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Bi-directional Titration of Yeast Gene Expression using a Pooled CRISPR Guide RNA Approach

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Author Contributions

E.K.B., M.D., H.A. and Y.Y. designed research, E.K.B. and M.D. performed research, J.F.C., R.E., E.O., and Y.Y constructed sgRNA library, E.K.B., M.D., and H.A. analyzed data, E.K.B., M.D. and H.A. wrote the paper.

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Abstract

Most classic genetic approaches utilize binary modifications that preclude the identification of key knockdowns for essential genes or other targets that only require moderate modulation. As a complementary approach to these classic genetic methods, we describe a plasmid-based library methodology that affords bi-directional, graded modulation of gene expression enabled by tiling the promoter regions of all 969 genes that comprise the ito977 model of *Saccharomyces cerevisiae*'s metabolic network. When coupled with a CRISPR-dCas9 based modulation and next-generation sequencing, this method affords a library based, bi-direction titration of gene expression across all major metabolic genes. We utilized this approach in two case studies: growth enrichment on alternative sugars, glycerol and galactose, and chemical overproduction of betaxanthins, leading to the identification of unique gene targets. In particular, we identify essential genes and other targets that were missed by classic genetic approaches.

Significance Statement

Traditional genetic modulation approaches are typically restricted to binary perturbations, single-sided titrations (either graded up or down regulation), or individual gene expression modulation. Here, we utilize a library approach that is able to simultaneously modulate gene expression in a metabolism-wide manner. This library, when coupled with next-generation sequencing, allows for the identification of novel gene perturbations that would have been missed by classic approaches. For the examples tested, this library identified targets that improved growth on alternative carbon sources as well as improved production of betaxanthins.

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Main Text

Introduction

Classic genetic approaches for identifying gene targets have traditionally been limited to binary modifications consisting of either deletion or strong over-expression (1-3). This approach is not ideal for identifying all targets, including essential genes that need to be repressed or any genes whose expression only requires modest modulation. Moreover, even most emerging genetic tools including CRISPR / CRISPRi, TALEN, and RNAi still mainly invoke binary gene expression modulation (4-6). While these approaches have been successful in ascribing functional annotation and identifying dominant gene targets, they are often insufficient when seeking to optimize cellular metabolic function, or when attempting to identify other salient genetic targets. For example, deletion library screens are unable to identify essential genes whose knockdown may in fact be positively correlated with phenotype. Moreover, it has long been recognized that optimal expression of genes resides at intermediate expression levels between the extremes of complete gene deletion and strong over-expression (7). Traditional approaches fail to identify these targets that are only effective at intermediate expression levels. Thus, new approaches are needed to both enable high-throughput identification of targets and complement the limitations of most genetic screens.

The use of transcriptional element libraries (such as promoter libraries (8)) have traditionally been used to enable graded gene expression for individual pathway applications for phenotypes such as small molecule production (9) and the consumption of alternative carbon sources (10, 11). However, these techniques are often not accessible to genome-wide, high-

throughput implementation. Recent advances to establish graded expression level libraries have utilized RNAi approaches with either micro-RNAs or full-length complementary mRNAs to afford two levels of gene knockdown (12). More recently, a CRISPR interference (CRISPRi) based sgRNA library was implemented in mammalian cells to create a broader spectrum of down-regulation to identify causative genetic targets for fitness phenotypes (13). In addition, one group utilized dCas9 fused to VPR to identify finely-tuned, ideal levels of gene expression for 168 different genes, by tiling each one 21 times (14). While these approaches are all working toward an accessible high throughput library for graded expression ranges, they fall short of achieving both knockdown and overexpression capacity and thus lack the full range of possible gene expression perturbations.

Here, we showcase the implementation and utility of a novel, plasmid-based library methodology that affords bi-directional titration of yeast gene expression in a manner that is complementary to traditional genetic approaches (**Figure 1a**). We leverage our previously reported STEPS approach to design a panel of single guide RNAs that tile promoter regions for all 969 genes represented in the ito977 genome-scale metabolic model (15). These sgRNAs were synthesized in a pooled format through collaboration with the Joint Genome Institute (JGI) Synthesis Science Program. By coupling this sgRNA library with a previously established CRISPR-dCas9 system, we can take advantage of dCas9 fusions with Mxi1 for heterochromatin formation or VPR for recruitment of the mediator complex, thus creating graded up and down regulations, respectively (16).

Results and Discussion

Construction of an sgRNA Library for Metabolism-wide, graded expression

The aforementioned sgRNA library was designed in a pooled format to target broad classes of metabolic function including carbohydrate and lipid metabolism (Pool 1), energy and cofactor metabolism (Pool 2), amino acid and nucleotide metabolism (Pool 3), and housekeeping/other (Pool 4) (**SI Appendix, Data Set 1A-D**). More specifically, oligos containing unique sgRNAs were designed to tile upstream of the promoter region of 969 metabolic enzyme genes in *S. cerevisiae*. This DNA was synthesized in collaboration with the Joint Genome Institute and was then amplified and cloned into both the dCas9-Mxi1 and -VPR backbone (**SI Appendix, Fig.1**). These libraries were then transformed and either propagated in broth or on plates in order to amplify the libraries. Plasmids were then harvested to obtain 100ug of DNA and subsequently sequenced via PCR amplification of the guide RNA region followed by Next Generation Sequencing from both Broth-based and Agar plate-based transformations (**SI Appendix, Fig. 2, and Table 1**) to validate coverage. Plate-based transformations were originally theorized to allow for an equal representation of all sgRNAs as this process does not have the same out-growth step as in liquid-broth propagation that could bias representation through potential competitive growth. However, these experiments demonstrated that broth-based transformations actually resulted in the best representation of the individual library members and thus purified DNA from this condition was used for subsequently screening experiments (**SI Appendix, Fig. 1).** To demonstrate the utility of this approach for the identification of unique targets as well as their optimal expression levels, we evaluated several growth and production phenotypes as proof-of-concept experiments.

Library enrichments on alternative carbon sources identify novel targets

As the first set of case studies, we utilized growth-based enrichments on alternative carbon sources using the common laboratory strain of *S. cerevisiae,* BY4741. Here, we chose to select enrichments on both glycerol and galactose due to their industrial relevance (galactose comprises a significant portion of marine biomass and glycerol is produced in large quantities as a by-product of biodiesel transesterification (17, 18)) as well as extensive prior studies on these carbon sources using classic approaches (19). Prior to conducting a deep sequencing analysis of enrichment using these pools, we first validated the enrichment capacity of these libraries using a repeated sub-culturing, colony isolation, and sequence analysis approach (**Figure 1b, SI Appendix, Fig. 3**). In these trials, individual beneficial guide RNAs were isolated and confirmed to not only show enrichment over time, but also confer a growth advantage when re-transformed.

Following these validation tests, a full-scale growth enrichment process accompanied by deep sequencing analysis was used to globally identify targets along with their optimal expression levels (**Figure 1b**). To do so, we chose a partial subculture condition to detect both enrichment and depletion as well as preventing over-enrichment by a few dominant targets. Macroscopic analysis of statistical enrichment and depletion of guide RNAs within the library illustrates that the majority of guides were depleted in the post-enrichment pools indicating that most perturbations to gene expression are outcompeted in this assay when growing on glycerol and/or galactose (**Figure 1c**, **SI Appendix, Sheet 2**).

Given the high-resolution aspect of this dataset (*i.e.,* having both target identifications along with their optimal expression levels), multiple modes of analysis are possible. For example, a cluster analysis allows for a full mapping of gene expression-level phenotype enhancement for both carbon sources (**Figure 1c**, **2a**, **SI Appendix, Fig. 4**). Each major cluster links together targets

whose optimal expression profile and patterns are similar. Initial evaluation of these trends and patterns indicates an overrepresentation of guide enrichment at moderate levels of expression (both for knockdown and overexpression) (**Figure 1b, 2a, 2b, SI Appendix, Fig. 4**). At the onset, this data illustrates the complementary nature of this approach to coarse-level, binary modification of gene expression. Several gene targets emerge whereby moderate knockdown greatly enhances growth relative to the complete knockout. As examples, knockdown of *IPK1* and *TPS2* resulted in improved growth on glycerol, but the complete deletion of these targets results in substantially reduced growth (**Figure 2b**). Additional examples include identifying guides targeting *FUN26* whose growth showed far improvement over the counterpart deletion of this gene (**SI Appendix, Fig. 5**). On the opposite end of the spectrum, glycerol-growth enhancing targets like *GRS2* overexpression are only optimal/functional when targeted at modest overexpression sgRNA localization regions. This point is especially poignant when comparing growth with both lower and higher levels of expression for this target (**Figure 2c**). Examples such as these illustrate the depth of new targets identifiable with this approach.

Beyond visually confirming the premise that different expression levels are required for different subsets of genes, these clusters can be analyzed to determine underlying metabolic trends for these growth phenotypes. For example, through Gene Ontology (GO) analysis, a significant number of phosphate-related metabolic genes were seen to be enriched for the medium knockdown level in galactose selection (utilizing the SGD Gene Ontology analysis tool) (**SI Appendix, Fig. 6**). More specifically, the most represented genes within this knockdown level were associated with phosphorous (44.4%) metabolic processes. Gene expression clusters for glycerol consumption indicated significant (P-value <0.05) enrichment of genes associated with organic acid synthesis (42.9%) in the case of the highly up-regulated cluster (VPR -150). Phosphorous

metabolism is also important for glycerol catabolism, wherein we observed the Mxi1 -500 cluster enriched with genes significantly (P-value < 0.05) associated with phosphorous metabolic processes (50%) and a further enrichment of genes associated with carbohydrate phosphorylation (28.6%) in the low knockdown cluster, VPR +80. Thus, large-scale trends of metabolism can be extracted through this analysis.

