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When size matters: FVIII construct optimization leveraging ceDNA, a non-viral gene therapy platform

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When size matters: FVIII construct optimization leveraging ceDNA, a non-viral gene therapy platform

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Generation Bio, Cambridge, MA

Development of successful gene therapies requires strategies for optimization of transgene expression that navigate the vast combinatorial complexity of sequence design space. Strategies that assume functional independence of genetic elements are attractive as they allow for independent screening of distinct genetic element modules, followed by combinatorial screening of only the best performers from each module. However, violation of this assumption increases the risk that either improved combinations are not identified or that unanticipated interactions between components limit the effectiveness of modular combinations. Additional constraints on the exploration of sequence design space are encountered by viral gene therapies, such as AAV, due to intrinsic limitations on the size of genetic elements that can be encoded. Meeting these challenges will likely require both new platform technologies and construct optimization strategies.

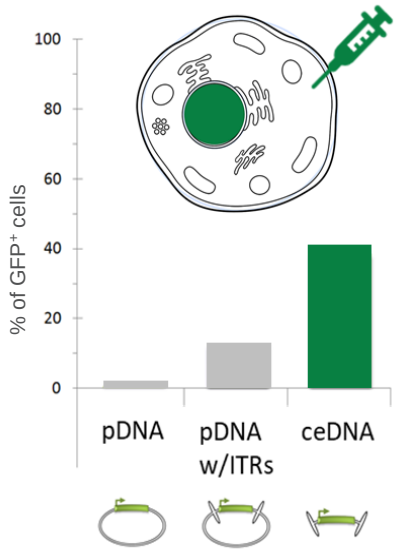
We have developed a non-viral gene therapy platform to deliver and durably express therapeutic proteins systemically *in vivo*. It is comprised of ceDNA, an engineered, double-stranded, linear, covalently closed-ended DNA construct, formulated in a cell-targeted lipid nanoparticle delivery system, ctLNP. This platform permits transgene optimization largely free from the size limitation of viral platforms. We have taken advantage of this feature to perform a semi-combinatorial optimization of FVIII expressing ceDNA for the treatment of Hemophilia A. Here we report identification of increases in construct expression across a range of genetic elements, including codon optimization, promoters, introns, secretion signals, 5' and 3' UTRs. Together, these optimizations resulted in marked improvements in FVIII peak expression *in vitro*, which translated to improved potency of ctLNP-ceDNA in mouse models of Hemophilia A.

We also discovered numerous instances of sequence context dependence that impacted performance of individual genetic elements, highlighting the limitations of modularity as a framework for construct optimization. For instance, the performance of 5' UTR sequences was shown to be highly dependent on the codon optimization of the open reading frame, which was not anticipated based on current mechanistic understanding of translation initiation. Uncovering important interaction effects between genetic elements also offers the chance to develop design rules that allow focusing of optimization efforts without sacrificing design complexity. In this regard, we uncovered important design principles governing the function and impact of intronic sequences on transgene performance.

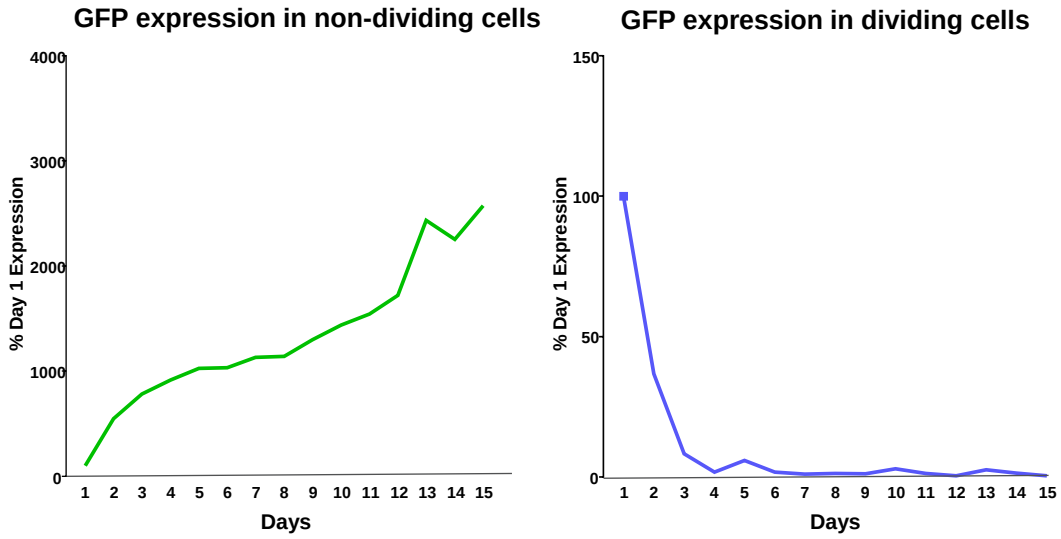
Optimization of constructs for gene therapy applications will need to meet new challenges as the field develops, such as incorporation of more complex regulation to enable better response of gene therapies to individual disease presentation. Towards that end, we have shown how relaxed constraints on the size of cis-regulatory elements can open the opportunity for different dimensions of optimization in the future.

