Poster 897

MEIRAGTx

Differential Usage of Transcription Factor Binding Sites to Boost Synthetic Promoter Activity

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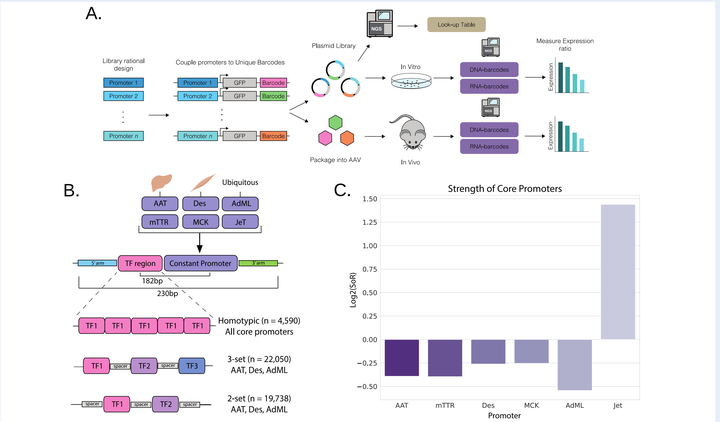
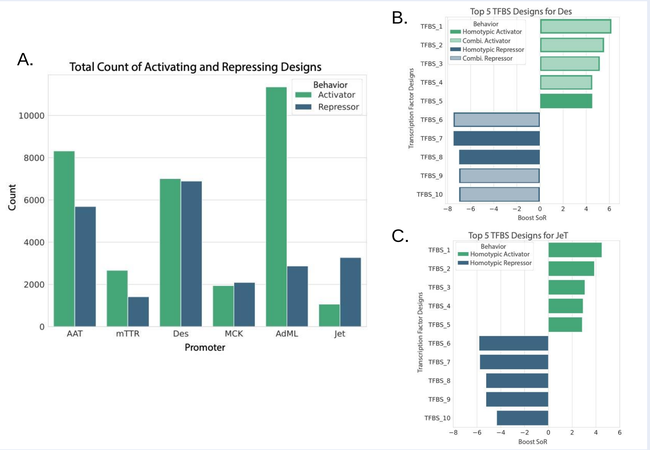
1. Using Transcription Factor Binding Site (TFBS) Shuffling to Design Strong Mini Promoters via Barcode-Driven Screening in Mouse N2A Cell Lines



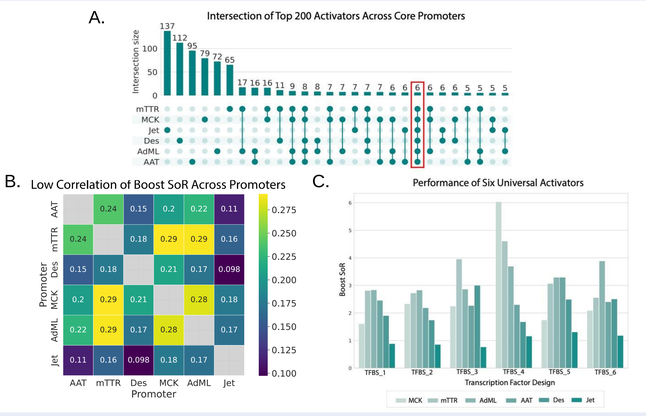
MPRA screens quantify transcriptional activity of different TFBS design strategies across small synthetic promoters. (A) Massively parallel reporter assays (MPRA) are high-throughput methods that assess the regulatory activity of thousands of DNA sequences simultaneously by linking each promoter design to a unique barcode and measuring its expression [1]. **(B)** Three design strategies (Homotypic, 3-set, and 2-set) were explored to assess transcriptional strength of 165,000 synthetic promoter sequences transfected into mouse Neuro2A (neuroblastoma) cell lines. **(C)** Six core promoters were used across designs: two liver-specific (AAT and mTTR), two muscle-specific (Desmin and MCK), and two ubiquitous (AdML and JeT). Promoter activity was quantified as the Sum of Ratios (SoR), representing transcriptional activity (RNA) normalized by transfection efficiency (DNA) for each sequence. Basal SoR (log_2) refers to the transcriptional activity of each core promoter in the absence of added TFBS.

2. Characterization of TFBS Behavior Reveals Strong Activating and Repressing Effects on Promoter Activity



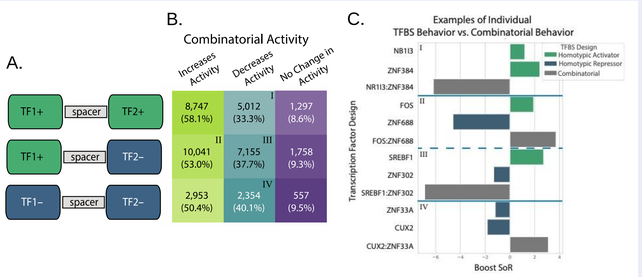
Strong Activating and Repressing TFBS designs identified across core promoters. Boost SoR is a log-based metric representing the proportional increase or decrease in promoter activity relative to the basal SoR [2]. **(A)** Distribution of all TFBS designs categorized as activators (Boost SoR ≥ 0.1, *n* = 89,302; 54.1%), repressors (Boost SoR ≤ –0.1, *n* = 59,245; 35.9%), or having minimal effect (Boost SoR between –0.1 and 0.1, *n* = 16,284; 10%). **(B)** Top five Activating and Repressing TFBS designs in muscle-specific Des and **(C)** ubiquitous JeT promoters. Shaded bars represent combinatorial designs; unshaded bars represent homotypic designs. The top activating design for Des increased activity by 6-fold relative to baseline, whereas the top repressing design reduced activity by up to 8-fold.