The approaches described here are indeed complementary to more classic genetic approaches such as gene deletion libraries, especially in the capacity to identify essential genes as critical targets for gene knockdowns. The sgRNA library synthesized here contained 86 essential genes. Within this set, 8 of these targets are identified as downregulation targets in galactose and 10 of these genes we identified as downregulation targets in glycerol (with an additional 10 of these genes enriched as overexpression targets) (**SI Appendix, Data Set 2 and Fig. 7**). These examples in particular are poignant demonstrations of the benefit of an expression titration library as these targets would not be identified in a full knockout collection. As examples, knockdowns were identified for both *DIM1* and *GPI18,* both of which are essential genes. The knockdown condition substantially improved growth rates in their respective alternative carbon sources whereas the complete deletion of these genes is lethal (**Figure 2d**). Thus, this approach identified a unique set of targets for which galactose and glycerol-improvement phenotypes were not previously ascribed.

Finally, overlap of the targets identified here with previously identified literature targets that serve to improve or hinder growth on alternative carbon sources was evaluated and found to be limited (**SI Appendix, Fig. 7)**. Of particular note, this study utilized a competitive growth enrichment assay whereas most targets in literature were isolated from knockout collections and utilized individual target analysis. Nevertheless, there is some overlap of targets. For example,

HXT17 is a known overexpression target for improving growth on galactose (20) and this same target was identified through this screen. In some cases, other well-known individual overexpression targets for improved growth on galactose, including *PGM2*, were not identified as statistically enriched likely due to being out competed in this assay. However, sgRNAs associated with down regulation of PGM2 were in fact significantly depleted, thus demonstrating the implication of this enzyme on galactose consumption in general **(SI Appendix, Table 2)**. Additional known targets such as the detrimental effect of *aim10* and *gal10* deletions growth on galactose (21) are instead complemented by the enrichment for only moderate levels of downregulation in our library. Collectively, the coupling of our sgRNA library with a competitive growth enrichment adds a dimension of enriching for fitness and optimal expression levels. Thus, while we have identified some known targets, we predominately highlight here the novel knowledge of optimal expression levels. As with any screening process, the mode of library screening is important for target identification and this library is likewise suitable for single clone at a time analysis as has been conducted with the gene deletion libraries.

Identifying novel targets for improved betaxanthins production

As our second overall case study, we sought to utilize a production-based screen rather than growth selection to demonstrate the versatility of this library. In this regard, we evaluated targets that would improve product secretion of betaxanthins in a *S. cerevisiae* strain background (BX3) over-expressing the CYP76AD5 tyrosine hydroxylase from *B. vulgaris* and L-Dopa dioxygenase (DOD) from *M. jalapa (22)* in addition to the negative feedback resistant mutant of DAHP synthase ($ARO4_{K229L}$)(23) (**SI Appendix, Fig. 8**). Using this strain and the fluorescent nature of this product, we were able to employ a parallel microplate-based approach suitable for

quantifying total (rather than simply intracellular) betaxanthin—a divergence of work in the literature. With this approach, our roughly 10^3 screening capacity demanded a two-layer screening approach that first looked for high variability in each of the four synthesized sgRNA pools followed by a deeper analysis of the most variable pools (**Figure 3a**). For example, an initial screening of the Mxi1 library highlighted that Mxi1 Pool 1, Pool 2, and Pool 4 produced similar variation in fluorescence whereas Pool 3 (amino acid and nucleotide metabolism) displayed minimal variation above the pool median (**Figure 3a**). As a result, we performed a deeper screen across Pool 1 by evaluating over 400-500 colonies (equivalent to roughly 70-80% of the coverage).

A total of 4 unique targets (in either expression level or identity) were identified from the analysis of Pool 1. To investigate interactions between these isolated targets, we constructed combinations of these sgRNAs and tested them against their individual sgRNA targets (**Figure 3b**). While almost all combinations produced fluorescence values that approached the best sgRNA in the pair, the combination of the *ZWF1-RKI1* sgRNAs showed positive epistasis and produced the highest fluorescence of any single or double sgRNA combination and values that exceed improvements seen in the literature (24).

As with the growth selections, the expression titration afforded by this pooled approach was able to identify novel targets not previously ascribed to this phenotype by more traditional approaches. For example, as *RKI1* is an essential gene, its utility as a perturbation target would be missed under classical approaches. Likewise, intermediate expression is often optimal as can be seen in the direct comparison of individual guides targeting *PDR11* and *LAC1* that resulted in increases in fluorescence compared to the parent strain, while their respective knockouts actually decreased fluorescence when compared to the parent strain (**Figure 3b**). These targets would have been missed in a traditional knockout screen. Perhaps the target that best exemplified this

optimality is *ZWF1*, a gene identified as a slight knockdown target. Indeed, by establishing a panel of sgRNAs associated with repression of *ZWF1,* the intermediate knockdown (sgRNA targeting the -371 region) resulted in the greatest improvement whereas strong knockdown had a strongly negative phenotype (**Figure 3c**). In this instance, we hypothesized that an intermediate level of *ZWF1* expression would properly balance NADPH and precursor availability for L-DOPA production, whereas too strong of a knockdown would simply improve 3-DHS levels, as previously validated (16). The isolated *ZWF1* gene has been previously characterized as an important target to control precursor flux into the shikimate pathway (25). While *zwf1* deletion properly balances the ratio of phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) needed for enhanced flux into the shikimate pathway, it also compromises flux by limiting the amount of NADPH available for the conversion of 3-DHS to shikimate. Our library identified an intermediate level of *ZWF1* expression that led to an increase in betaxanthins production. This level of intermediate expression would have been missed in a knock-out screen. This example showcases the importance of evaluating multiple expression levels as many beneficial targets can be missed otherwise.

Finally, deep screening of 384 clones from VPR Pools 1 and 4 indicated several novel targets requiring modest overexpression, including *DIT2*, *FBA1*, and the alpha-1,2 mannosyltransferase, *GPI10*. These non-pathway specific targets provide additional key insight into pathway regulation. Therefore, this library not only can identify novel targets and levels of expression, but could be used as a discovery tool to learn more about pathway regulation and metabolic flux.

Conclusion

Taken together, these two case studies highlight the importance of utilizing bidirectional titration of gene expression for identification of novel gene targets as well as their optimal level of expression. This approach is highly complementary to classic genetic approaches that tend to only create binary changes in gene expression. In many cases, we find gene knockdowns of essential genes that greatly impact phenotypes of interest yet have been missed thus far due to a reliance on binary genetic changes. Likewise, we identify targets for which only moderate levels of modulation are optimal. The unique ability to screen for novel targets as well as their optimal level of expression using this library provides a powerful tool for studying genotype-phenotype relationships. We foresee that the new knowledge gained using this tool will push forward a second wave of genetic analysis in the yeast *S. cerevisiae*.

Materials and Methods

Data Availability

All Next Generation Sequencing data can be found at NCBI under accession number: SUB7272763. In addition, all code used for analysis can be found at the following link: https://github.com/emkbowman/Bi-directional-Titration-NGS-Analysis. Analyzed NGS data is available under **SI Appendix, Dataset 2**. Transformed or purified sgRNA library may be acquired upon request.

Strain Design

DH10β was used to propagate all yeast expression vectors including those described by Mumberg (26) and pJED103-based (27). To amplify plasmids, *E. coli* strains were cultivated in LB or SOB media (Teknova) supplemented with 50 µg/mL ampicillin or kanamycin (Sigma) with 225 RPM

orbital shaking at 37 °C. Yeast strain BY4741(EUROSCARF) was cultured in yeast synthetic complete (YSC) medium containing roughly 6.7 g/L of yeast nitrogen base (Difco), 20 g/L glucose (MP Biomedicals) and 1x CSM-URA, CSM-URA-LEU, or CSM-URA-LEU-HIS (MP Biomedicals) depending on the required auxotrophic selection. The *S. cerevisiae strain* BX3 (URA3::pCCW12-MjDOD-tADH1-pTDH3- BvCYP76AD5-tTDH1-pTEF1-ScARO4K229L-tENO2) was constructed from BY4741 (Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) by digestion of the pCMC0759 integration vector, provided as a gift from the Dueber Lab (24)**.**

Transformations

In order to transform Gibson cloning reactions, 3 µL of 3-fold diluted Gibson reaction (NEB, 254 ng backbone) was mixed with 50 µL of electrocompetent E. coli DH10β and electroporated (2mm Electroporation Cuvettes) with a BioRad Genepulser Xcell at 2.5 kV. For transformation of ligations, 3 µL ligation mix (NEB) was directly added to 50 µL competent cells and transformed as above. For ampicillin-marked plasmids, transformants were re-suspended in 500 µL of SOB, plated on LB agar supplemented with 50 µg/mL ampicillin, and incubated at 37°C overnight. Transformants of kanamycin-marked plasmids were recovered in 500 µL SOB for 30 minutes at 37°C and then plated. Individual clones were picked into SOB media containing 50 µg/mL antibiotics and incubated at 37°C overnight. Plasmids were then mini-prepped (GeneJET Plasmid Miniprep Kit, Thermo Scientific) and sequence verified via Sanger sequencing. The Frozen EZ Yeast Transformation II Kit (Zymo Research) was used to transform plasmids into yeast. Briefly, between 200 ng and 1 µg of plasmid was mixed with 20 µL chemically competent cells prepared by manufacturer's instructions, and 200 µL EZ Solution III followed by incubation at 30 °C for 45 minutes. Transformations were then plated on YSC+agar plates containing either CSMURA,

CSM-URA-LEU, or CSM-URA-LEU-HIS, and incubated at 30 °C for 2 days. Individual colonies were randomly picked in triplicate into 1 mL of YSC media and incubated at 30 °C for another 2 days. For long-term storage, all yeast strains with the exception of transformed libraries were stocked in 15% glycerol and kept at -80 °C in sterile flat-bottomed micro-titer plates (Corning) covered with an adhesive aluminum foil seal (Thermo Scientific) and plastic lid.