ceDNA is a closed-ended, linear, duplex DNA vector whose structure imparts key features for non-viral gene therapy

ceDNA ITR structure is key to accessing the nucleus



ceDNA expression is consistent with non-integrating episomes



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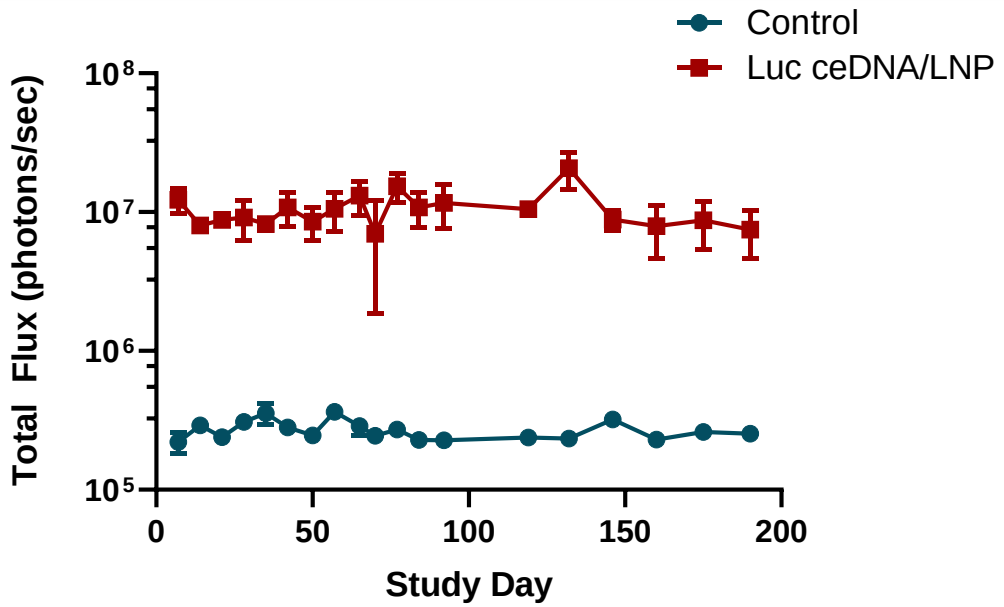
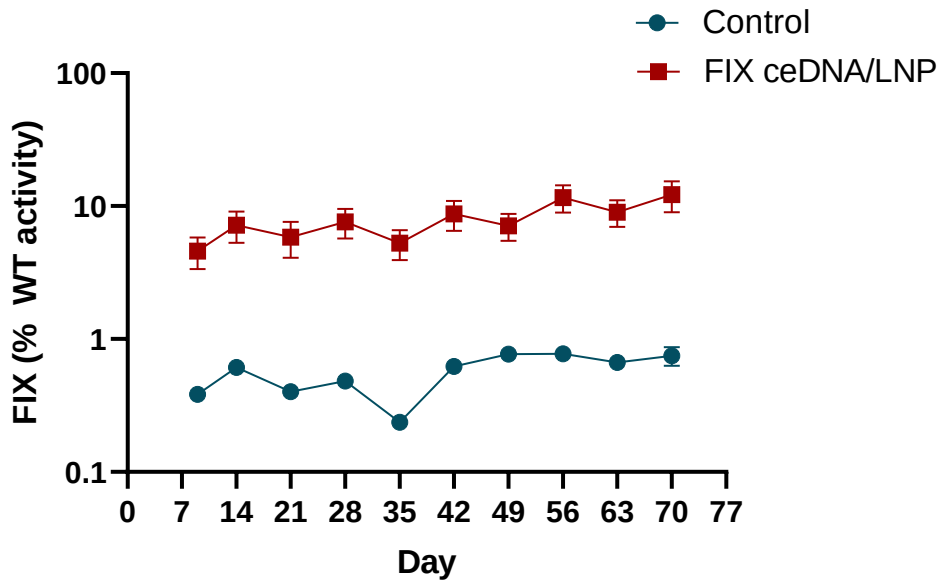
ceDNA-LNP shows durable expression in immunocompetent mice after a single IV administration

Luciferase



- Single IV administration at study day 0
- Total flux measured by IVIS *in vivo* imaging

Factor IX



- Single IV administration at study day 0
- Factor IX activity calculated from protein ELISA

FIX used as surrogate for durability and redosing in wildtype mice because this human protein does not raise neutralizing antibodies in mice, unlike human FVIII

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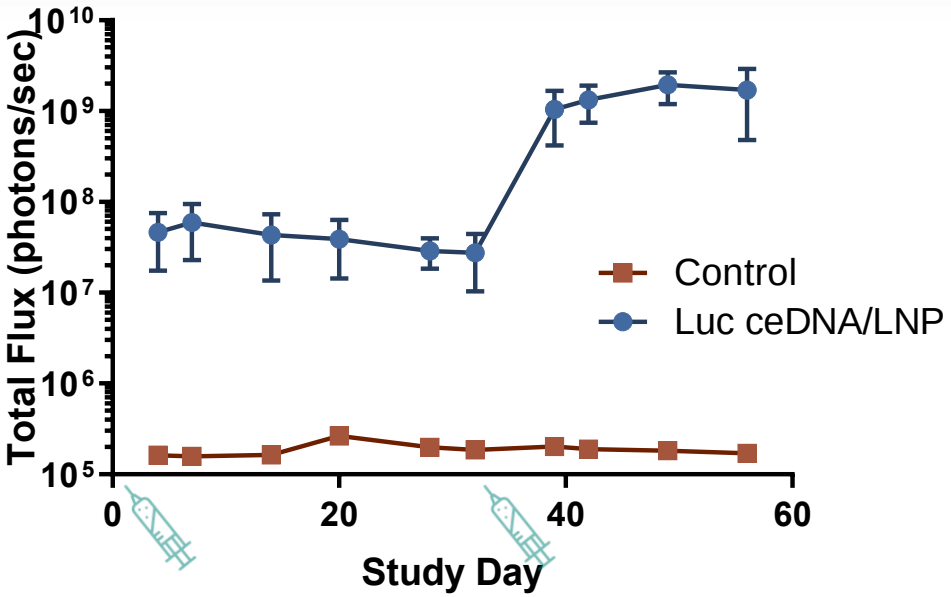
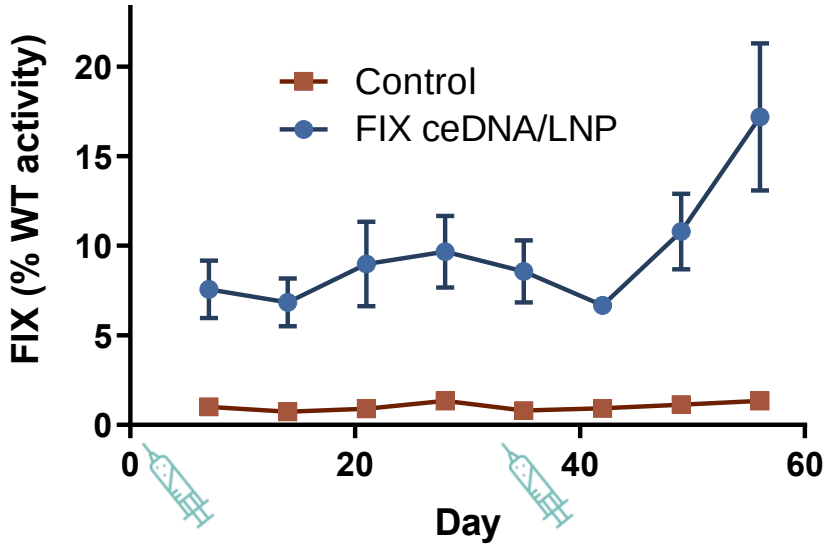
ceDNA-LNP redosing achieves increased expression in immunocompetent mice

