Genome Orthogonal Zinc Finger Proteins for the Development of Genomic Medicines

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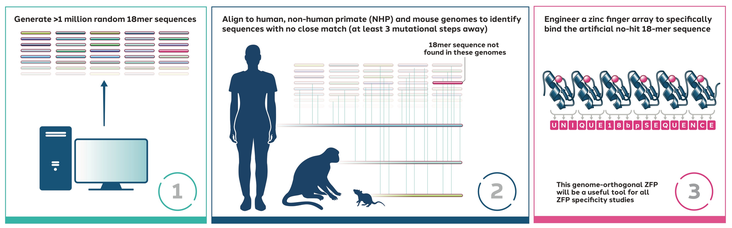
Abstract

Introduction: Zinc finger proteins (ZFPs) are the most abundant DNA-binding proteins found in the human genome[1]. Sangamo Therapeutics has developed a proprietary ZF library that allows us to engineer 100s of ZFPs that target a given genetic locus with high precision. Fusing a ZFP to an effector domain of a transcription factor (zinc finger-transcription factors, ZF-TFs) enables up- or down-regulation of a target gene[2]. ZF-TFs hold great promise as genomic medicines due to their high potency, tunability, specificity and compact size, which allows for packaging into adeno-associated virus (AAV) for delivery. A key requirement for effective genomic medicines is specificity – the ability to regulate a target gene without perturbating other genes. Transcriptional profiling methods such as microarray platforms and RNA sequencing (RNA-seq) are powerful techniques used to measure transcriptome wide ZF-TF specificity. Specificity is measured by comparing ZF-TF–treated samples to a control ZF-TF, which binds at a defined alternative genomic locus. However, a ZF-TF designed to not target any genomic site (genome orthogonal) is a more appropriate control for specificity analysis.

Methods: To identify ZFPs that do not bind to preclinically or clinically relevant genomes, we designed a panel of engineered ZFPs to specifically target DNA sequences several mismatched bases away from any sequence found in the mouse, cynomolgus and rhesus macaque, or human reference genomes. The engineered ZFPs were screened for DNA binding by enzyme-linked immunosorbent assay (ELISA) with potent binding to their intended target sites observed for 60% of the designed ZFPs.

Results/Conclusions: Using the Affymetrix microarray platform, we measured the transcriptome-wide specificity of a panel of ZFPs fused to the Krüppel-associated box (KRAB) repressor domain from the human KOX1 transcription factor in human fibroblasts and found a subset of ZF-TFs with minimal to no off-target transcriptional activity. Their specificity was further evaluated in mouse primary neurons and human induced pluripotent cell (iPSC)-derived neurons transduced with AAVs encoding the engineered ZF-TFs. Minimal to no dysregulation of any genes in the AAV-transduced neurons was observed by microarray and RNA-seq, further demonstrating the exquisite specificity of these genome orthogonal ZF-TFs. These proteins will be valuable controls for all ZFP specificity assessments and will also find use as controls in preclinical in vivo studies to distinguish between the effect of expressing an exogenous ZFP and the effect of modulating a specific target.

Designing highly specific, genome orthogonal ZFPs for specificity analysis

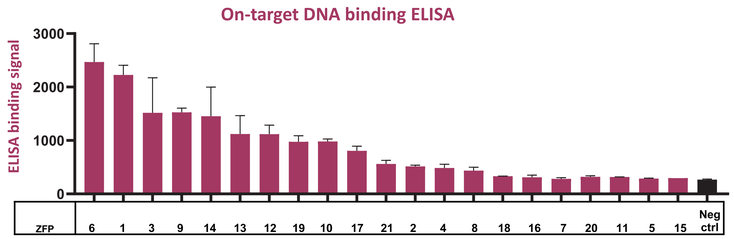


Design Strategy:

1. Generate a pool of >1 million random 18mer DNA sequences
2. Align each of the >1 million 18mer DNA sequence to the mouse, cynomolgus and rhesus macaque and human genomes to identify sequences at least 3 mismatches away from any genomic DNA site present in these genomes
3. Engineer ZFP arrays (6-fingers) to target and bind each synthetic, genome orthogonal 18mer DNA sequence
4. Screen the panel of ZFPs for on-target activity using ELISA, and off-target activity (when fused to the KRAB domain of human KOX-1) using microarray analysis and total RNA-seq, to determine their usability as research tools for measuring ZFP specificity in preclinical off-target studies

Results

Genome orthogonal ZFPs display a range of in vitro binding activities



In vitro-expressed ZFPs were evaluated for binding to their corresponding designed 18mer target DNA sequences by ELISA. The screen identified ZFPs with a broad range of on-target DNA binding signals.