Cloning Procedures

Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Sequences and annotations can be found in **SI Appendix, Table 3**. PCR and anneal/extend double-stranding reactions were performed with Q5 DNA Polymerase from New England Biolabs according to the manufacturer specifications. Digestions were performed according to manufacturer's (NEB) instructions. PCR products and digestions were cleaned with a QIAquick PCR Purification Kit (Qiagen). All vectors were de-phosphorylated with Antarctic Phosphatase (NEB) according to the manufacturer's instructions and heat inactivated for 15 min at 65°C.

All plasmids for expression of dCas9 were derived from the pJED103 vector series acquired from AddGene catalog #46921(27). All RGR plasmids were derived from the dCas9-Mxi1and dCas9-VPR plasmids previously reported (16). To construct new RGRs, the dCas9-Mxi1 and dCas9-VPR RGR cloning vectors were linearized at the 5' end of the sgRNA scaffold using the SpeI enzyme and then a 100 bp fragment containing a variable HH-sgRNA sequence was inserted via Gibson assembly to create the full TEF1p-HH-sgRNA-HDV-TKC27t cassette. The 100 bp insert fragments were constructed by an anneal/extend PCR using two 60-bp oligos (IDT) with 20 bp overlaps at their 3' ends. Multiplexed sgRNAs were constructed using PCR with

primers MD1522/MD1523 followed by Gibson Assembly into Mxi1 sgRNA vectors linearized with EcoRI.

Library Enrichment on Alternative Carbon Sources

BY4741 was transformed in a pooled format utilizing a scaled-up Geitz transformation. These libraries were cultivated in 2% Raffinose, CSM-L, YNB to maintain the plasmid and prevent any errant glucose-based enrichment. Libraries were stocked in 1 mL aliquots and mixed 1:1 with 40% glycerol and stored at -80 °C . For use, these aliquots were thawed and grown in a 50 mL flask containing 2% Raffinose YSD-L until the OD_{600} was over 1.0. Libraries were then diluted down to an OD of 0.1 and re-suspended into 50mL of media comprised of CSM-L, YNB, and either 6% Glycerol or 4% Galactose. Biological duplicates were used for library enrichment (i.e. two separate enrichments were performed for each condition). The 6% Glycerol media was adjusted to a pH of 4.0. These cultures were monitored for growth and once the OD_{600} reached 1.0, they were serially diluted into a fresh flask containing new media with a starting OD_{600} of 0.1 for 3 times before conducting a yeast mini prep followed by NGS analysis.

NGS Sample Prep and Analysis

Yeast mini preps were used as a template and amplified utilizing Q5 polymerase and following manufacturers' instructions (Primers in **SI Appendix, Table 3**). To avoid sequence bias, only 20 amplification cycles of PCR were used. The product was purified via gel electrophoresis and extracted utilizing a gel purification kit from Qiagen prior to being submitted for library construction and analysis at the UT FBS Sequencing Core. All raw NGS data can be found at NCBI under accession number: SUB7272763. Analysis of sequencing reads was performed by

obtaining individual read counts and aligning to the sgRNA library, utilizing code that can be found at https://github.com/emkbowman/Bi-directional-Titration-NGS-Analysis (**SI Appendix, Table 1**). These were normalized to the total number of reads and then averaged. A two-tailed T-test was run on each guide to confer significance of enrichment, determined by a p-value of <0.05. Finally, the log fold change was calculated for each guide utilizing Excel and clustered using Cluster 3.0, with city-block distance and complete linkage settings. This analysis was visualized in Java TreeView, with pixel settings centered at log 1.0.

Growth Analysis of Select Guides

Guides identified via either Sanger sequencing or NGS were either re-transformed (if isolated via Sanger sequencing to confirm enrichment of beneficial guides) or re-cloned utilizing cloning procedures mentioned above and transformed utilizing the EZ yeast transformation according to manufacturer's instructions and plating on synthetic selective media (CSM-L, 2% Raffinose). Three clones were picked from each transformation and grown in 2% Raffinose synthetic defined media and glycerol stocked. Glycerol stocks were taken out and grown overnight in 2% Raffinose synthetic defined media and then diluted down to an OD_{600} of 0.1 in the same 6% Glycerol or 4% Galactose enrichment media from which they were identified. OD measurements were collected roughly every 24 hours.

Plate Reader Measurement of Betaxanthin Fluorescence

All betaxanthin-producing strains were characterized using the Cytation 3 MicroPlate Reader (BioTek Instruments, VT). At minimum, biological triplicates were used for each strain and each condition, with up to 6 replicates used for more sensitive fluorescence assays. Strains

were grown from glycerol stock for 30 °C at 72 h in CSM-URA-LEU media and then back-diluted 100x into CSM-URA-LEU supplemented with 10 mM ascorbic acid and 50 mM beta-alanine. Strains were grown until fluorescence no longer increased from day-to-day (typically around 72-120 h). To measure bulk fluorescence, cultures were diluted either 20x or 40x in PBS (to ensure the detector was not saturated). To measure supernatant fluorescence, cultures were spun down at 1600 g for 4 minutes and the supernatant was diluted 20x in PBS. To measure intracellular fluorescence, cells were spun down and washed once with PBS, re-suspended in 100 uL PBS, and diluted 20x. Fluorescence was normalized to OD_{600} for intracellular fluorescence measurements.

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Competing Financial Interests

The authors declare no competing interests.

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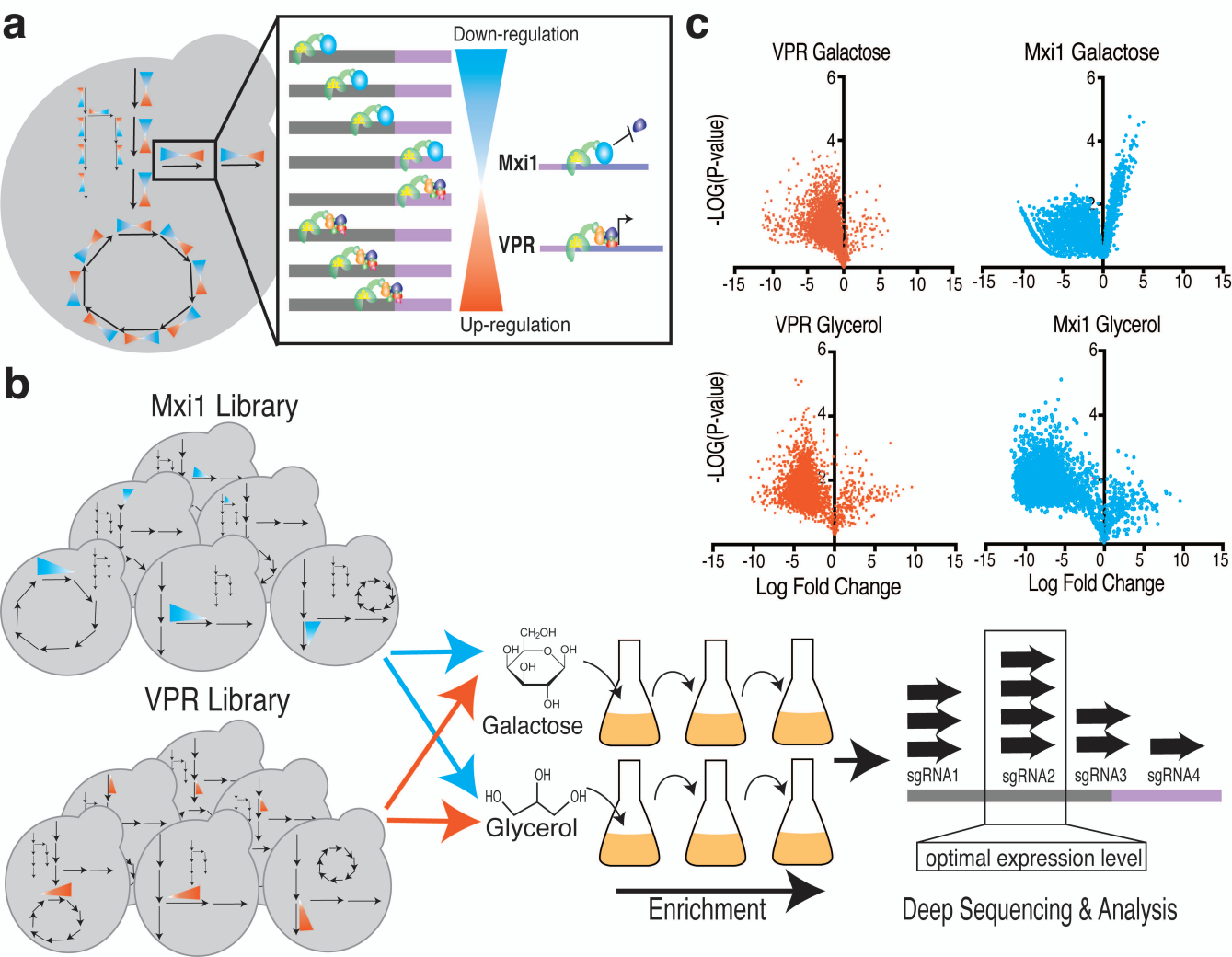
Figure Legends