Luciferase



- Single IV administration at study day 0
- Re-dosed at day 35 with 10X higher dose
- Total flux measured by IVIS *in vivo* imaging

Factor IX



- Single IV administration at study day 0
- Re-dosed at day 36 at same dose level
- Factor IX activity calculated from protein ELISA

FIX used as surrogate for durability and redosing in wildtype mice because this human protein does not raise neutralizing antibodies in mice, unlike human FVIII

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Hemophilia A

Redosable childhood intervention; titration to correct level in each patient

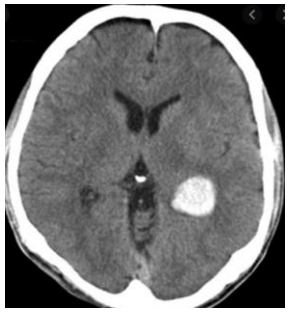
DISEASE

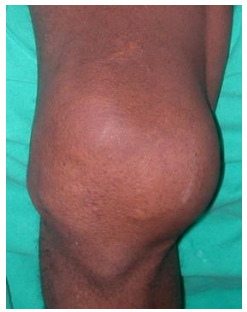
~16,000 patients in US

Diagnosis in early childhood

Bleeding disorder caused by deficiency in clotting factor VIII

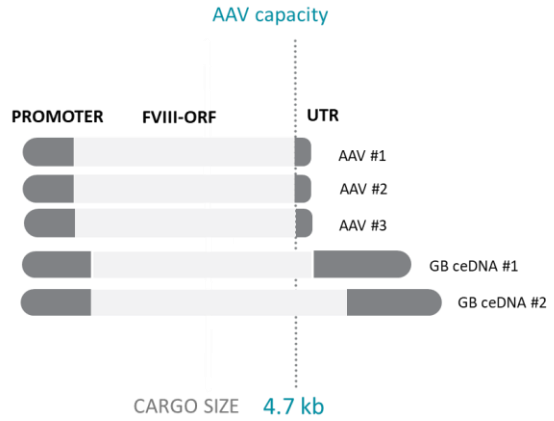
Gene therapy in development limited to adults, efficacy is variable and waning



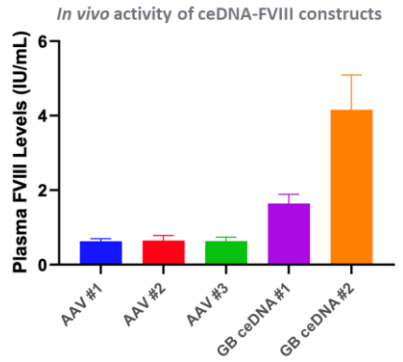


Atalay et al., Ital. J. Med. 9(3): 290-293 (2015)
AAAS EurekAlert! 30 Aug 2018 Univ. Witwatersrand

OUR APPROACH



ceDNA capacity enables optimized Factor VIII constructs



POTENTIAL THERAPEUTIC OPPORTUNITY

- ★ Greater potency based on larger genetic payload
- ★ Titration to target expression level for each patient
- ★ Dosing in childhood before disease progression
- ★ Redosing to extend benefit over a lifetime

