial high hematocrit levels**

Results

Reliable *in vivo induction of* Erythropoietin (Epo) using the engineered type III HHR system



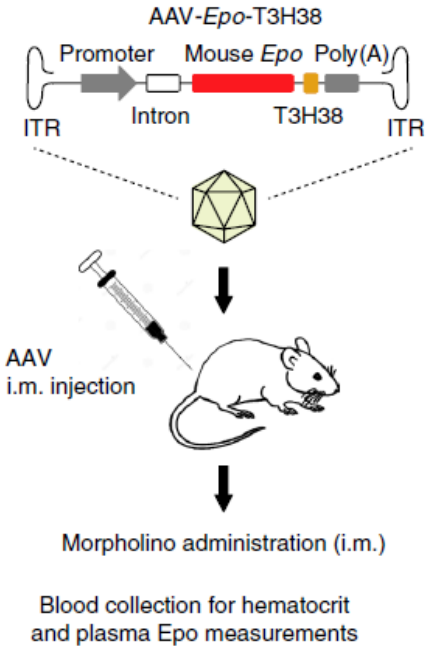


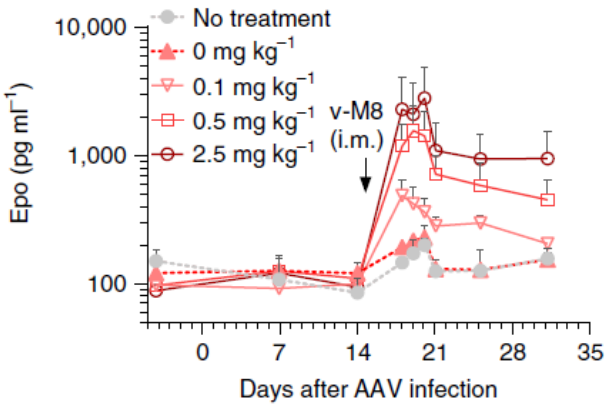


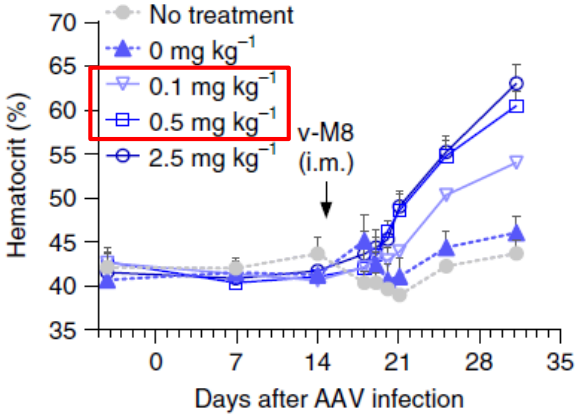
→ **Physiological hematocrit levels seem to be possible**

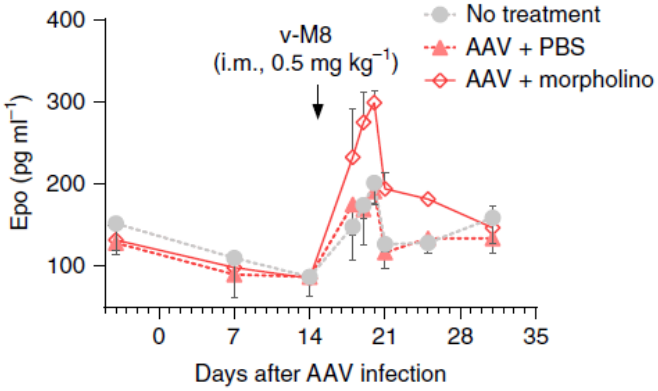
Results

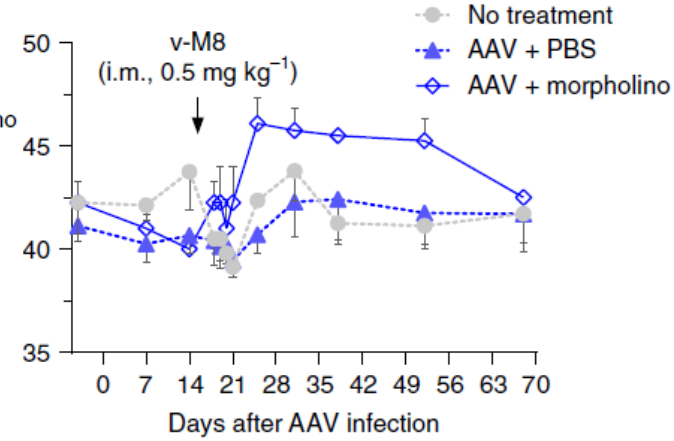
Reliable in vivo induction of Erythropoietin (Epo) using the engineered type III HHR system