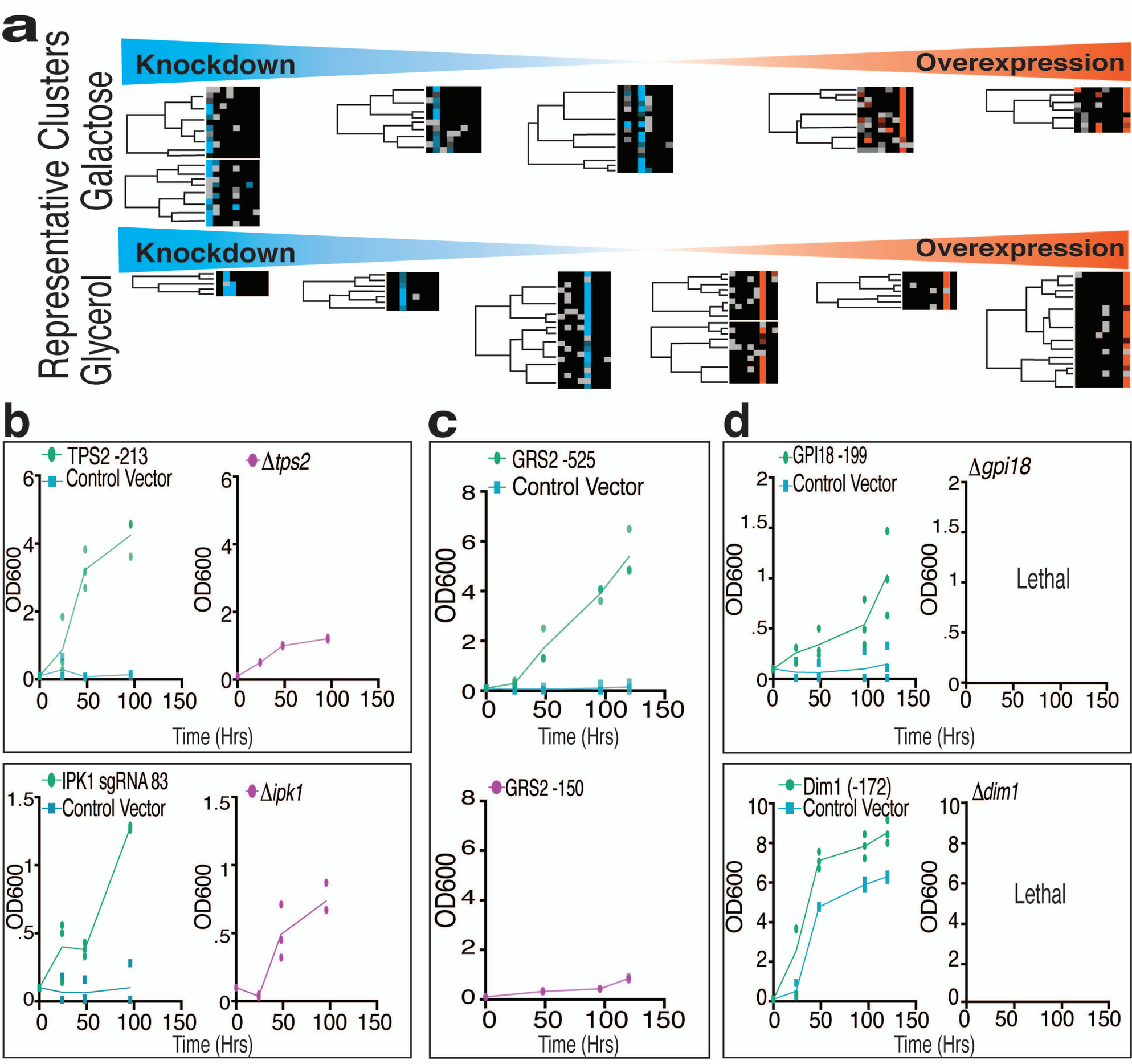
Figure 1. *Enrichment of a Bi-directionally Titrated Metabolism-Wide Library.* **a)** A metabolism-wide, bi-directional titration panel of sgRNAs was synthesized based on the ito977 model of metabolism and enabled via dCas9 fused to either Mxi1 or VPR. These fusions allow for graded knockdown or overexpression of targeted genes of interest, respectively. **b)** Enrichments were performed on the alternative carbon sources of galactose and glycerol using the Mxi1 and VPR libraries independently to allow for ease of deep sequencing. Final populations from each enrichment were deep sequenced to identify statistically significantly enriched guides. **c)** Volcano plots of guide enrichment for each condition. These plots show that the majority of guides in the library were depleted, indicating that most perturbations were outcompeted in growth on alternative carbon sources.

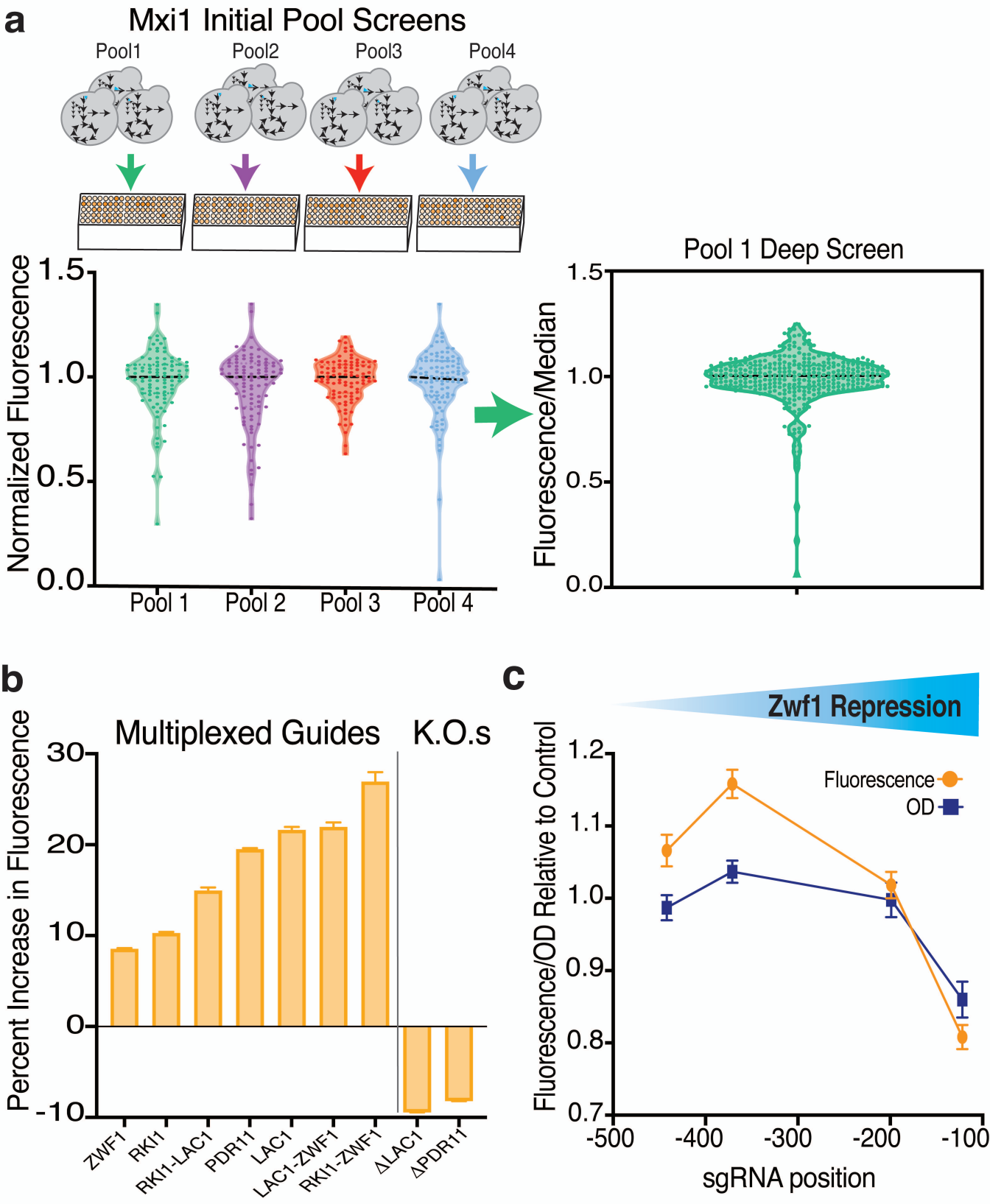
Figure 2. *Cluster analysis and Subsequent Ideal Guide Confirmation of Glycerol and Galactose Enrichments*. **a)** A representative set of clusters for enriched guides are illustrated for both galactose and glycerol. In this representation, gene ID is on the vertical axis and the bidirectional titration is on the horizontal axis going from strong knockdown to strong overexpression. The predominant level of expression in each cluster is highlighted by blue or orange. Results illustrate a strong enrichment of moderate expression levels across these conditions. **b)** An example of intermediate knockdown targets identified from glycerol enrichment are compared with the knockout demonstrating that moderate regulation was optimal. **c)** As an example of a moderate expression target, GRS2 overexpression is highlighted wherein the guide furthest upstream (i.e. slightest up-regulation) was significantly enriched following serial culturing and provided a far improved growth over the strong expression guide and the wild-type. This ability to tune gene

expression to specific levels of overexpression is not seen in traditional overexpression libraries. **d)** Essential genes were uniquely identifying with this method as down-regulation targets. As examples, *gpi18* was identified from the glycerol enrichment and *dim1* from the galactose enrichment. Both guides were re-cloned and showed improved growth whereas the deletion is lethal, and thus these represent new targets unseen with traditional approaches.