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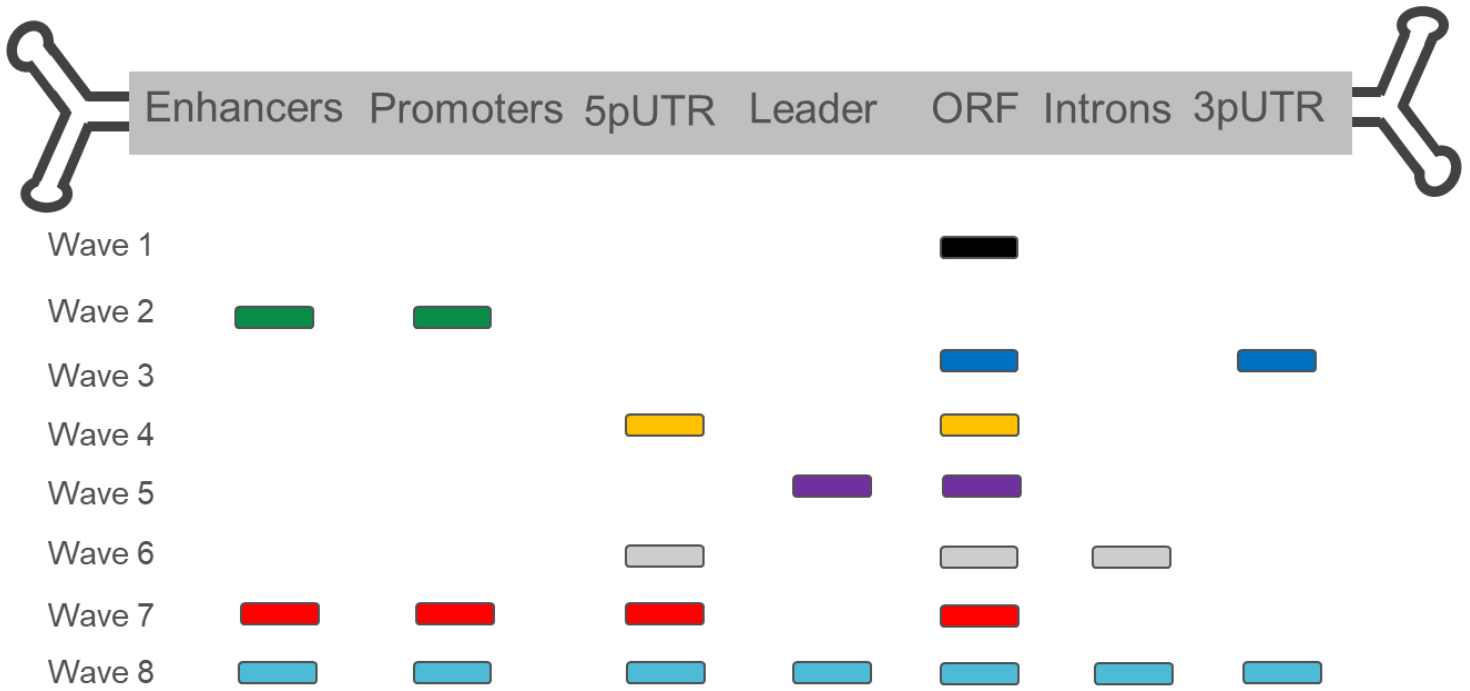
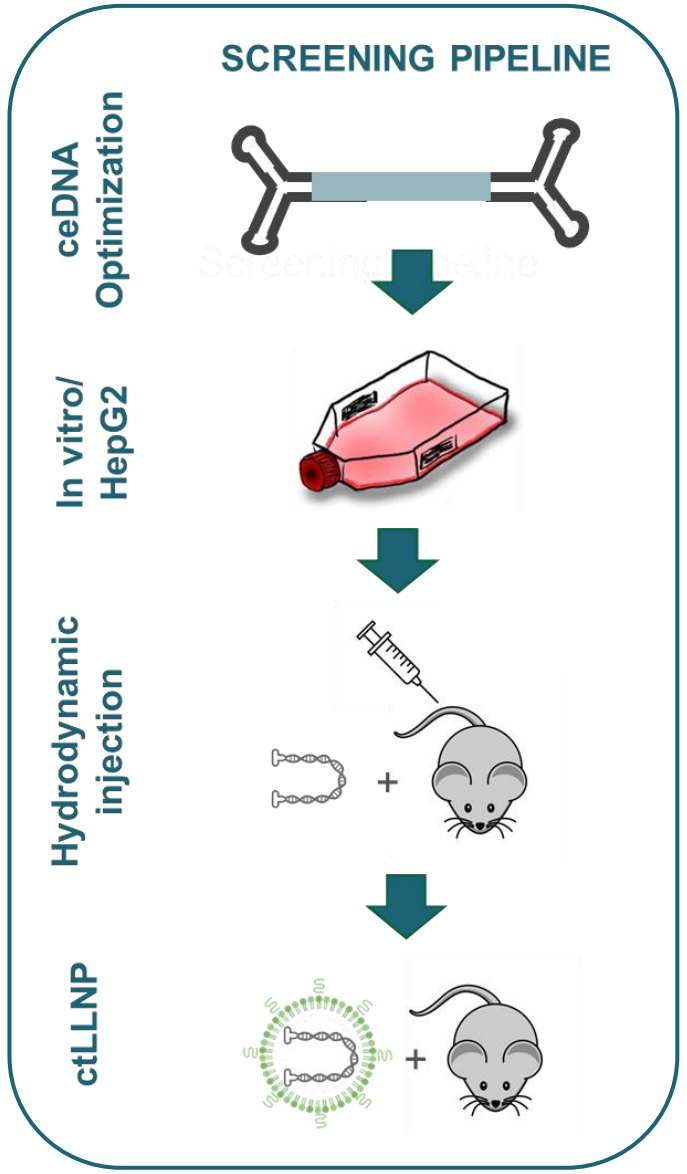
FVIII ceDNA optimization using a semi-combinatorial construct screening strategy

- Rational design utilizes design rules and assumptions of module independence to efficiently screen construct variants
- We generated and screened a semi-combinatorial library of > 300 constructs with following objectives:
  - Leveraging the increased capacity of ceDNA to improve FVIII expression
  - Testing specific design principles to improve library design and efficiency of rational design strategies
  - Identifying and characterizing genetic interactions between modules that impact performance



Schematic of semi-combinatorial screening strategy. Each wave of designs combines different types and numbers of genetic modules as denoted by colored rectangles.