→ **Physiological hematocrit levels up to 70 days after AAV infection**

Summary Paper 1

+

- Successful implementation of a promotor independent, well controllable (ON-switch) *in vivo* transgene expression system
- System allows to delay transgene expression well until AAV-induced innate immune responses subside and may prevent emergence of the anti-transgene antibodies observed with other AAV-based systems
- Regulatory element only 63bp
- Local administration and induction allow two or more therapeutics in the same individual
- Morpholinos have been approved for human in from the FDA up to 50 mg/kg
- Long lasting induction of transgene upon Morpholino administration, beneficial for short half-life proteins like Epo.
- Single and well tolerated Morpholino doses can be administered
- Engineered ribozyme allowed the reduction of 'leakage' (protein expression the absence of antisense oligo)

-

- Only one transgene (Epo) tested in the study
- Large genes (>4.8kb are unsuitable for standard AAV vectors

Paper 2

Dose-dependent activation of gene expression is achieved using CRISPR and small molecules that recruit endogenous chromatin machinery

Anna M. Chiarella[1], Kyle V. Butler[2], Berkley E. Gryder[3], Dongbo Lu[1], Tiffany A. Wang[1], Xufen Yu[2], Silvia Pomella[3,4], Javed Khan[3], Jian Jin[2*] and Nathaniel A. Hathaway[1*]

Nature Biotechnology, 2019

Introduction

- Genes can be activated or suppressed using CRISPR-Cas9 systems. However, tools that enable dose-dependent activation of gene expression without the use of exogenous transcription of regulatory proteins are lacking.

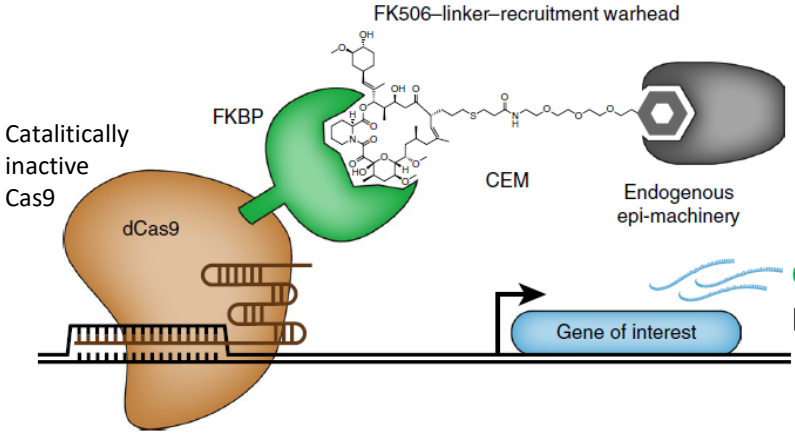
- Problem: **Dose-dependent activation** of (trans)gene expression using small molecules

- Goals:
  (a) Create a novel tool which **harnesses small molecules** for gene expression regulation
  (b) **Reversible and competeable** system
  (c) Potentially useful for *in vivo* studies
  (d) **Activation of endogenous genes** should be **similar to established CRISPRa systems** (e.g. d Cas9-VPR)

