Enhancing ZFN Expression Construct and Nuclease Activity Leads to Improvement of *In Vivo* Genome Editing Platform

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Abstract

Sangamo Therapeutics currently has open three clinical trials in the first ever evaluation of in vivo genome editing. The goal of these trials is to utilize a ZFN-mediated genome editing strategy to permanently modify patient liver cells through insertion of a corrective transgene at the Albumin locus, following systemic AAV2/6 delivery. We are currently evaluating this approach using donor constructs encoding the genes that are defective in Hemophilia B and mucopolysaccharidosis (MPS) types I and II.

Therapeutic transgene insertion into the Albumin locus and co-opting its high transcriptional activity could potentially provide long-term expression of the corrective transgene in stably-modified hepatocytes. Stable insertion also avoids any potential issues associated with non-integrating gene therapy approaches, which is particularly important for treating pediatric diseases with liver-directed therapies. During growth and development there is significant hepatic cellular division and potential for loss of episomal genomes.

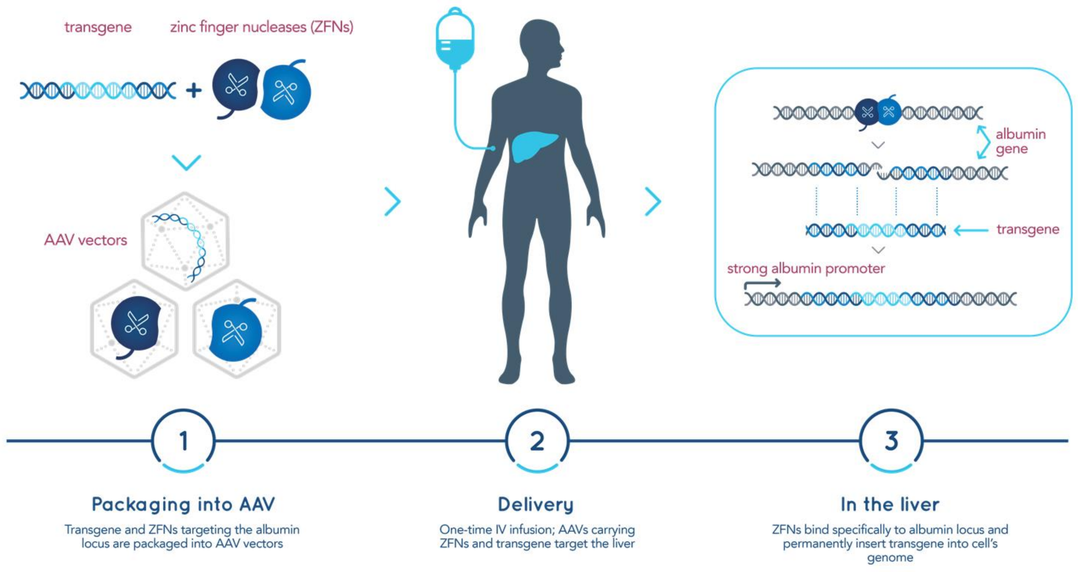
This in vivo genome editing approach depends upon effective ZFN expression and nuclease activity in patient hepatocytes. The work described here highlights the potential for next-generation in vivo genome editing constructs.

These optimized constructs improve both ZFN expression and nuclease activity through the rational enhancement of (a) the AAV-ZFN expression construct backbone and (b) the coding ZFN sequences by modulating both the DNA-binding and nuclease domains of the ZFNs.

Importantly, selective substitution of ZFN amino acid residues at the protein-DNA interface allows for increased ZFN activity, the ability to tolerate a SNP in the ZFN Albumin target site, and greatly increased specificity. These improvements were achieved while preserving the original ZFN target site, which allows use of the original transgene donor construct.

These optimizations further highlight the advantages of using ZFNs as a tool for the correction of monogenic disease via in vivo genome editing.

