Highly Specific Zinc Finger Proteins with Synthetic Target Sites Enable Self-Regulated Expression of Dosage-Sensitive Transgenes

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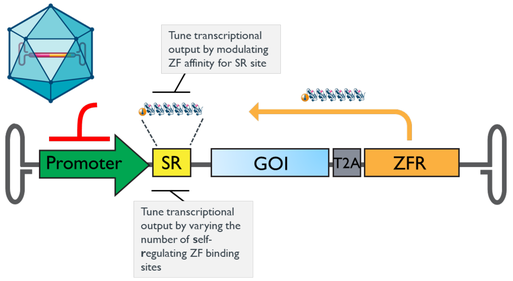
Sangamo THERAPEUTICS

Poster # 722

Introduction

- Limiting overexpression of AAV-delivered cargo is an important safety consideration particularly for indications with dosage sensitive genes which require transgene expression in a precise therapeutic range to balance efficacy and toxicity.
- To address this therapeutic challenge, we developed a self-regulating expression technology using zinc finger repressors (ZFR) that serve as vector elements to precisely control transgene expression.
- The combined ZFR and target site payload is <1kb, does not require exogenous components and should be compatible with any promoter and transgene construct for which fine control of expression is required.



Identification of highly specific, genome orthogonal ZFs for self-regulated expression

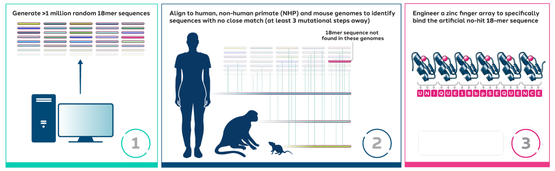


Figure 1. Design strategy for identifying ZFs for self-regulated expression.

- A pool of over 1 million random 18mer DNA sequences was generated and screened to identify unique 18mer DNA sequences at least 3 mismatches away from any genomic site present in the human, cynomolgus and rhesus macaque and human genomes.
- A proprietary design algorithm was used to generate 6-finger ZF arrays targeting a panel of synthetic, genome orthogonal DNA sequences.
- A panel of ZFs were evaluated for binding activity, specificity and tolerability to identify the top candidates for self-regulated transgene expression.

ZFRs with potent binding activity and exquisite specificity

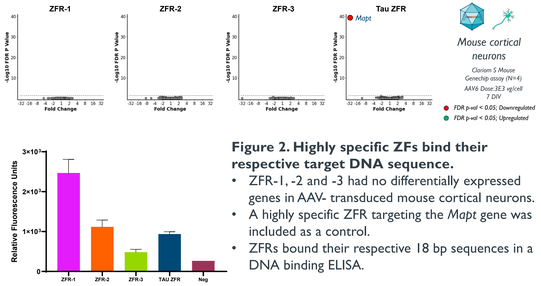


Figure 2. Highly specific ZFs bind their respective target DNA sequence.

- ZFR-1, -2 and -3 had no differentially expressed genes in AAV- transduced mouse cortical neurons.
- A highly specific ZFR targeting the *Mapt* gene was included as a control.
- ZFRs bound their respective 18 bp sequences in a DNA binding ELISA.

IV-administered ZFRs are well-tolerated in the mouse brain

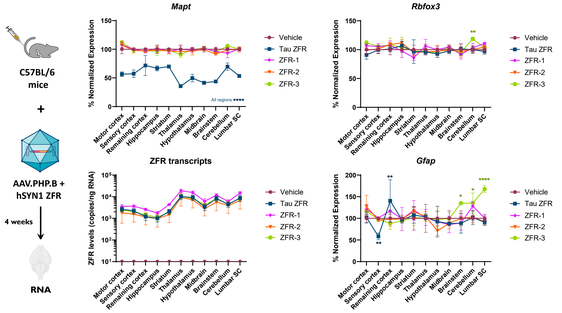


Figure 3. IV administered ZFRs are expressed and well tolerated in vivo.

- AAV-ZFRs were administered intravenously at 1E14 vg/kg into mice (n=4 mice per group) under the control of a neuron-specific promoter (human Synapsin I). Total RNA was isolated from several brain regions and analyzed by RT-qPCR.
- **(Left)** A *Mapt*-targeted ZFR was included as a dosing control. *Mapt* gene expression was normalized to the mean of *Atp5b* and *Eif4a2*, and transgene expression normalized to total RNA input. **(Right)** *Rbfox3* and *Gfap* gene expression was normalized to the mean of *Atp5b* and *Eif4a2*. All data are shown as mean +/- SD.
- No neuronal loss or neuroinflammation was observed across all brain regions in ZFR-1 & ZFR-2 treated mice. ZFR-3 treated animals displayed significantly elevated *Gfap* levels in several brain regions and the spinal cord.