Methods

Rationale/Idea

Using chemical epigenetic modifiers (CEMs) to increase gene activation



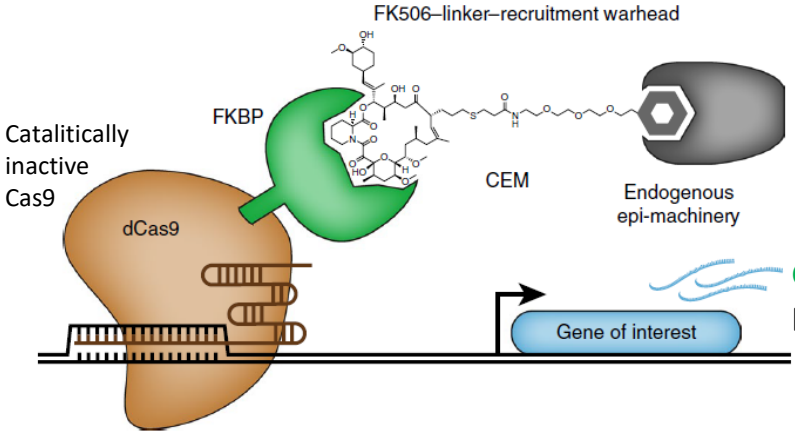
GFP reporter gene under TRE3G promotor

Endogenous genes

Methods

Rationale/Idea

Using chemical epigenetic modifiers (CEMs) to increase gene activation

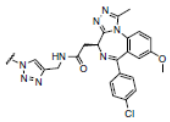
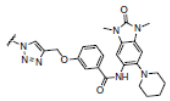
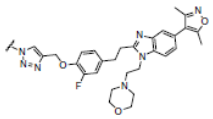


GFP reporter gene under TRE3G promotor

Endogenous genes

CEMs with their recruitment protein

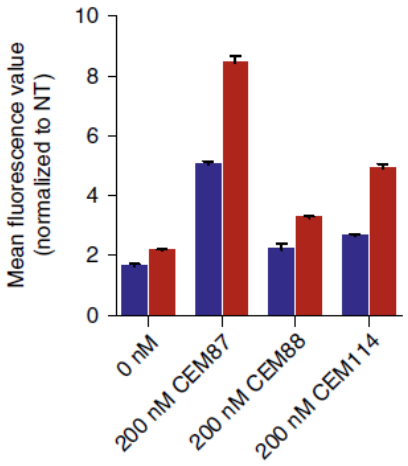
Endogenous chromatin machinery

CEM87		BRD4	Associates with chromosomes during mitosis and targets chromatin
CEM88		BRPF1	Forms a complex with Moz/Morf-Hbo1 and targets chromatin to regulate transcription (acetylation)
CEM114		CBP/p300	Interacts with CRE (cAMP response element) and enhances transcription target genes

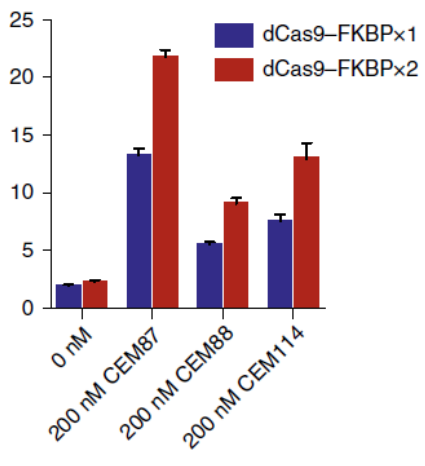
Results

Characterisation of dCas9-FKBP system

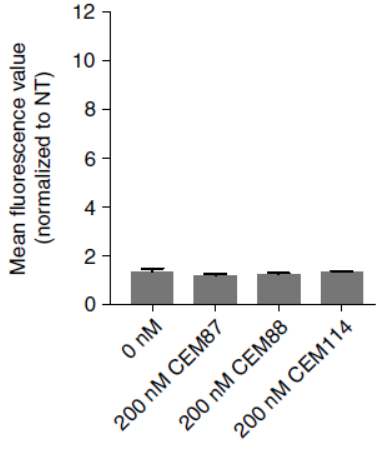
GFP



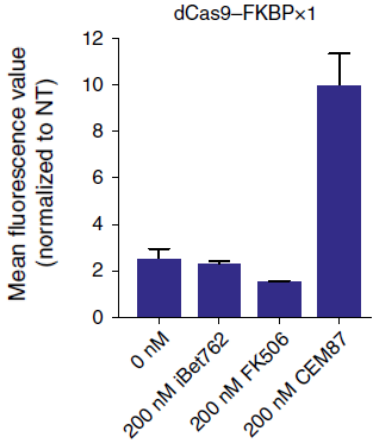
GFP



No GFP



Treatment with individual recruitment components

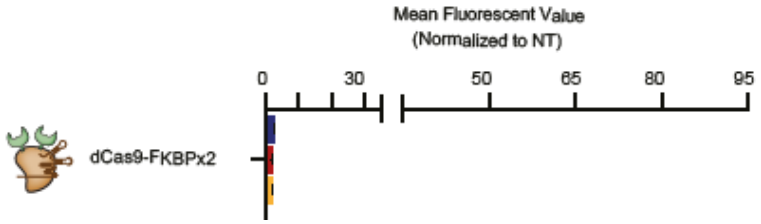


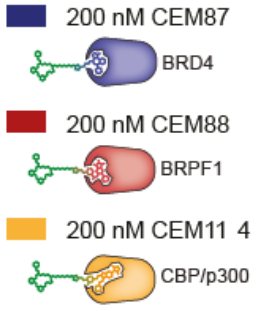
→ **No GFP expression with iBet762 and FK506**