Figure 3. *Betaxanthins Production Phenotype Improvement Utilizing a Pooled Screening Approach.* **a)** Initial, pool-based screening of Mxi1 pools was conducted using a plate-based assay followed by deep screening, as shown with Pool 1. Characterization of identified sgRNAs imparting an improved fluorescence were identified via sanger sequencing, and re-cloned into the parent strain, BX3. **b)** Identified targets when analyzed and in multiplex showcase several unique targets achieving up to 30% increase in fluorescence over the parent strain. In addition, the knockouts of the two, best performing, single guides were made in BX3 and their fluorescence measured indicating that these targets would not have been identified through a traditional approach. **c)** Tiling of the ZWF1 promoter region with guide RNAs showed an ideal level of expression (i.e., more fluorescence) at the medium level of knockdown. This ideal mid-level of expression is uniquely identified in a titratable expression library.


a
Down-regulation
Mxi1
VPR
Up-regulation
c
VPR Galactose
Mxi1 Galactose
VPR Glycerol
Mxi1 Glycerol
-LOG(P-value)
Log Fold Change
b
Mxi1 Library
VPR Library
Galactose
Glycerol
Enrichment
sgRNA1
sgRNA2
sgRNA3
sgRNA4
optimal expression level
Deep Sequencing & Analysis


a
Representative Clusters
Galactose
Knockdown
Overexpression
Glycerol
Knockdown
Overexpression
b
TPS2 -213
Control Vector
Δtps2
OD600
Time (Hrs)
IPK1 sgRNA 83
Control Vector
Δipk1
OD600
Time (Hrs)
c
GRS2 -525
Control Vector
OD600
Time (Hrs)
GRS2 -150
OD600
Time (Hrs)
d
GPI18 -199
Control Vector
Δgpi18
Lethal
OD600
Time (Hrs)
Dim1 (-172)
Control Vector
Δdim1
Lethal
OD600
Time (Hrs)


a
Mxi1 Initial Pool Screens
Pool1
Pool2
Pool3
Pool4
Normalized Fluorescence
1.5
1.0
0.5
0.0
Pool 1
Pool 2
Pool 3
Pool 4
Pool 1 Deep Screen
Fluorescence/Median
b
Multiplexed Guides
K.O.s
Percent Increase in Fluorescence
30
20
10
0
-10
ZWF1
RKI1
RKI1-LAC1
PDR11
LAC1
LAC1-ZWF1
RKI1-ZWF1
ΔLAC1
ΔPDR11
c
Zwf1 Repression
Fluorescence
OD
Fluorescence/OD Relative to Control
1.2
1.1
1.0
0.9
0.8
0.7
-500
-400
-300
-200
-100
sgRNA position

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Supplementary Information File for
Bi-directional Titration of Yeast Gene Expression using a Pooled CRISPR Guide RNA Approach

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Contents

Supplementary Table 1
NGS data of library coverage for each pool.

Library	Pool	Number of Variants	% reads match variants (100% match)	Missing
VPR	Pool 1	1203	70.7	5
	Pool 2	588	73.3	0
	Pool 3	720	72.4	0
	Pool 4	1320	72.6	1
Mxi1	Pool 1	1203	82.8	0
	Pool 2	588	83.7	0
	Pool 3	720	82.4	0
	Pool 4	1320	83	0

NGS data of library coverage for each pool. Most pools were fully in-tact with the majority matching guide sequences perfectly (100% match).

Supplementary Table 2

Galactose consumption associated genes with corresponding sgRNA library enrichment information.

Name	LFC	Guide Location	Library	Carbon Source
HXT17	2.5	-200	VPR	Galactose
GAL10	2.5	-300	Mxi1	
AIM10	2.5	-300		
HXT10	3.75	-300		
HXT9	2.1	-150		
GAL7	-9	80	VPR	
GAL1	-8.75	-300	Mxi1	
PGM2	-8	-150		
ALD4	-8.5	-150		
HUT1	-8	-300		
GPD1	5.5	-500	Mxi1	Glycerol
TPI1	2.5	-150		
STL1	3	-300		
GUP1	3.5	-300		
GPD2	6	-300		

List of genes identified in library enrichment that have been associated with galactose or glycerol consumption as identified by *yeastminer* (yeastmine.yeastgenome.org).
Log fold change values have been rounded to the closest quarter for ease of reading.

Supplementary Table 3

List of primers used in this study

Name	Purpose	Sequence
MD1518	Fwd AE Gibson sgRNA ZWF1 -442	AAGTTTACCCCATCGATCAATTGTTCTGATGA GTCCGTGAGGACGAAACGAGTAAGCTCG
MD1519	Rev AE Gibson sgRNA ZWF1 -442	GCTATTTCTAGCTCTAAAACGTAGCGCTACTA TTATTGTTGACGAGCTTACTCGTTTCGT
MD1520	Fwd AE Gibson sgRNA ZWF1 -199	AAGTTTACCCCATCGATCACGCCACCTGATGA GTCCGTGAGGACGAAACGAGTAAGCTCG
MD1521	Rev AE Gibson sgRNA ZWF1 -199	GCTATTTCTAGCTCTAAAACTACATTGAAGAA TTCGCCACGACGAGCTTACTCGTTTCGT
MD1522	Fwd primer PCR of TEF-RGR cassettes to Gibson into Mxi1-RGR plasmids	TAATACGACTCACTATAGG
MD1523	Rev primer PCR of TEF-RGR cassettes to Gibson into Mxi1-RGR plasmids	GGAGTAGAAACATTTTGAAGCTATTAGACTGT TTGAAAGATGATACTCTTTATTCCTAC
MD1631	Fwd anneal/extend to make sgRNA LAC1 -460	aagtttaccccatcgatcATTCCTTCTGATGAGTCCGTG AGGACGAAACGAGTAAGCTCG
MD1632	Fwd anneal/extend to make sgRNA LAC1 -346	aagtttaccccatcgatcACATGATCTGATGAGTCCGTG AGGACGAAACGAGTAAGCTCG
MD1633	Fwd anneal/extend to make sgRNA LAC1 -160	aagtttaccccatcgatcACGGGTTCTGATGAGTCCGTG AGGACGAAACGAGTAAGCTCG
MD1634	Fwd anneal/extend to make sgRNA GRS2 -525	aagtttaccccatcgatcATCGAAGCTGATGAGTCCGTG AGGACGAAACGAGTAAGCTCG
MD1635	Fwd anneal/extend to make GRS2 -393	aagtttaccccatcgatcAACATCACTGATGAGTCCGTG AGGACGAAACGAGTAAGCTCG
MD1636	Fwd anneal/extend to make PDR11 -500	aagtttaccccatcgatcAGGAGAGCTGATGAGTCCGTG AGGACGAAACGAGTAAGCTCG
MD1637	Fwd anneal/extend to make PDR11 -341	aagtttaccccatcgatcAAGTTGTCTGATGAGTCCGTG AGGACGAAACGAGTAAGCTCG
MD1638	Fwd anneal/extend to make PDR11 -218	aagtttaccccatcgatcACCTATGCTGATGAGTCCGTG AGGACGAAACGAGTAAGCTCG
MD1639	Fwd anneal/extend to make RKI1 -447	aagtttaccccatcgatcAATAGCTCTGATGAGTCCGTG AGGACGAAACGAGTAAGCTCG
MD1640	Fwd anneal/extend to make RKI1 -200	aagtttaccccatcgatcATGAAATCTGATGAGTCCGTG AGGACGAAACGAGTAAGCTCG
MD1641	Fwd anneal/extend to make RKI1 60	aagtttaccccatcgatcATACAGACTGATGAGTCCGTG AGGACGAAACGAGTAAGCTCG
MD1642	Rev anneal/extend to make sgRNA LAC1 -460	GCTATTTCTAGCTCTAAAAC**GAAAAGACAGT TGTTTCCTT**GACGAGCTTACTCGTTTCGT
MD1643	Rev anneal/extend to make sgRNA LAC1 -346	GCTATTTCTAGCTCTAAAACCGTTATTTGTCTA GCATGATGACGAGCTTACTCGTTTCGT
MD1644	Rev anneal/extend to make sgRNA LAC1 -160	GCTATTTCTAGCTCTAAAACATGTAGCAGAAA AACGGGTTGACGAGCTTACTCGTTTCGT
MD1645	Rev anneal/extend to make sgRNA GRS2 -525	GCTATTTCTAGCTCTAAAACTACTCTTTCAGC AATCGAAGGACGAGCTTACTCGTTTCGT
MD1646	Rev anneal/extend to make sgRNA GRS2 -393	GCTATTTCTAGCTCTAAAACTGTACCAAAATC ACACATCAGACGAGCTTACTCGTTTCGT
MD1647	Rev anneal/extend to make PDR11 -500	GCTATTTCTAGCTCTAAAACTCCCTTAGAGAC TAGGAGAGGACGAGCTTACTCGTTTCGT