ZF-TFs display minimal to no off-target transcriptome changes in different cell types

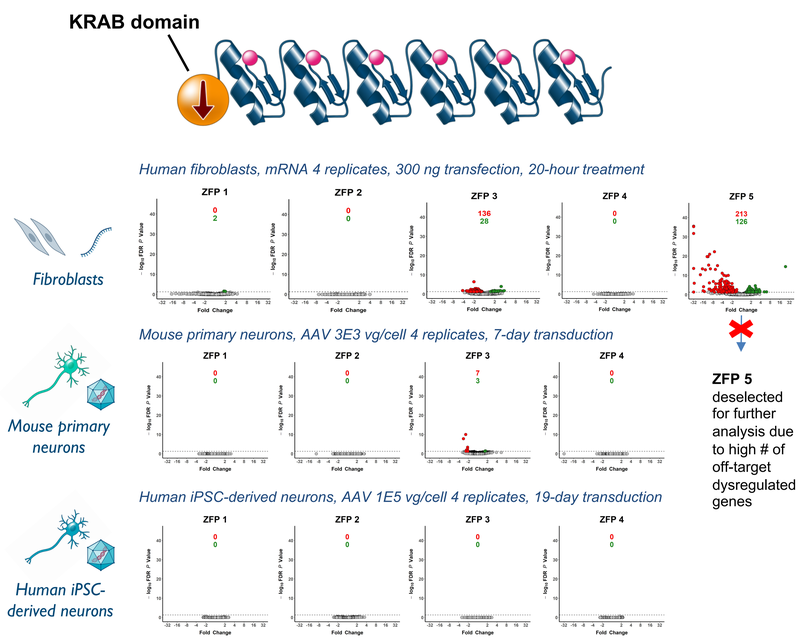


Image created with biorender.com

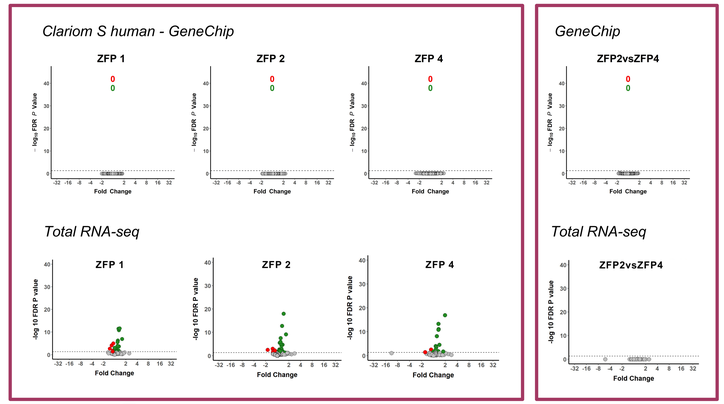
Genome orthogonal ZFPs were fused to a KRAB domain to measure specificity using the Affymetrix microarray platform. Human fibroblasts transfected with ZF-KRAB mRNA and mouse primary neurons or human iPSC-derived neurons transduced with AAV6 expressing the indicated ZF-KRAB showed minimal to no off-target gene regulation for the majority of ZF-TFs evaluated.

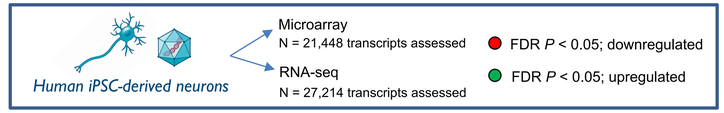
FDR, false discovery rate; vg, vector genomes

- FDR $P < 0.05$; # genes downregulated
- FDR $P < 0.05$; # genes upregulated

High-powered microarray analysis and total RNA-seq revealed the exquisite specificity of a panel of genome orthogonal ZF-TFs

Human iPSC-derived neurons, AAV 1E5 vg/cell 6 replicates, 19-day transduction





A high-powered microarray analysis which included 6 treatment replicates of each genome orthogonal ZF-TF further confirmed no off-target genes were regulated by these proteins in human iPSC-derived neurons when compared to a control ZF-TF targeting an intronic region of a gene not expressed in neuronal cells. Furthermore, a comprehensive analysis using total RNA-seq revealed minimal perturbation of coding or noncoding RNA species when compared to the control ZF-TF. Volcano plots on the **right** panel comparing ZFP 2 to ZFP 4 revealed no off-target genes were dysregulated, suggesting the off-target genes up-regulated in the total RNA-seq volcano plots on the **left** panel were regulated by the control ZF-TF rather than off-target effects of ZFP 2 or ZFP 4.

Summary and Conclusions

- Multiple ZFPs were identified with on-target DNA binding activity in vitro
- Affymetrix microarray analysis revealed several ZF-TFs had minimal to no off-target activity in mRNA-transfected human fibroblasts, AAV-transduced primary mouse neurons or human iPSC-derived neurons
- Total RNA-seq revealed minimal to no coding or non-coding transcripts were perturbed by ZF-TFs in human iPSC-derived neurons
- This work demonstrates the exquisite specificity of genome orthogonal ZF-TFs which will be used as controls in specificity assessments of clinical ZF-TF candidates
- Furthermore, these ZFPs will also serve as useful controls in preclinical studies to understand any effects of exogenous ZFP expression in vivo
- A subset of the most highly specific ZF-TFs will be assessed for tolerability and specificity in future in vivo studies

References

1. Klug A. The discovery of zinc fingers and their applications in gene regulation and genome manipulation. *Ann. Rev. Biochem.* 2010;79(1):213-31.
2. Wegmann S, DeVos SL, Zeitler B, et al. Persistent repression of tau in the brain using engineered zinc finger protein transcription factors. *Sci Adv.* 2021;7(12):eabe1611.

Disclosures

- All authors are or were employees of Sangamo Therapeutics.

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