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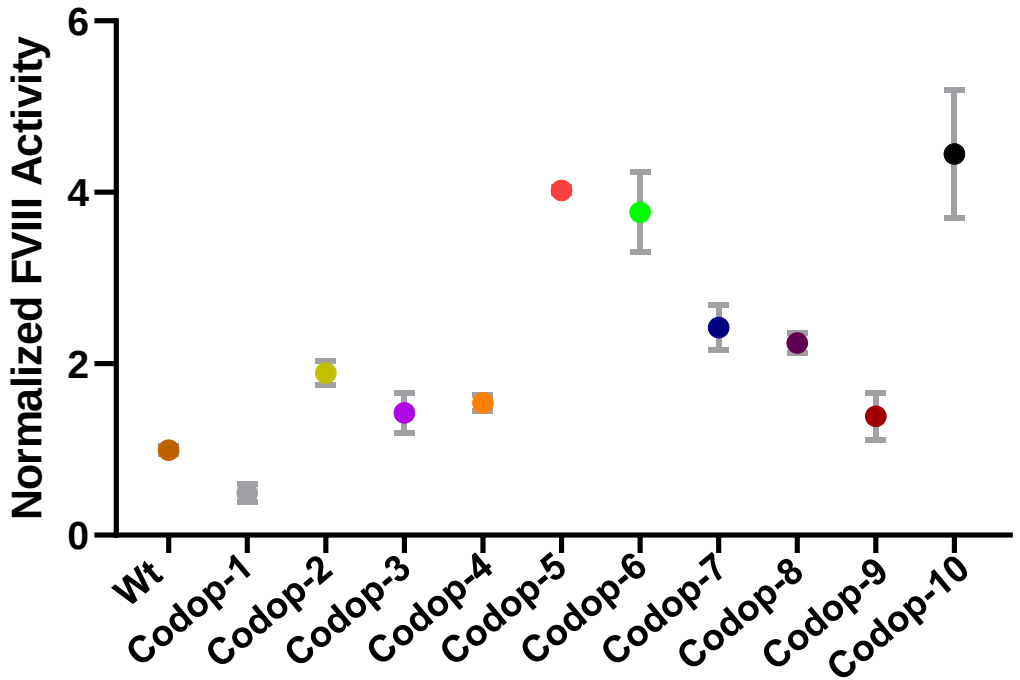
Codon optimization improves FVIII expression & highlights value of comprehensive empirical screening of multiple algorithm outputs

- Codon selection can greatly impact protein expression, but the ability of current algorithms to computationally identify optimal solutions is not well understood.
- FVIII ORFs with different codon optimizations were synthesized and assessed for FVIII activity when expressed using a standardized ceDNA chassis.
- In vitro performance varied considerably for optimized ORFs, spanning > 4-fold differences in activity compared to a control with Wt codon usage
- In vitro assays were a good predictor of performance in vivo

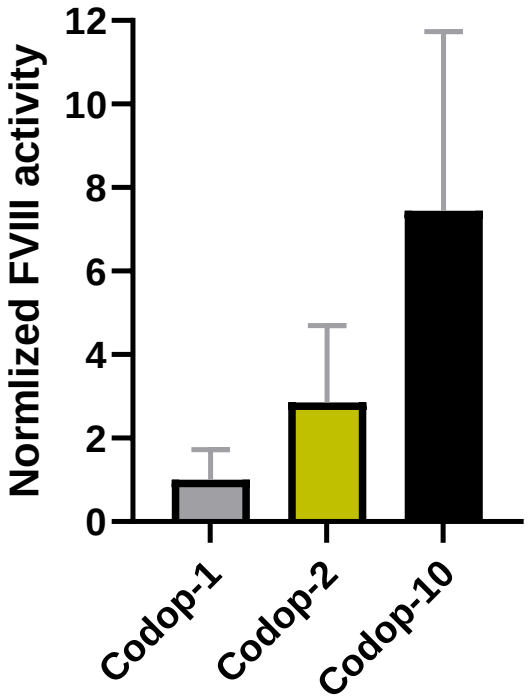
TAKE HOME

- Codon optimization algorithms allow efficient survey of a large design space but should not be utilized as a deterministic optimization tool.
- Comprehensive empirical screening is required to maximize value from codon optimization strategies

A)



B)



A) In vitro analysis of codon optimized FVIII constructs. HepG2 cells were transfected with ceDNA using lipofectamine. FVIII activity was measured in the cell culture supernatant using a two-stage chromogenic assay 72h after transfection. Data is the mean & standard deviation of triplicate wells from a representative experiment.

B) In vivo analysis of codon optimized FVIII constructs. ceDNA was administered to CD-1 mice by Hydrodynamic injection. FVIII activity was measured in plasma using a two-stage chromogenic assay 72h post administration. Data is the mean and standard error from 4 mice.

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Intron inclusion is beneficial to FVIII expresion, but subject to complex design rules

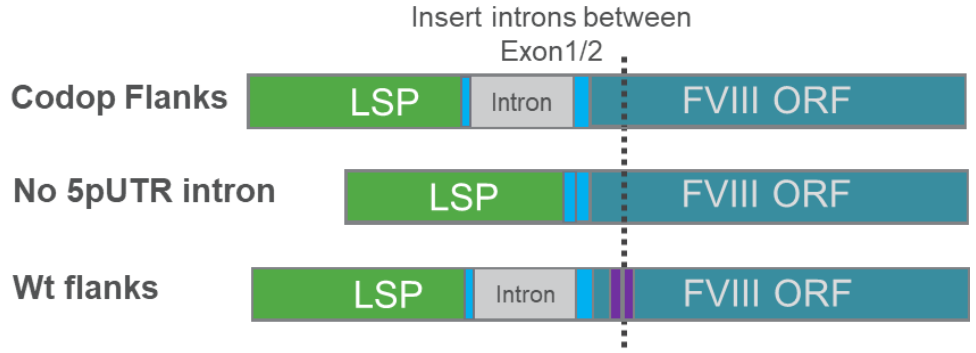
- Introns can impact multiple aspects of gene expression (e.g. RNA export & stability)
- ceDNA's increased size capacity was used to test intron design constraints and impact on FVIII expression