MD1648	Rev anneal/extend to make PDR11 -341	GCTATTTCTAGCTCTAAAACAACGATAGAGTG GAAGTTGTGACGAGCTTACTCGTTTCGT
MD1649	Rev anneal/extend to make PDR11 -218	GCTATTTCTAGCTCTAAAACTAGTATCCATTTT GCCTATGGACGAGCTTACTCGTTTCGT
MD1650	Rev anneal/extend to make RKI1 -447	GCTATTTCTAGCTCTAAAACAGGTTTCTCGTA AAATAGCTGACGAGCTTACTCGTTTCGT
MD1651	Rev anneal/extend to make RKI1 -200	GCTATTTCTAGCTCTAAAACAATTACGAACCA GATGAAATGACGAGCTTACTCGTTTCGT
MD1652	Rev anneal/extend to make RKI1 60	GCTATTTCTAGCTCTAAAACAGAGAGCTGCAG CATACAGAGACGAGCTTACTCGTTTCGT
EKB0065	GPI18 Fwd A/E sgRNA -522	GCTATTTCTAGCTCTAAAACGATGATGACGAG CTTACTCGTTTCGT
EKB0066	GPI18 Fwd A/E sgRNA -332	GCTATTTCTAGCTCTAAAACGCAGTCGACGAG CTTACTCGTTTCGT
EKB0067	GPI18 Fwd A/E sgRNA -199	GCTATTTCTAGCTCTAAAACATAACTGACGAG CTTACTCGTTTCGT
EKB0068	GPI18 Fwd A/E sgRNA 68	GCTATTTCTAGCTCTAAAACTACATCGACGAG CTTACTCGTTTCGT
EKB0069	FUN26 -542 A/E Fwd	GCTATTTCTAGCTCTAAAACCCTTAGGACGAG CTTACTCGTTTCGT
EKB0070	DIM1 -503 Fwd A/E	GCTATTTCTAGCTCTAAAACTGGTATGACGAG CTTACTCGTTTCGT
EKB0071	DIM1 -419 Fwd A/E	GCTATTTCTAGCTCTAAAACGAAGAAGACGA GCTTACTCGTTTCGT
EKB0072	DIM1 -172 Fwd A/E	GCTATTTCTAGCTCTAAAACATGGATGACGAG CTTACTCGTTTCGT
EKB0073	DIM1 58 FWD A/E	GCTATTTCTAGCTCTAAAACTCAGTAGACGAG CTTACTCGTTTCGT
EKB0074	GUS1 87 Fwd A/E	GCTATTTCTAGCTCTAAAACTAAGTTGACGAG CTTACTCGTTTCGT
EKB0075	ALG12 -361 FWD A/E	GCTATTTCTAGCTCTAAAACTGAAGAGACGAG CTTACTCGTTTCGT
EKB0076	COR1 -500 FWD A/E	GCTATTTCTAGCTCTAAAACTCTAACGACGAG CTTACTCGTTTCGT
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EKB0078	GRS2 -393 FWD A/E	GCTATTTCTAGCTCTAAAACACATCAGACGAG CTTACTCGTTTCGT
EKB0079	GRS2 -170 FWD A/E	GCTATTTCTAGCTCTAAAACAGGTTAGACGAG CTTACTCGTTTCGT
EKB0080	GRS2 69 FWD A/E	GCTATTTCTAGCTCTAAAACGGTGGTGACGAG CTTACTCGTTTCGT
EKB0081	GPI18 Rev A/E sgRNA-522	aagtttaccccatcgatcATTCAGCAACGGACAGATGAT CTGATGAGTCCGTGAGGACGAAACGAGTAAG CTCG
EKB0082	GPI18 Rev A/E sgRNA -332	aagtttaccccatcgatcATCATCGTCGTCATGGCAGTC CTGATGAGTCCGTGAGGACGAAACGAGTAAG CTCG
EKB0083	GPI18 Rev A/E sgRNA -199	aagtttaccccatcgatcAATAAATAGGAAAGTATAACT CTGATGAGTCCGTGAGGACGAAACGAGTAAG CTCG
EKB0084	GPI18 Rev A/E sgRNA -199	aagtttaccccatcgatcAATTAGGCAGTTTGATACATC CTGATGAGTCCGTGAGGACGAAACGAGTAAG CTCG

EKB0085	FUN26 -542 A/E Rev	aagtttaccccatcgatcAAATAGCCCACGCACCCTTAG CTGATGAGTCCGTGAGGACGAAACGAGTAAG CTCG
EKB0086	DIM1 -503 Rev A/E	aagtttaccccatcgatcAAGTGGCAACGTCAGTGGTAT CTGATGAGTCCGTGAGGACGAAACGAGTAAG CTCG
EKB0087	DIM1 -419 Rev A/E	aagtttaccccatcgatcATGGTGTTTACTAGTGAAGAA CTGATGAGTCCGTGAGGACGAAACGAGTAAG CTCG
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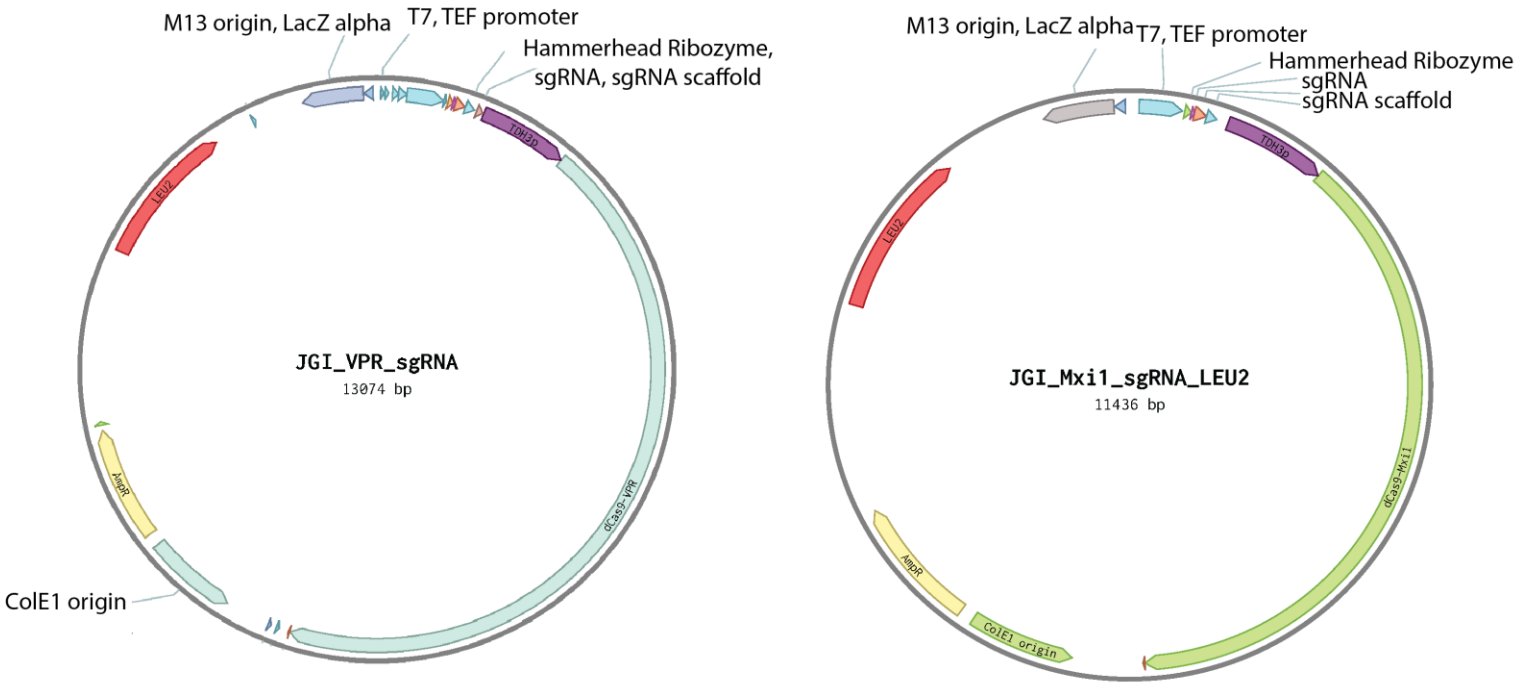
EKB0090	GUS1 87 Rev A/E	aagtttaccccatcgatcAAACTCCATAGCTATTAAGTT CTGATGAGTCCGTGAGGACGAAACGAGTAAG CTCG
EKB0091	ALG 12 -361 REV A/E	aagtttaccccatcgatcATGCCTCGTAACAGGTGAAGA CTGATGAGTCCGTGAGGACGAAACGAGTAAG CTCG
EKB0092	COR1 -500 REV A/E	aagtttaccccatcgatcAAAGTAACGCAATTATCTAAC CTGATGAGTCCGTGAGGACGAAACGAGTAAG CTCG
EKB0093	GRS2 -525 REV A/E	aagtttaccccatcgatcATACTCTTTCAGCAATCGAAG CTGATGAGTCCGTGAGGACGAAACGAGTAAG CTCG
EKB0094	GRS2 -393 REV A/E	aagtttaccccatcgatcATGTACCAAAATCACACATCA CTGATGAGTCCGTGAGGACGAAACGAGTAAG CTCG
EKB0095	GRS2 -170 REV A/E	aagtttaccccatcgatcATTTATATTCTGTCAAGGTTAC TGATGAGTCCGTGAGGACGAAACGAGTAAGC TCG
EKB0096	GRS2 69 REV A/E	aagtttaccccatcgatcACGTTTGAGATATATGGTGGT CTGATGAGTCCGTGAGGACGAAACGAGTAAG CTCG