→ **CEM linker-dependent activation**

Results

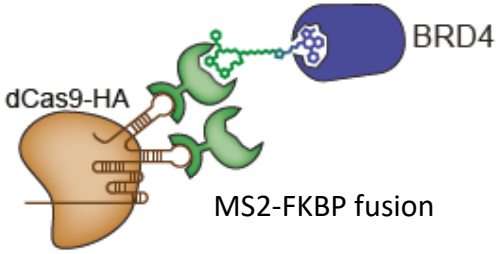
Optimisation and characterisation of dCas9 recruitment strategies





Results

Ms2-tagging (gRNA containing MS2-compatible stem loops)

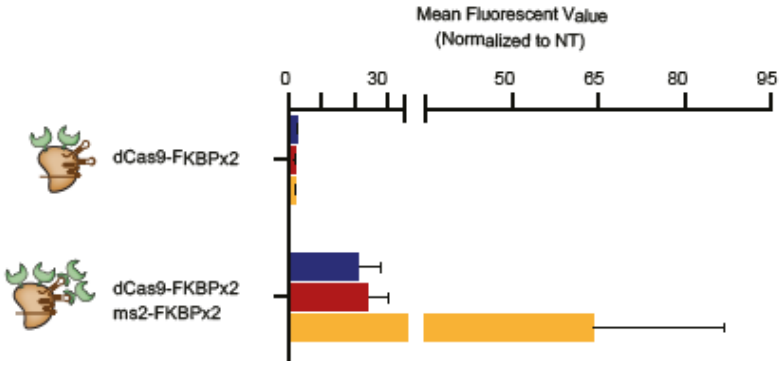


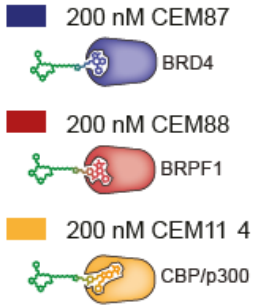
- Natural RNA-Protein interaction of MS2 bacteriophage coat protein with a stem loop structure from viral RNA to repress viral replicase in noninfected cells
- Used to monitor RNA at the site of translation