DESIGN VARIABLES CONSIDERED:

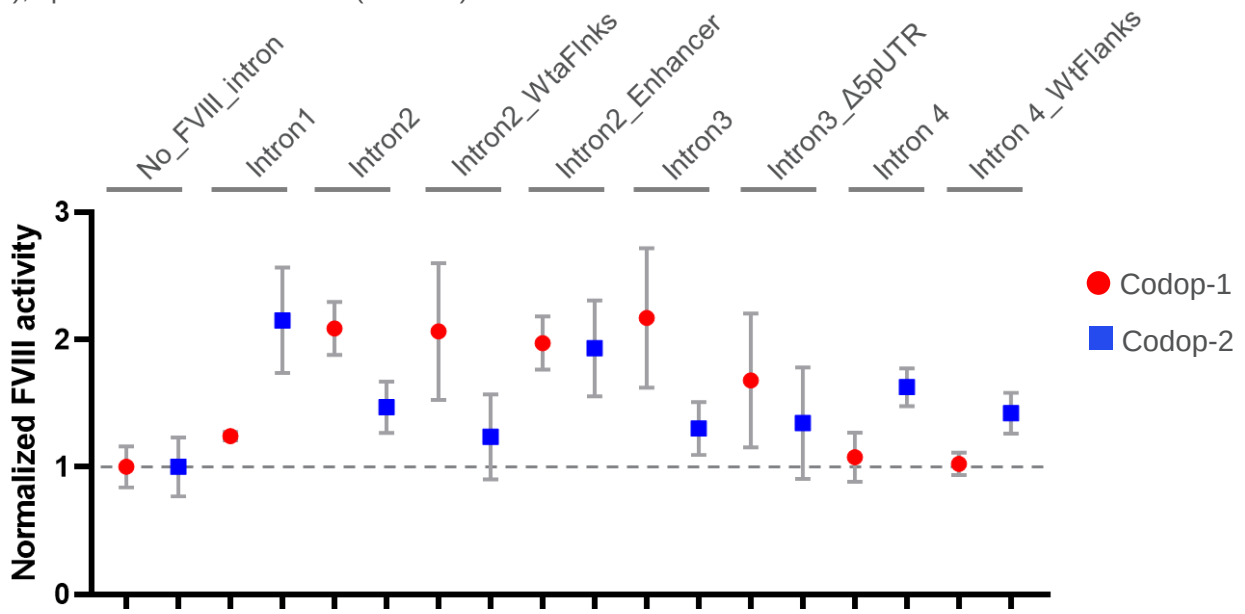
1. Intron sequence
2. Intron embedded enhancers
3. Codon optimized ORF sequence
4. ORF Sequence flanking SD/SA sites
5. Presence of intron in 5pUTR

TAKE HOME

- Introns were generally beneficial, however the level of benefit depended on the specific intron sequence
- Performance of an intron was most sensitive to the global codon optimization of the ORF and relatively insensitive to the local sequence flanking the intron
- Imbedding enhancers within introns has the potential to confer additional gains in function
- Introns within the FVIII ORF conferred advantages in addition to the 5pUTR intron often present in promoters



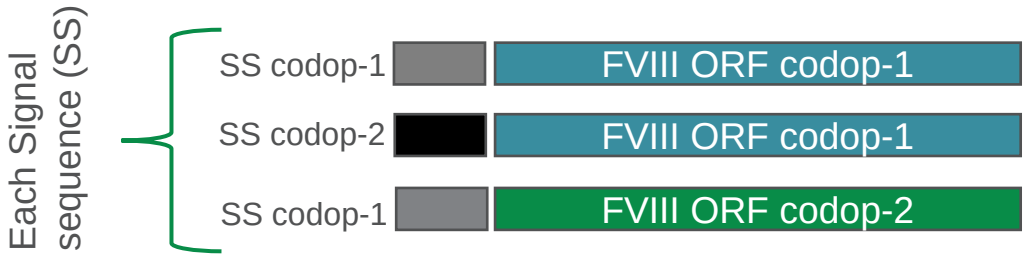
FVIII intron design strategy. Introns inserted in two different codon optimizations of FVIII. Exon flanking sequences are derived from codon optimized or WT cDNA sequence (purple bars). Liver Specific Promoter (LSP), 5pUTR with/ without intron (blue bar)