ZFRs are well-tolerated after hippocampal delivery in vivo

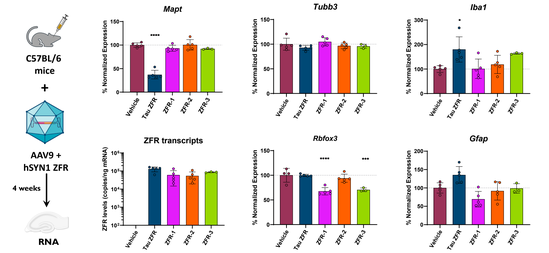


Figure 4. Intrahippocampally administered ZFRs are expressed and well tolerated in the mouse brain.

- The same panel of ZFRs were administered intrahippocampally into both hemispheres in mice (n=5 mice per group) at 3E10 vg per hemisphere. Total RNA was isolated from the hippocampus and analyzed by RT-qPCR.
- ZFR transcript expression levels were ~100-fold higher in this ROA than in the hippocampus of IV administered ZFRs (Figure 3).
- ZFR-2 had the best tolerability profile of all candidate ZFRs evaluated with no changes in neuronal or neuroinflammatory markers observed.

Tuning down expression by increasing the number of ZFR self-regulating sites

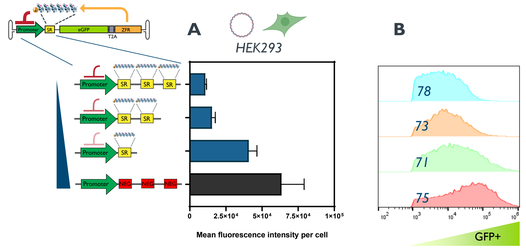


Figure 5. ZFR self-regulated expression is tuned by the number of ZFR binding sites in an expression cassette.

- HEK293 cells were transfected with a panel of ZFR-1 expression plasmids and analyzed for GFP fluorescence by flow cytometry 24hr post transfection.
- Mean fluorescence intensity (four biological replicates) indicated the number of self-regulating (SR) sites directly influenced GFP expression levels in transfected cells compared to a non-self-regulated (NEG) control (A).
- HEK293 cells with the highest GFP fluorescence decreased in all self-regulated plasmids compared to the non-self-regulated control (B). However, the % of GFP+ cells (shown on histogram) did not significantly decrease compared to the non-self-regulated control.

ZF affinity for a self-regulating site tunes transcriptional output

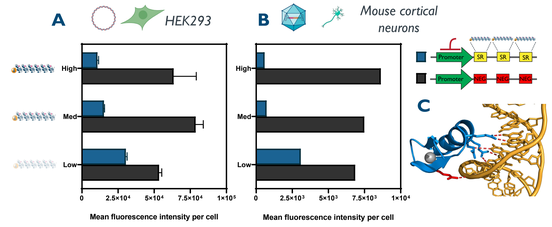


Figure 6. Modulating ZF affinity for a given target site directly influenced transcriptional output.

- MFI was measured by flow cytometry in (A) HEK293 cells 24hr post transfection with plasmid DNA and (B) in mouse cortical neurons 7 DIV post AAV transduction.
- ZFR-1 affinity for target self-regulating sites was modulated by mutating arginine residues (C)(shown in red) which non-specifically contact the DNA phosphate backbone.

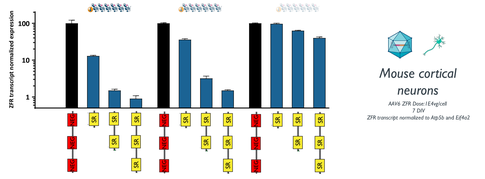


Figure 7. ZFR self-regulated expression is tuned by perturbing both the number of SR sites and ZF binding affinity for each SR site.

- Transcriptional output from AAV transduced mouse cortical neurons was measured by RT-qPCR. The % of ZFR transcripts expressed relative to the non-self-regulated ZFR control is shown.
- ZFR-1 transcript levels directly correlated with ZFR-1 affinity for target self-regulating sites present in the vector genome, with multiple SR sites further reducing ZFR expression.

Conclusions

- We created a self-regulated transgene expression platform using highly specific ZFRs targeting artificial DNA sequences.
- Highly specific ZFR candidates ZFR-1 and ZFR-2 were well tolerated in C57BL/6 mice.
- ZFR self-regulated expression is directly tuned by the number of ZF binding sites and affinity for a given SR site.
- Further development and in vivo validation will be performed to evaluate this ZF platform as a solution for safer, effective gene therapies with dose-sensitive transgenes.

Disclosures

This work was funded by Sangamo Therapeutics. All authors are current employees of Sangamo Therapeutics.

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Presented at ASGCT 2024