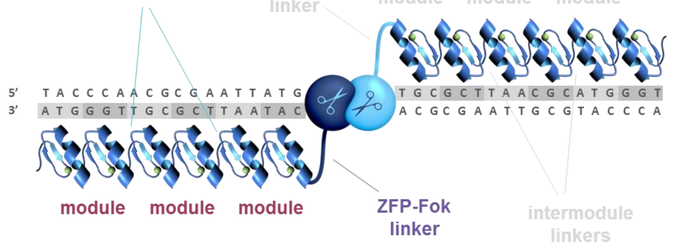
In vivo genome editing platform



Objective: Enhance platform by focusing on the ZFN expression construct

(1) Increased ZFN expression and nuclease activity
(2) Increased specificity (with decreased off-target cleavage)

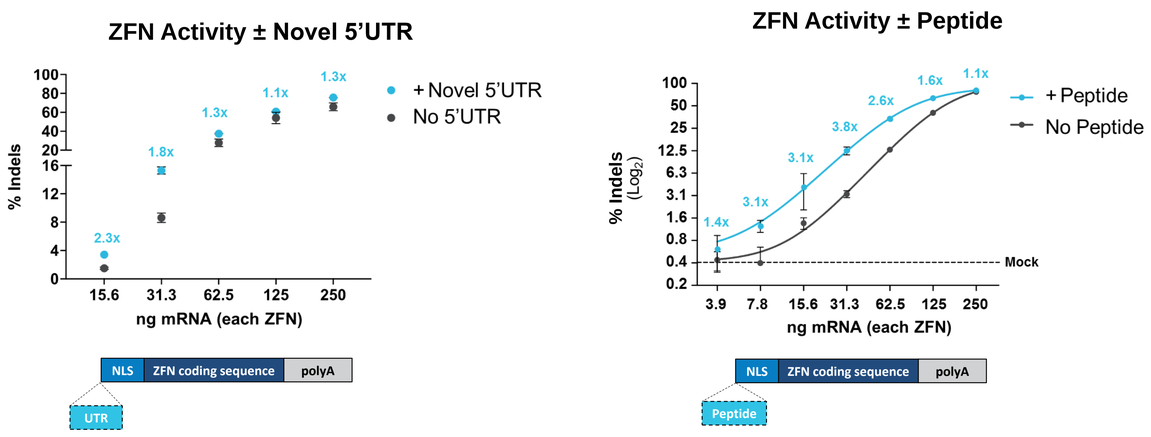
ZFN technology enhancements for optimal precision, efficiency and specificity



- New linkers for configuring DNA-binding modules allow reversing ZFP-Fok domain order and skipping up to 2 bases to overcome SNPs and increase design options 300-fold
- New dimer architectures yield DNA editing efficiency as high as 99.5%
- Phosphate contact tuning via replacement of key residues results in >1000 fold reduction in off-target cleavage (to undetectable levels)

Improvements to ZFN expression & activity

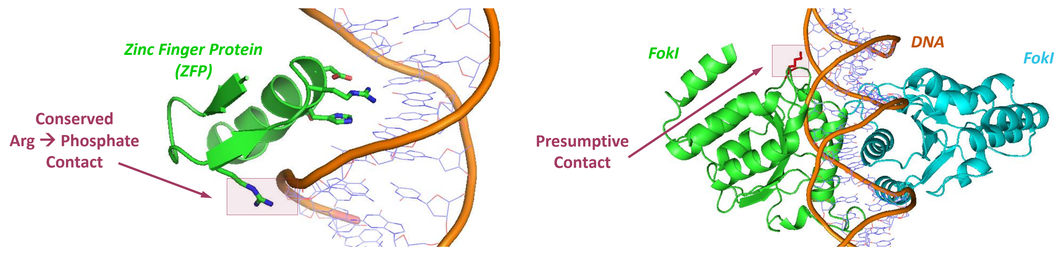
Addition of a novel 5'UTR & N-terminal peptide **each independently lead to 2-3x ZFN activity**, which correlated with increased ZFN protein expression (not shown)



K562 cells were transfected with Albumin-targeting ZFNs as indicated and cells were assessed for ZFN activity (% indels) by deep sequencing after 24 hr.

