# Genome-Orthogonal Gene Circuitry: Improved Synthetic Enhancer-Transcription Factor Systems with Minimal Off-Target Effects

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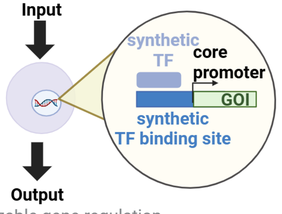
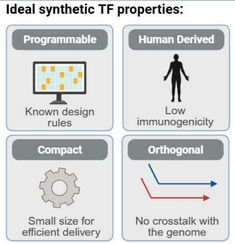
## Orthogonal ZFTF Expression Circuit

**Cell therapies which synthesize therapeutics precisely when and where they are needed hold promise for addressing numerous challenging diseases. However, to fully realize this potential, synthetic regulators are required that can drive therapeutic gene expression without perturbing endogenous transcriptional programs. Zinc finger-based transcription factors (ZFTFs) are highly programmable, human-derived proteins well suited for therapeutic use, given their specificity, small size, and compatibility with small molecule or SynNotch-based control [2]. In this project we designed novel mammalian genome-orthogonal enhancers and ZFTFs that serve as potent transcriptional activators with minimal impact to endogenous gene networks.**

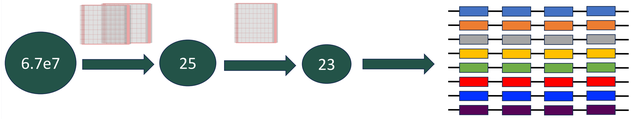



**Synthetic transcription factors play a central role in therapeutically relevant gene circuits.** Engineered transcriptional regulators act downstream of extracellular cues such as antigen recognition or small molecule treatment, translating these cues into customizable genetic responses, thereby enhancing the specificity, modularity, and safety of next-generation therapeutic platforms.

**Zinc finger DNA binding domains enable synthetic transcription factor (ZFTF) implementation** because their design rules are well understood, they are human derived, compact, and **can be designed to be orthogonal to the human genome**

## Design of DNA Binding Sites for Genome Orthogonal ZFTFs




**1) Generate random 19mers containing 6x GNN tri-nucleotides plus one final N.**

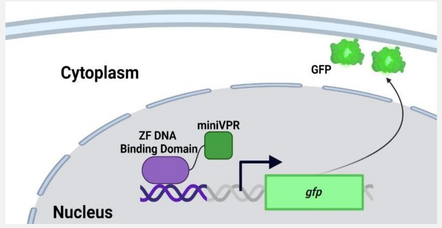
**2) Filter out sequences that match the human genome or have only 1-2 mismatches**

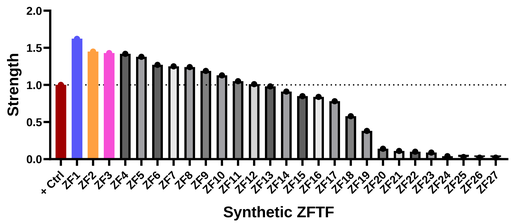
**3) Remove any remaining sequences that contain transcription factor binding sites**

**4) Generate synthetic enhancers with 4x repeats of the 19mer with 10bp spacers free of TFBS between each repeat. 2 TFs were designed for each synthetic enhancer.**

## Arrayed Screening of Candidate ZFTFs for Activation of Gene Expression

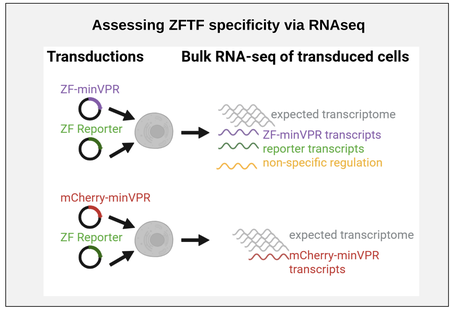
**Assessing zinc finger transcription factor strength:** ZFs were fused to a transcriptional activator and co-transduced with their cognate reporters (containing 4x ZF binding sites linked to a core promoter driving GFP expression)

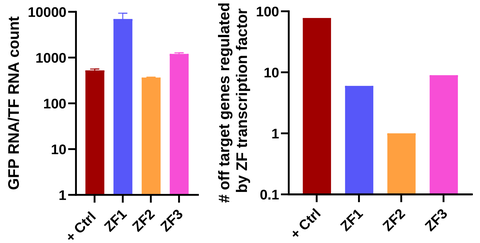



**Top 3 synthetic zinc finger expression circuits demonstrate high strength.** Arrayed screen in HEK cells revealed 3 candidates that outperform literature control Israni et al. [1] in transcriptional strength (>1.5 fold).