- Here: interaction of MS2 protein and gRNA (stem loops) from CRISPR system

Results

Optimisation and characterisation of dCas9 recruitment strategies

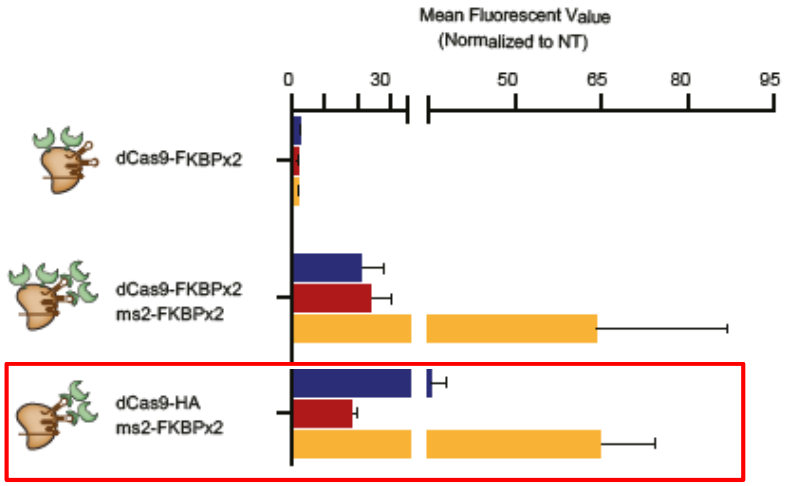




→ **FKBP fusions must be strategically chosen not just increased in numbers**

Results

Optimisation and characterisation of dCas9 recruitment strategies



200 nM CEM87
BRD4
→ worked

200 nM CEM88
BRPF1

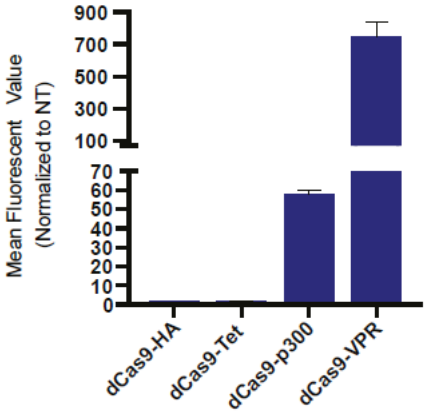
200 nM CEM11 4
CBP/p300
→ worked

→ FKBP fusions must be strategically chosen not just increased in numbers

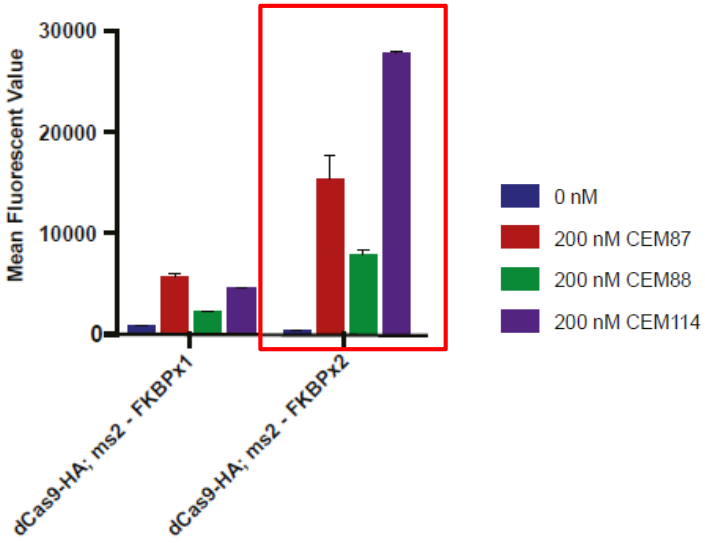
Results

Optimisation and characterisation of dCas9 recruitment strategies

dCas9 direct fusion



ms2-FKBP comparison

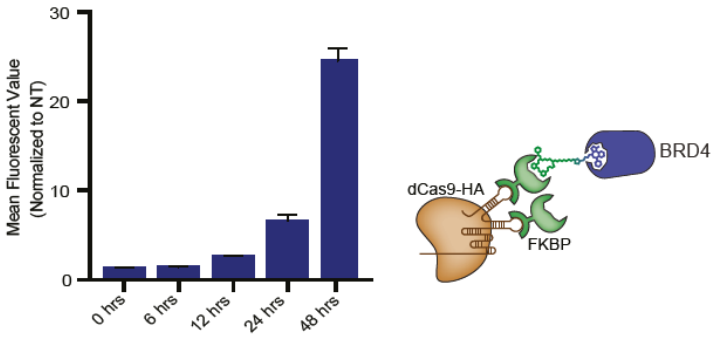


Results

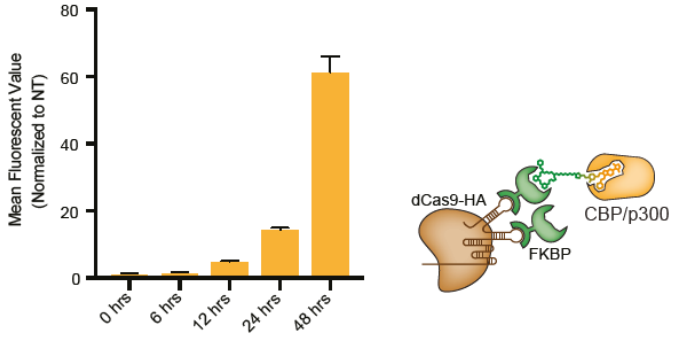
Optimisation and characterisation of dCas9 recruitment strategies