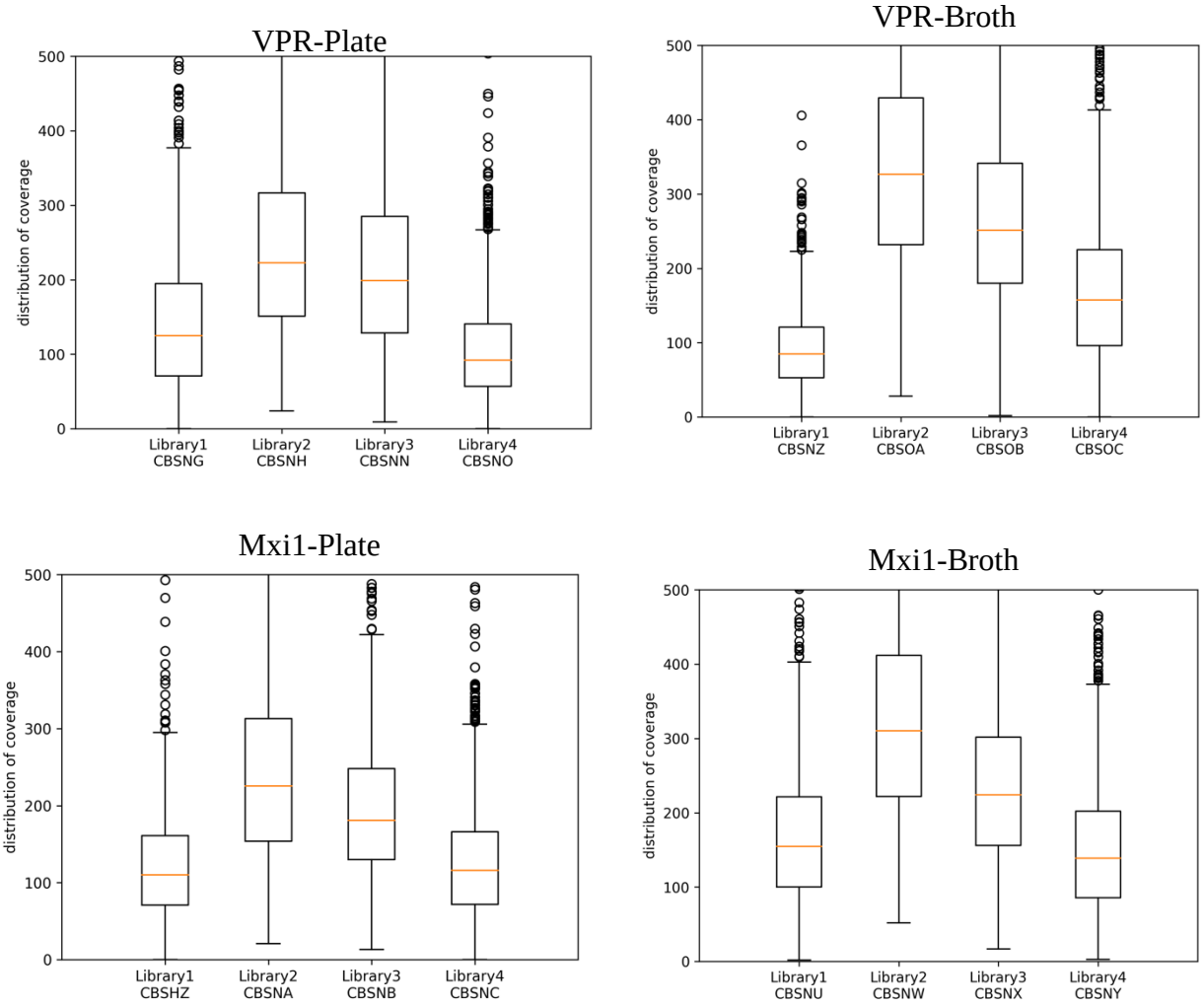
Supplementary Figure 1

Maps of dCas9-Mxi1 and dCas9-VPR library plasmids with placeholder for sgRNA. This is the only region of diversity in the library, allowing for ease of identification of gene target and expression level.



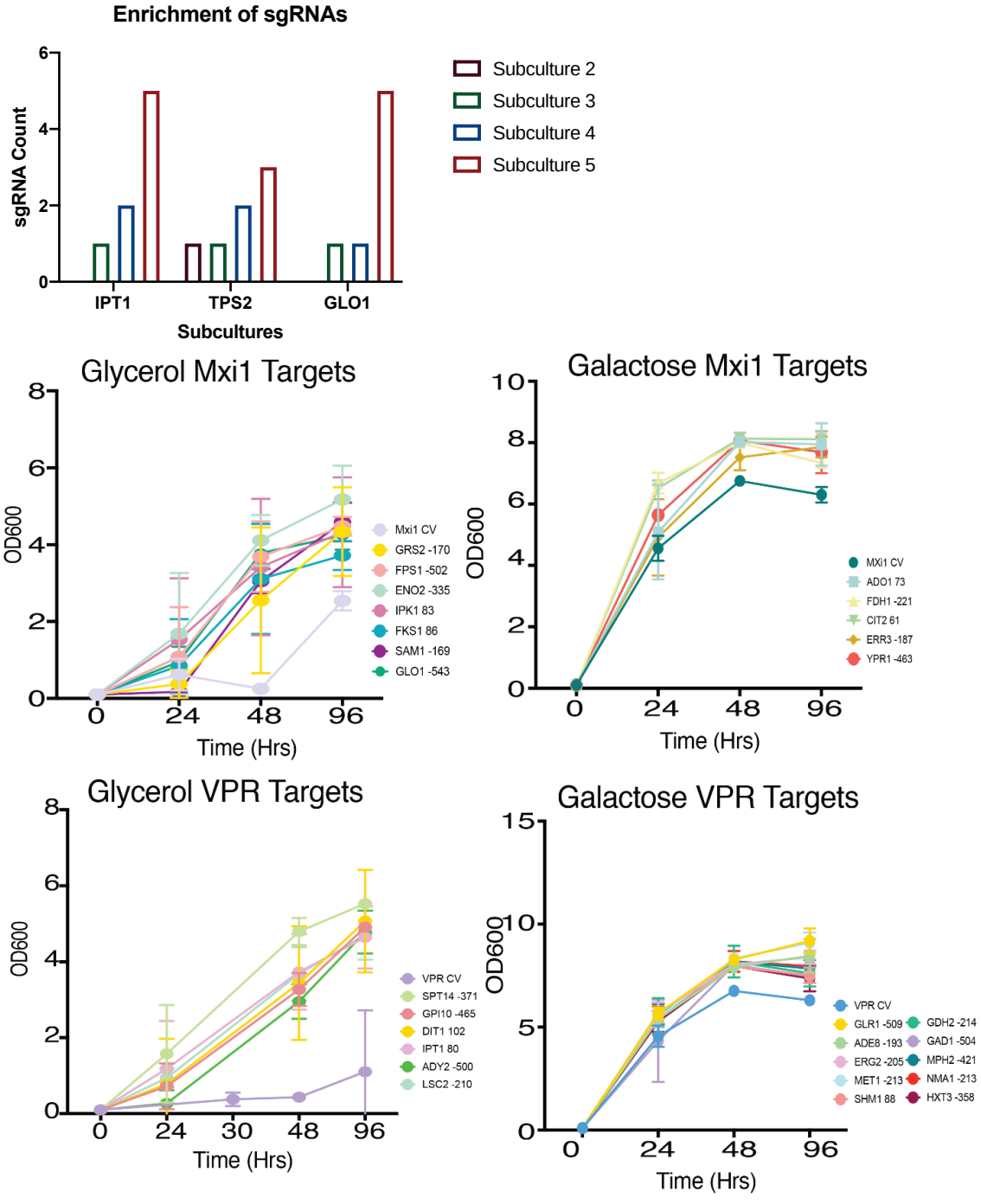
Supplemental Figure 2

Box and whisker plots representing (a&b) VPR and (c&d) Mxi1 library distribution of coverage for each pool (library1=pool 1, etc…). The broth protocol for amplification of each library shows a better overall distribution of the guides, and was therefore used throughout this study.