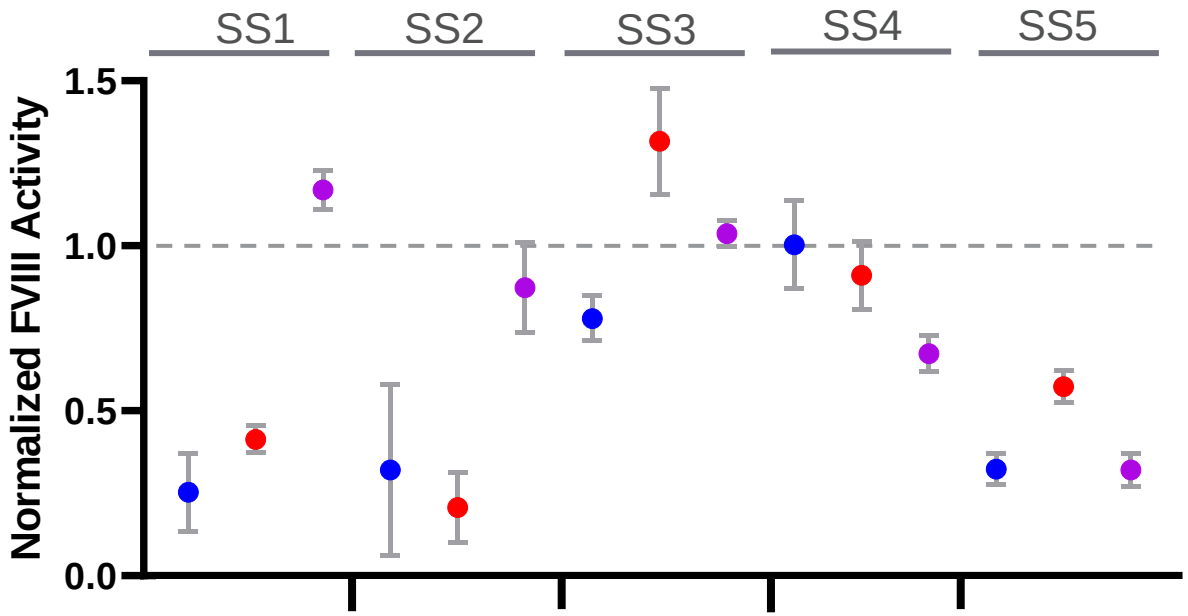
In vitro analysis of FVIII intron constructs. HepG2 cells were transfected with pDNA using lipofectamine. FVIII activity was measured in the cell culture supernatant using a two-stage chromogenic assay 72h after transfection. Data is the mean & standard error of triplicate wells from a representative experiment. Data is normalized to a construct with the same FVIII ORF codon optimization but without an intron.

Heterologous signal sequence function is highly influenced by codon usage

- Signal sequences encode short N-terminal peptides that are required for efficient trafficking of proteins to the ER and into the secretory system
- FVIII is known to be inefficiently secreted – therefore we assessed a panel of heterologous signal sequences for impact on FVIII activity
- We evaluated two sources of sequence interactions for effects on signal sequence performance – signal sequence codon choice and FVIII ORF codon choice.



Design Strategy for incorporation of heterologous signal sequences. Three constructs were designed for each heterologous signal sequence to test the contribution of FVIII ORF codon usage and signal sequence codon usage. **1)** FVIII ORF codop-1 with signal sequence codop-1, **2)** FVIII ORF codop-1 with signal sequence codop-2, **3)** FVIII ORF codop-2 with signal sequence codop-1.



In vitro analysis of FVIII constructs with heterologous signal sequences (SS. HepG2 cells were transfected with pDNA using lipofectamine. FVIII activity was measured in the cell culture supernatant using a two-stage chromogenic assay 72h after transfection. Data is the mean & standard error of triplicate wells from a representative experiment. FVIII ORF codop-1 with signal sequence codop-1. FVIII ORF codop-1 with signal sequence codop-2, FVIII ORF codop-2 with signal sequence codop-1. Data normalized to appropriate ORF codop with WT signal sequence.

TAKE HOME

- Heterologous signal sequences generally resulted in lower or equivalent FVIII expression
- Codon usage of the FVIII ORF & the signal sequence impacted expression to differing degrees depending on the signal sequence

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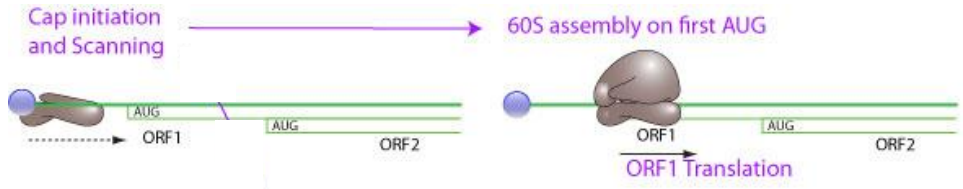
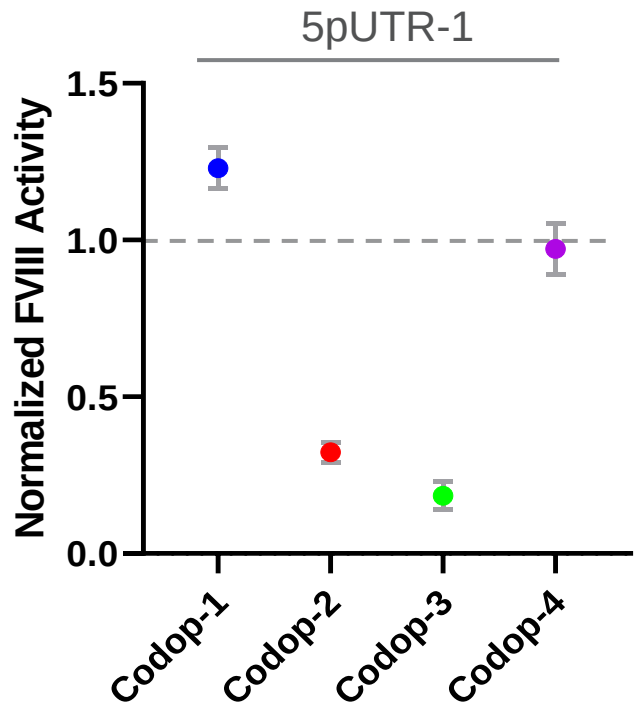
Modularity & independence of 5pUTR function is constrained by the ORF sequence

- 5pUTR sequences are often treated as a modular element that is optimized independent of the ORF sequence
- We evaluated a small panel of 5pUTR sequences for function in the context of different FVIII ORF sequences and found evidence for substantial sequence interactions that impacted 5pUTR performance.

Translation initiation in Eukaryotes utilizes a ribosome scanning mechanism



Schematic of translation initiation. The 40S ribosome subunit binds to the 5' cap and scans the mRNA for the first AUG codon. Assembly of the 60S ribosome subunit at the AUG site results in initiation of translation.