Time-course of final strategy with CEM87 and CEM114

CEM87 exposure



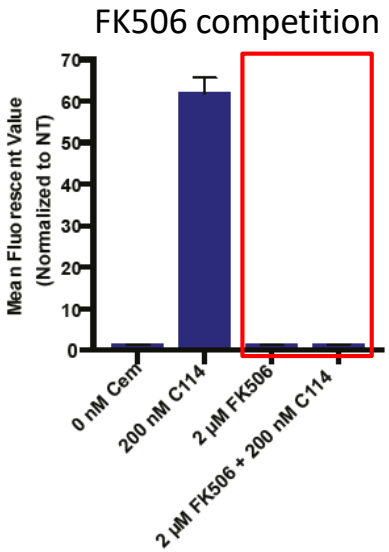
CEM114 exposure



→ GFP expression was highest 48 hours post CEM recruitment

Results

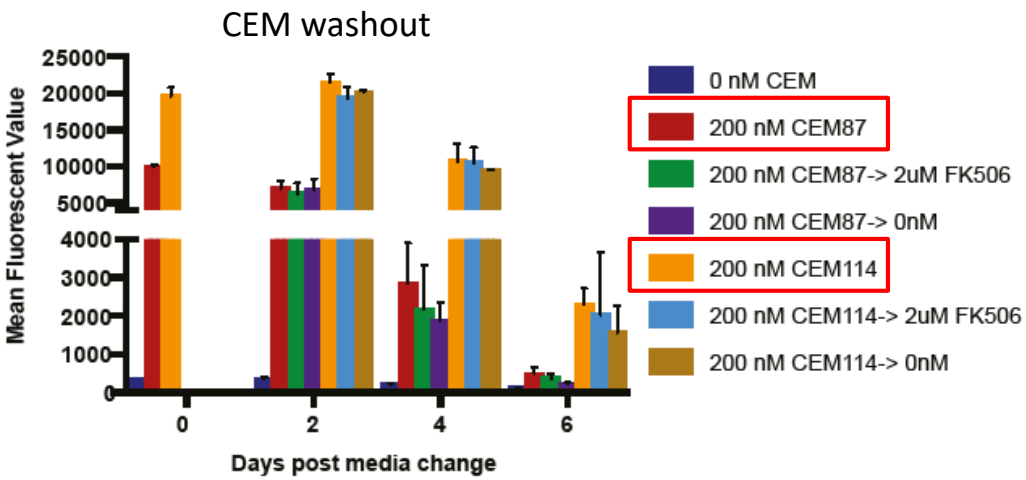
The dCas9-HA; ms2-FKBPx2 system is reversible



→ **Gene expression activation is competable**

Results

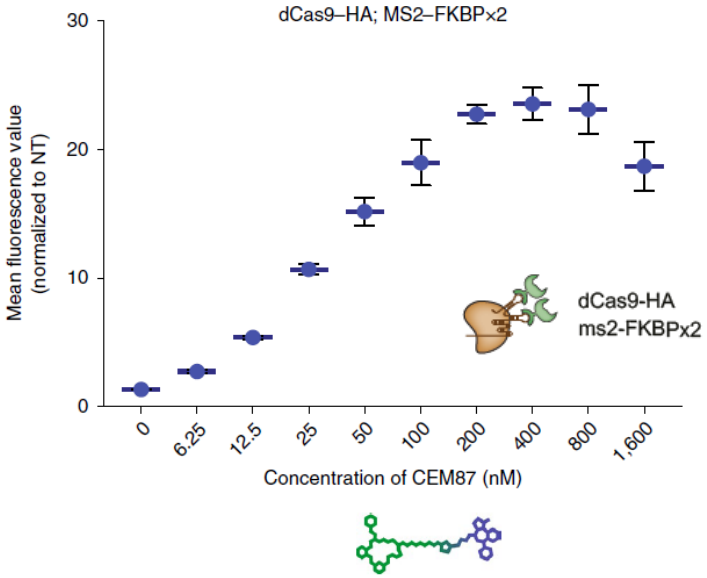
The dCas9-HA; ms2-FKBPx2 system is reversible