3. TFBS are Highly Selective Across Core Promoters in Homotypic Design



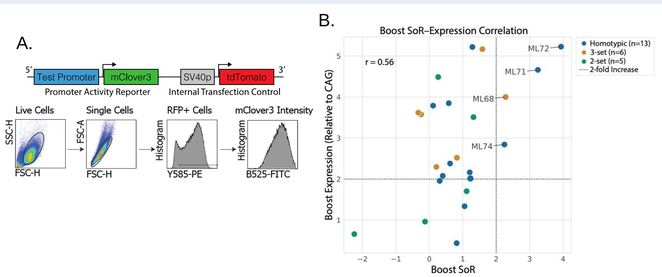
High degree of selectivity among homotypic TFBS designs. (A) UpSet plot of top 200 activating homotypic TFBS designs (ranked by Boost SoR) revealed only six TFBS overlapping across all core promoters. No overlap was observed among the top 200 repressing TFBS designs. The minimal overlap suggests a high degree of selectivity, indicating that TFBS–promoter interactions cannot be generalized based on TFBS activity in other contexts. **(B)** Correlation heatmap of Boost SoR values for homotypic designs across core promoters. Values represent Pearson correlation coefficients. The highest correlation (R^2=0.29) was observed between AdML and mTTR, and between AdML and MCK. On average, TFBS designs tested with JeT and MCK showed lower correlations with other promoters (R^2_{JeT}=0.14, R^2_{MCK}=0.23). **(C)** Performance of the six universal TFBS activators across core promoters. On average, these designs boosted transcriptional activity by at least 2-fold relative to the basal promoter activity.

4. Individual TFBS Behavior Does Not Predict Activity of its Combinatorial Designs



Homotypic TFBS behavior has little predictive power of combinatorial activity for dual-motifs. (A) Schematic of the three behavioral combinations in the 2-set TFBS design, with "+" and "–" indicating activating and repressing TFs, respectively. **(B)** Chi-squared contingency table showing the distribution of combinatorial activity (increase, decrease, and no change) across the different behavioral combinations. Counts and percentages are color-coded by magnitude (green=high, teal=medium, purple=low). While the difference between groups is statistically significant ($p=3.1\times10^{-29}$), the effect size is minimal (Cramér's V=0.0413), suggesting limited influence of individual TF behavior on combined activity. **(C)** Representative examples of each behavioral combination. Roman numerals correspond to combinations in Figure B.

5. Validation of TFBS Activity Using FACS-based Assay Shows Positive Correlation



Positive correlation between barcode-driven (RNA) and FACS-based (protein) assays. (A) Mini-promoter activity was assessed using a flow cytometry-based assay. Candidates were cloned upstream of mClover3 in a dual-reporter plasmid also expressing tdTomato as an internal transfection control. Activity was quantified as the mClover3/tdTomato median fluorescence ratio in live tdTomato⁺ cells. **(B)** Pearson's correlation coefficient (r=0.56, p=0.003) showed a positive correlation between computed Boost SoR and Boost expression relative to CAG (measured via flow cytometry) and proportionate to baseline activity. Notably, no promoters exhibited strong Boost SoR with weak expression relative to CAG, indicating consistent directionality between assays.

6. Conclusions

- MeiraGTx has a robust and predictive platform to evaluate the performance and transcriptional strength of hundreds of thousands of sequences using a high-throughput barcode-driven assay.
- Here, we characterized TFBS that modulate the activity of six core promoters, identifying elements that boost performance by up to a 6-fold increase and 8-fold decrease—including a 4-fold enhancement of the JeT promoter.
- TFBS in our library showed strong promoter-specific selectivity, enabling the identification of both promoter-enriching and activity-limiting sequences—key insights for designing potent synthetic promoters for gene therapy [3].
- Individual behavioral classification of a TFBS has little predictive power for the overall activity of combinatorial designs.
- Validation of TFBS designs demonstrated a significant positive correlation with *in-vitro* expression.
- Designing strong, compact promoters are critical for gene therapy, enabling efficient expression within the size limits of viral vectors like AAV.

6. Conclusions

1. van Arensbergen, J. (2019). High-throughput identification of human SNPs affecting regulatory element activity. *Nature genetics*, *51*(7), 1160–1169.
2. Martinez-Ara et al. (2022). Systematic analysis of intrinsic enhancer-promoter compatibility in the mouse genome. *Molecular Cell, 82(13), 2519–2531.e6.*
3. Li, C., & Samulski, R. J. (2020). Engineering adeno-associated virus vectors for gene therapy. *Nature reviews. Genetics*, *21*(4), 255–272.