Enhanced ZFN specificity

Removal of conserved zinc finger backbone and presumptive FokI non-specific contacts with DNA phosphate backbone provides a rapidly and broadly applicable method to increase ZFN specificity



ZFN Left		ZFN Right		Albumin (On-Target) 2000 ng	62.5 ng	31.3 ng	15.6 ng	Off-Target 1 2000 ng
Parent	Standard	Parent	Standard	78.9	21.1	1.9	0.2	1.23
Optimized	F1F3Q (ZFP) + N159D (FokI)	Optimized	F3Q (ZFP) + P95S (FokI)	80.9	53.9	20.6	4.6	0.01

- **Despite significantly increased on-target activity, the optimized ZFN pair (ZFN2.0) has only background levels of activity at the only known off-target for the parent ZFNs** (% indels observed in GFP-transfected sample = 0.01%)
- In addition to specified ZFP backbone and FokI changes, the ZFN2.0 Left/Right optimized constructs contain novel 5'UTR and WPRE elements to increase activity, and zinc finger changes to increase activity & specificity.
- K562 cells were transfected with Albumin-targeting ZFNs as indicated. Cells were assessed for ZFN activity (% indels) by deep sequencing after 24 hr.

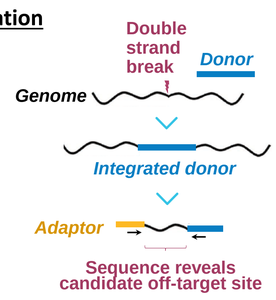
Unbiased, genome-wide assessment of ZFN 2.0 specificity

1 Candidate Off-Target (OT) Identification
Oligo-Duplex End-Capture Assay

Treat cells with ZFNs + donor oligonucleotide duplex

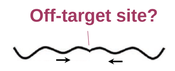
- K562 cells, delivery via nucleofection
- 50 ng mRNA / ZFN, 1 µM oligo duplex

Sequence genome segments adjacent to integrated donors



2 OT Confirmation
Deep Sequencing to Assess Actual ZFN Activity

Treat K562 or HepG2 cells with ZFNs. PCR-amplify candidate off-target sites. Assess for indels.



Levels of on-target modification during candidate OT identification (quadruplicate transfections)

	% Indels On-Target (ALB)	StDev	% Oligo Capture (ALB)	StDev
ZFN 2.0	76.36	3.6	10.38	0.33