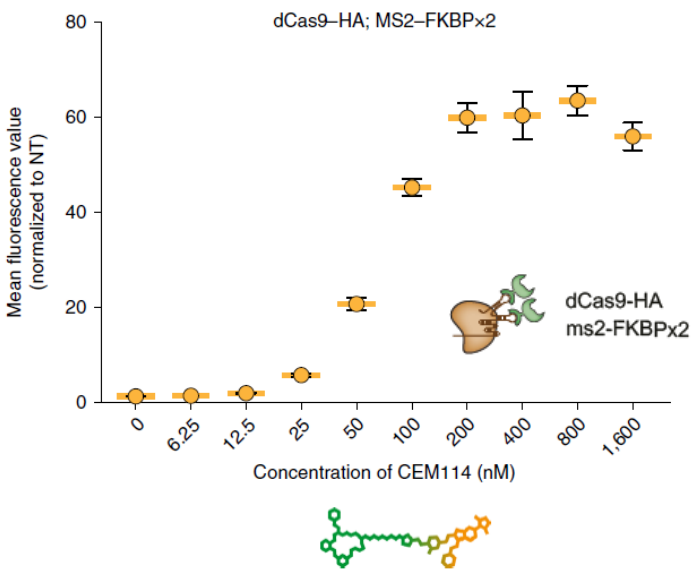


→ **Gene expression is reversible upon time (CEM washout)**

Results

Dose-dependent **transgene (GFP)** activation using dCas9-HA; MS2-FKBPx2 system



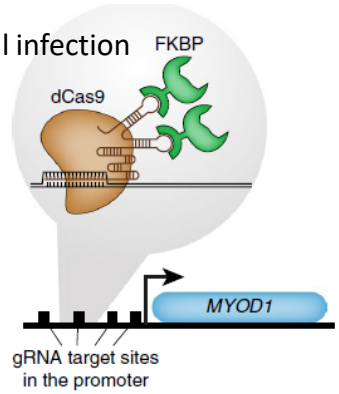


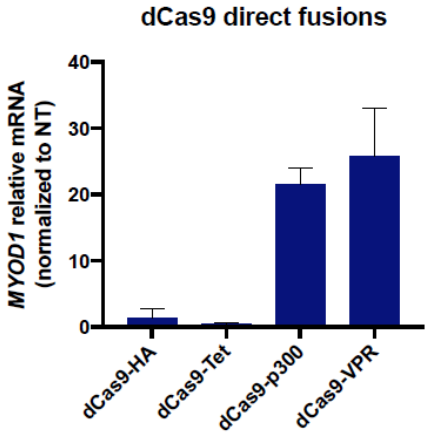
→ **Dose-dependent regulation of gene (GFP) activation between 6.25 and 200 nM of CEM87 and CEM114**

Results

Endogenous gene (MYOD1) targeted using optimized dCas9-CEMa system

Lentiviral infection

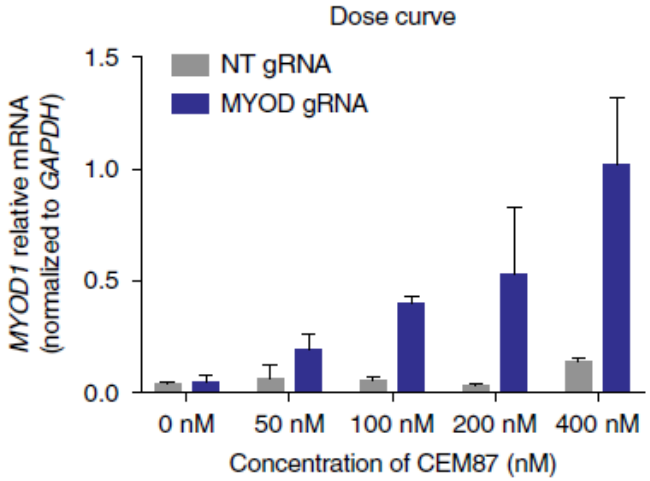




→ **dCas9-HA system has no MYOD1 expression without CEM87 (almost no leakage)**

→ **dCas9-p300 and dCas9-VPR systems activate MYOD1 expression upon transfection**

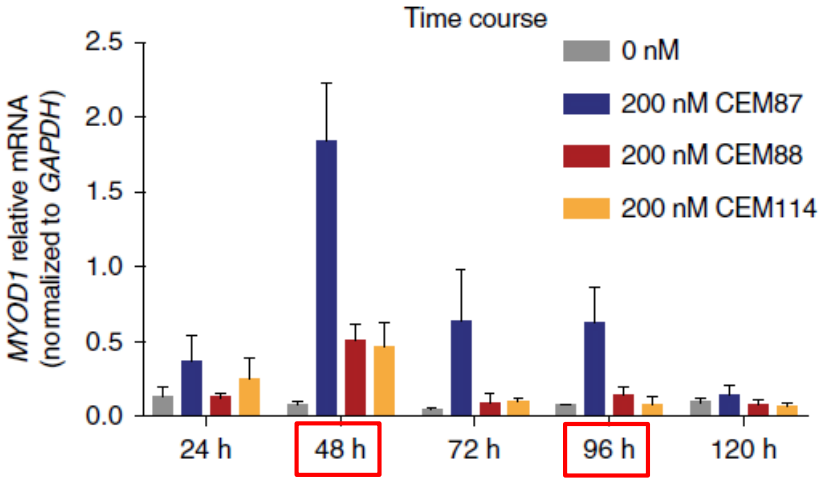
qRT-PCR



→ **dCas9-HA;ms2-FKBP activates MYOD1 gene expression upon CEM87 addition in a dose-dependent manner**