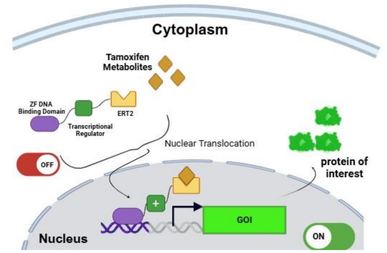
## Bulk RNA-Seq Analysis of ZF Genome Orthogonality



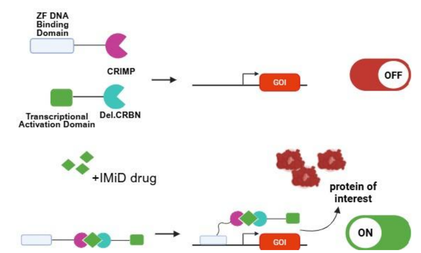




**Strength and specificity of top ZFTFs were analyzed by transcriptomic analysis.** Bulk RNA-seq in K562 cells transduced with ZFTFs and cognate reporters reveals top ZFTFs generate more GFP/TF RNA than the literature control [1] and activate fewer off target genes (7, 1, or 9 vs. 80).

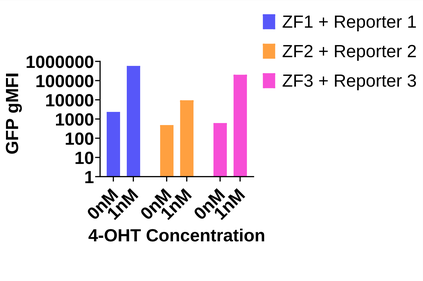
## Genome Orthogonal Drug Inducible Expression Switches



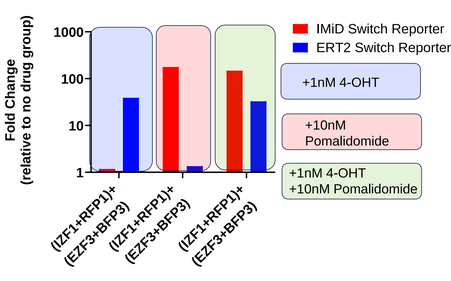

**Tamoxifen Switch Design.** A transcriptional switch responsive to 4-OHT (active tamoxifen metabolite) was built by fusing the ZFs to a modified version of ERT2 (drug binder) and a transcriptional activator (p65). Switch performance was evaluated using a GFP reporter assay.




**Pomalidomide Switch Design.** A transcriptional switch responsive to IMiD was built by fusing a ubiquitination deficient mutant of CRBN (del.CRBN) to a minVPR activator and fusing the ZF DNA binding domain to CRIMP, an IMiD co-binder derived from IKZF3 protein. Pomalidomide and Tamoxifen switches were analyzed within the same cell using dual mCherry and BFP reporters, respectively.




**Tamoxifen transcriptional switch functions in vitro.** 700-fold induction of reporter was detected in top tamoxifen drug switch in HEK cells treated with 1nM (physiologically relevant) concentration of tamoxifen metabolite (4-OHT) Binkhorst et al. [3].




**Tamoxifen- and pomalidomide-responsive transcriptional switches function orthogonally**. When co-expressed in HEK cells, each switch can be independently or simultaneously induced within the same cellular context.

## Conclusions and Next Steps

### Conclusions

- We identified three ZFTFs (ZF3, ZF2, and ZF1) that outperformed a previously published genome-orthogonal ZFTF [1] by >1.5-fold in transcriptional strength.
- Bulk RNAseq revealed that the top three ZFTFs (ZF3, ZF2, and ZF1) drove higher GFP expression per ZFTF RNA molecule than the published ZFTF, while affecting significantly fewer off-target genes (1, 7, or 9 vs. 80).
- The most potent ZFTF1 exhibits reduced off-target effects in two additional human cell types (T and liver cell lines) and in mouse melanoma cells.
- In the context of ERT2 and IMiD drug switches, ZF3 has best potency and specificity in an ERT2 switch and ZF1 has best potency and specificity in an IMiD switch.

### Next Steps

- Evaluate performance of orthogonal ZFTF expression circuit in drug regulated switch with therapeutically relevant proteins.

### References:

1) Morsut, L., et. al., (2016). *Cell*, 164(4), 780–791
2) Li, H.S., et. al., (2022). *Science, 378*(6625), 1227–1234.
3) Binkhorst, L., et. al., (2015). *Breast Cancer Research and Treatment, 152*(1), 119–128.