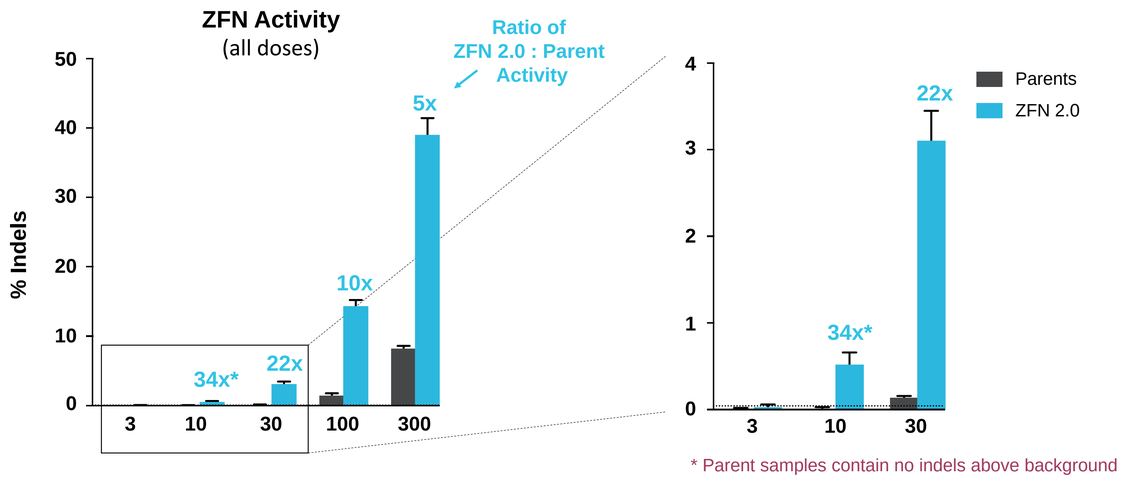
Candidate OTs / OT confirmation

	Gene/ Location	Capture events	K562 % indels	HepG2 % indels
	ALB (on-target)	3498	87.7%	98.2%
1	SKA2	18	ns	ns
2	Chr5 (intergenic)	12	ns	ns
3	Chr9 (intergenic)	11	ns	ns
4	AC079807.4	10	ns	ns
5	Chr1 (intergenic)	9	ns	ns
6	CSMD3	9	ns	ns
7	Chr18 (intergenic)	9	ns	ns
8	Chr19 (intergenic)	9	ns	ns
9	Chr1 (intergenic)	8	ns	0.08^
10	HECTD4	8	ns*	ns
11	Chr17 (intergenic)	8	ns	ns
12	Chr4 (intergenic)	8	ND	ND
13	CSMD3	8	ns	ns
14	Chr11 (intergenic)	8	ns	ns
15	Chr1 (intergenic)	7	ns	ns
16	AKAP6	7	ND	ND
17	Chr19 (intergenic)	7	ns	ns
18	OSBPL6	7	ns	ns
19	QPCT	7	ns	ns
20	RP11-269F21.3	7	ns	ns
21	LPHN3	7	ns	ns
22	CYFIP2	7	ns	ns
23	AUTS2	7	ns	ns
24	COL4A6	7	ns	ns
25	ChrX (intergenic)	7	ns	ns
26	ACAN	7	ns	ns

Legend
ns = not significant
ns* = indels not consistent with ZFN cleavage
^indels consistent with ZFN cleavage and non-significant p-value
ND: no data due to technical difficulties with PCR amplification

ZFN 2.0 is highly active in human hepatocytes

- The lead pair of ZFNs (ZFN2.0) were delivered as AAV2/6 and compared side-by-side with the parent ZFN pair in iPSC-derived human hepatocytes.
- Results show highly efficient, dose-dependent ZFN2.0 activity, with 5 to 20-fold higher levels of indels as observed for the parent pair.



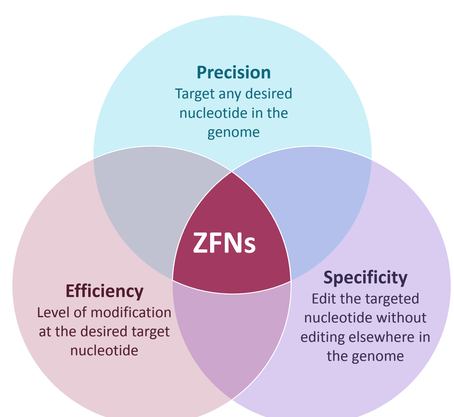
* Parent samples contain no indels above background

iPSC-derived human hepatocytes were transduced in triplicate with AAV2/6 encoding the parent or ZFN 2.0 Albumin ZFNs. ZFN activity (% indels) was determined by deep sequencing 7 days post-transduction. X-axis indicates multiplicity of infection (MOI) per ZFN. Dotted line indicates the level of modification observed in mock-treated samples. In addition to zinc finger changes to increase activity and specificity, the ZFN 2.0 pair incorporates the novel 5'UTR, N-terminal peptide, and WPRE into the expression construct.

Conclusions

Optimization of the AAV-ZFN expression construct and selective substitution of ZFN amino acid residues at the protein-DNA interface produced next-generation *in vivo* genome editing constructs for correction of monogenic disease.

- ZFN 2.0 is 5 to 20-fold as active as the parent ZFNs in human hepatocytes
- ZFN 2.0 eliminates a known off-target site and is highly specific genome-wide



Conflict of Interest Statement
All authors are full-time employees of Sangamo Therapeutics, Inc.