In vitro analysis of 5pUTR function dependence on ORF codon usage. HepG2 cells were transfected with pDNA using lipofectamine. FVIII activity was measured in the cell culture supernatant using a two-stage chromogenic assay 72h after transfection. For each different codon optimization, expression with an engineered 5pUTR is shown normalized to the base construct. Data is the mean & standard deviation of triplicate wells from a representative experiment.

TAKE HOME

- Optimization of the 5pUTR is best achieved in the context of the desired ORF due to genetic interaction effects
- Translation initiation may not be an isolated feature of the 5pUTR sequence

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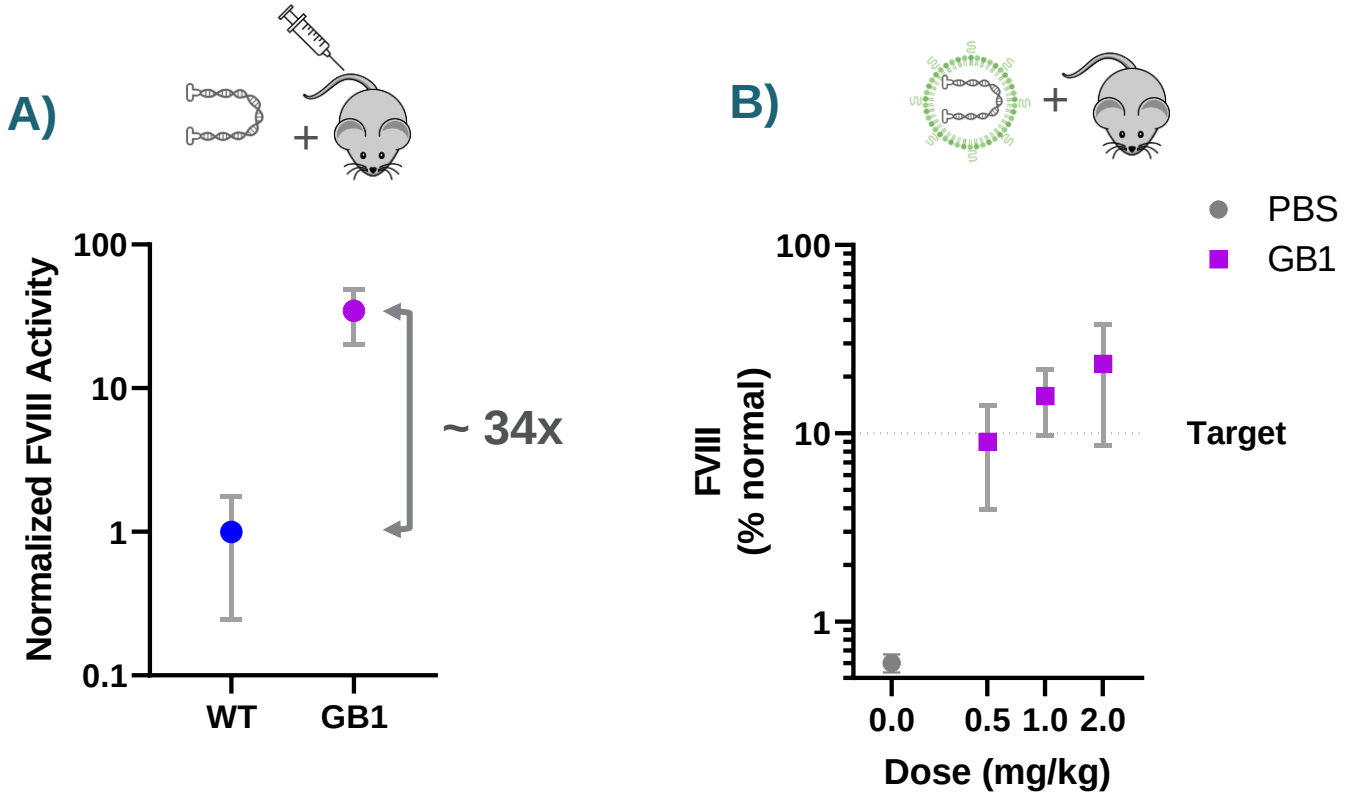
A semi-combinatorial optimization strategy leverages the larger size capacity of ceDNA to engineer construct with > 30-fold increase in FVIII expression in vivo



In vivo benchmarking of optimized FVIII ceDNA GB1. A) ceDNA was administered to CD-1 mice by Hydrodynamic injection. GB1 activity data is normalized to an unoptimized ceDNA expressing a FVIII ORF with WT codon usage. GB1 expressed ~34x more FVIII. **B)** ceDNA delivered systemically with ctLNP at three doses. FVIII activity shown at Day 10. A dose response was observed reaching > 20% normal at the highest dose.

TAKE HOME

- GB1 is the culmination of a semi-combinatorial construct optimization process using ceDNA
- Combinatorial screening was integral to countering the pervasive and impactful interaction effects between genetic modules
- GB1 expressed ~34x more FVIII than an unoptimized construct containing a Wt FVIII ORF sequence.
- GB1 supported target levels of FVIII activity (10% of normal) at a dose of 0.5mg/kg when delivered systemically with ctLNP.

Summary

- ceDNA combined with non-viral gene delivery opens new avenues for construct optimization – taking advantage of the increased cargo capacity to increase potency
- A semi-combinatorial screening approach identified GB1 – a FVIII ceDNA construct with ~ 34-fold higher expression than an analogous WT FVIII construct
- Genetic interactions between modules such as introns, 5pUTRs, signal sequences and ORFs was common and impactful on construct performance – questioning the utility of screening genetic modules as independent features.
- Better understanding of design elements driving sequence-specific context effects allowed more efficient rational design
- In general, construct optimization strategies would benefit from large-scale combinatorial screening instead of relying on assumptions of modularity to reduce screen complexity and design space