Results

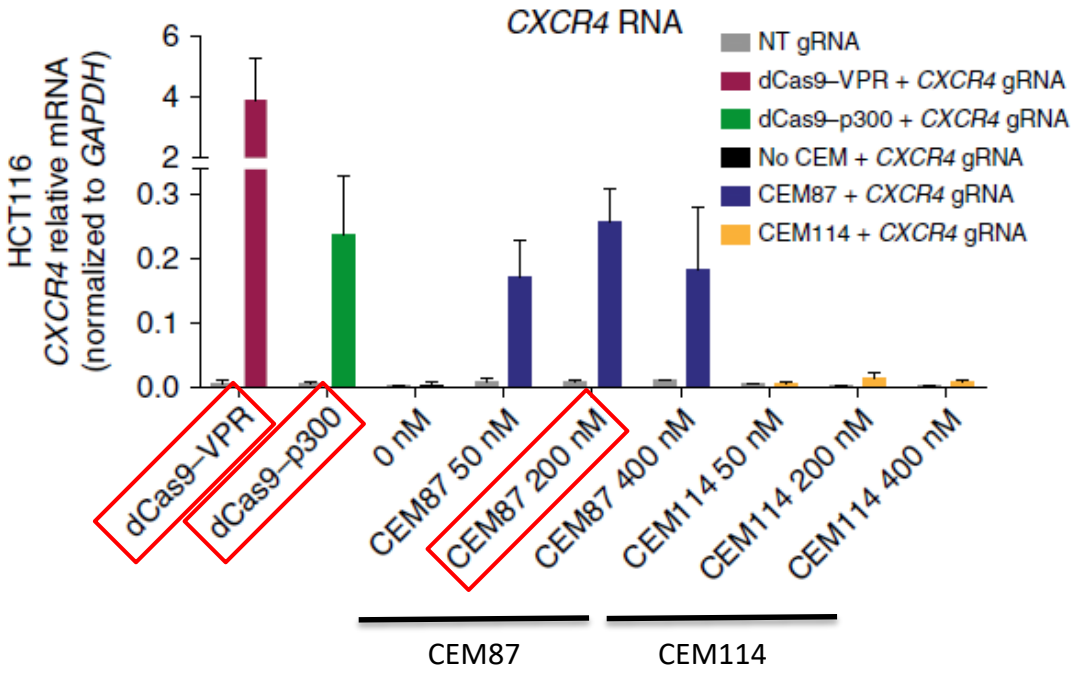
Endogenous gene (MYOD1) targeted using optimized dCas9-CEMa system



→ **After 96 hrs CEM87 activation of MYOD1 (in HEK293T cells) was no longer significant**

Results

Benchmarking the dCas9-HA;ms2-FKBP system to current dCas9 activating systems



→ **Similar activation to dCas9-VPR and dCas9-p300**
→ **CEM114 does not activate the CXCR4 gene**
→ **Low expressing genes: IL1RN, OCT4 (both significantly increased)**
→ **High expressed gene: 1 gRNA MYC1 (no significant CEM87-induced activation)**

→ **Groups created a set of gRNA targeting super-enhancer (SE) network controlling MYOD1 and there was a significant increase in MYOD1 expression (indirect activation)**

Summary Paper 2

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- Establishment of a tool using small molecules for gene activation *in vitro*
- Successful dose-dependent activation of endogenous/ transgene expression using CRISPR and CEMs
- Reversible and competeable system which may be useful for *in vivo* gene therapies
- Low expressing genes can be activated similarly to established CRISPR activators
- Possible indirect activation of genes (e.g. MYOD1)

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- No data regarding bioavailability/toxicity of CEMs since only *in vitro* data published
- Highly expressed genes are difficult to activate/control with the CEMa system

3.) Conclusions

- Successful implementation of reversible switches and regulators harnessing antisense oligonucleotides and small molecules

- Efficient and safe regulation of (trans)gene expression may allow the use of modern gene therapies in various (human) diseases in the future

Thank you for your attention