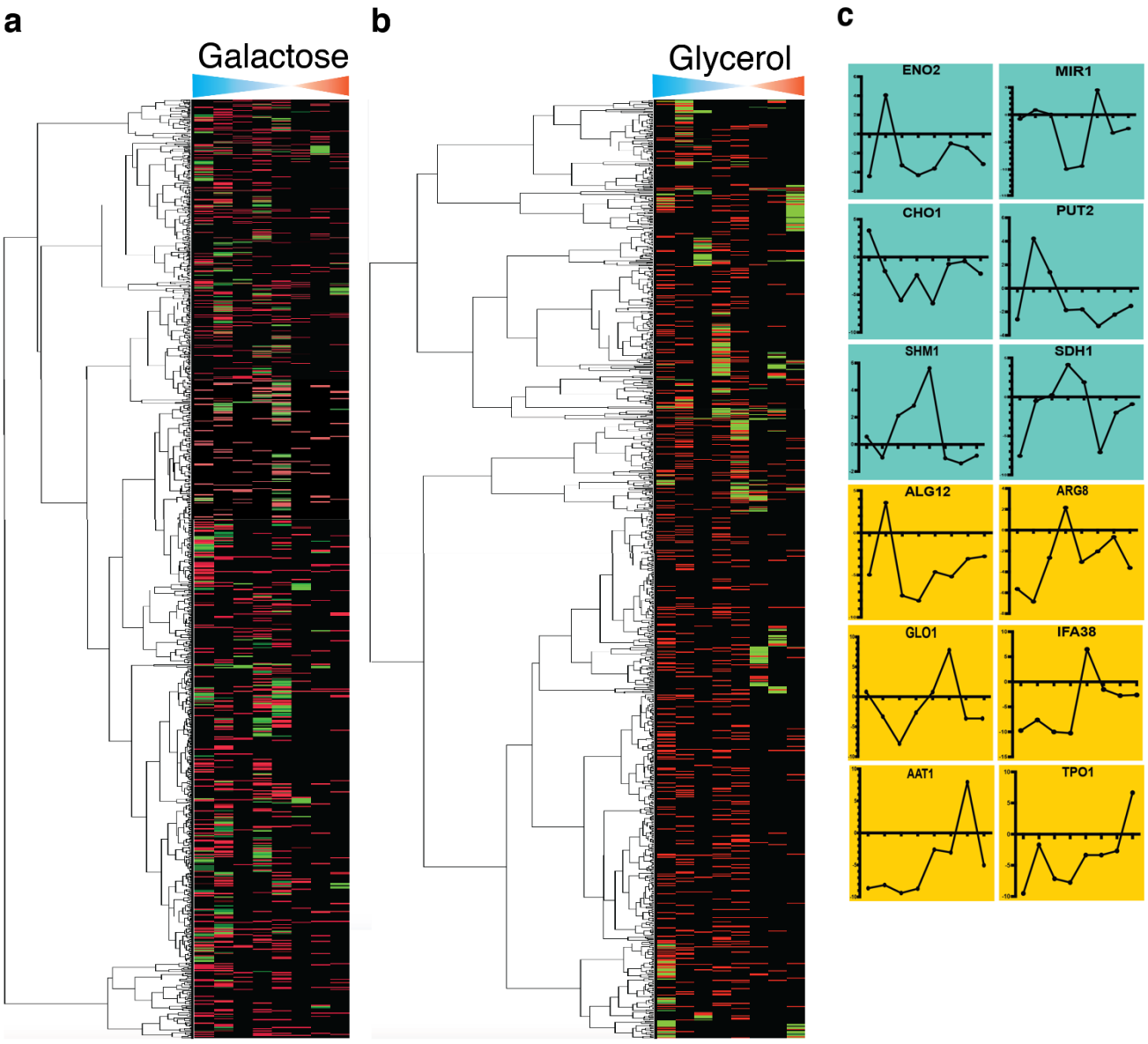
Supplementary Figure 3

Confirmation of Enrichment over time. Isolates were sequenced individually and plasmids with target guides re-transformed into BY4741. a) Bar graph showing number of reads of representative individual guides from isolates submitted. This indicated enrichment occurring over time. b) Isolates from bar graph – and others identified, for each library under each condition.



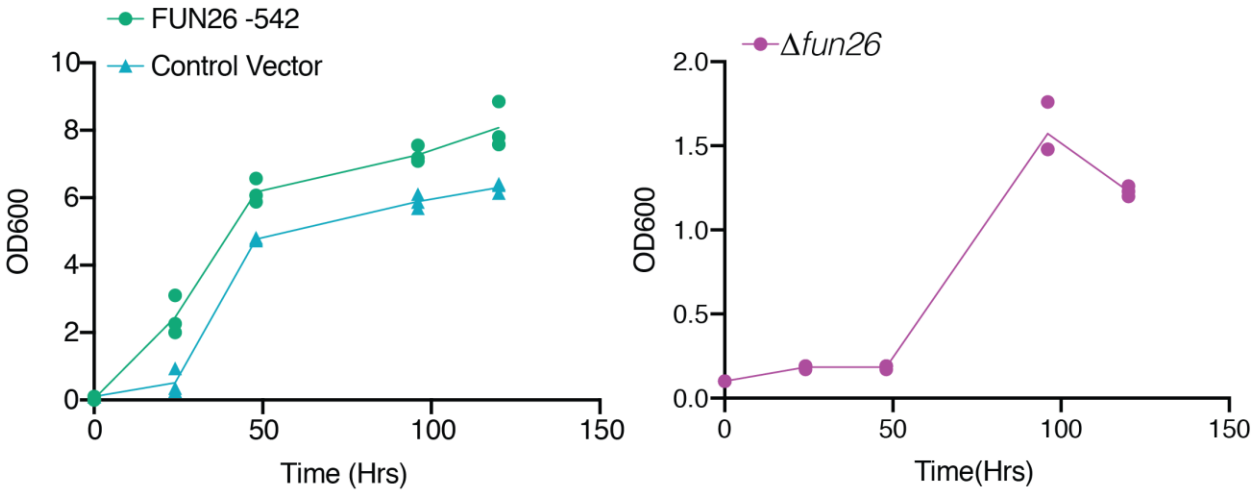
Supplementary Figure 4

a&b) Cluster analysis of all sgRNA library members using Euclidean Distance and complete linkage. Select individual nodes were used for GO analysis-see Figure 2. This analysis indicated clustering of optimal expression levels for particular pathways under different conditions. c) Individual gene targets pulled from NGS analysis. Teal indicates guides enriched under galactose consumption conditions, yellow indicates glycerol consumption enrichment. Enrichment of guides was performed in duplicates, with initial growth in raffinose, followed by dilution into alternative carbon source at an OD of 0.1. This enrichment of single guide RNAs was identified via next generation sequencing. These indicate primarily a single optimum level of expression, with some samples showing graded levels leading up to the optimum level (as seen for SHM1, SDH1 and PUT2).



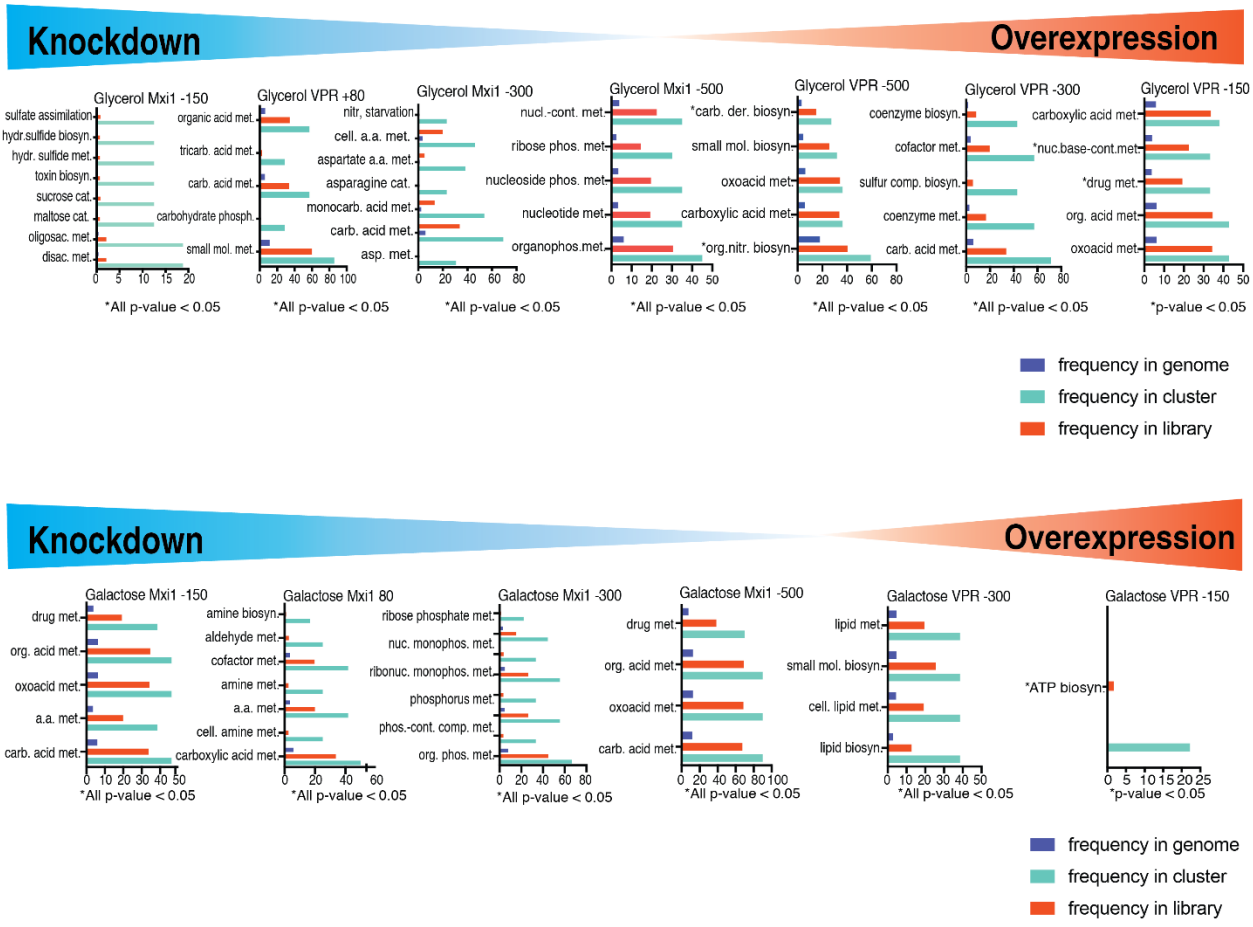
Supplementary Figure 5

Beneficial guide targeting FUN26 at -542 from the open reading frame. When compared to the knockout results in improved growth, versus worse growth from the knockout of *fun26*. Here, the phenotype from the guide RNA creates a beneficial effect on growth over the control.

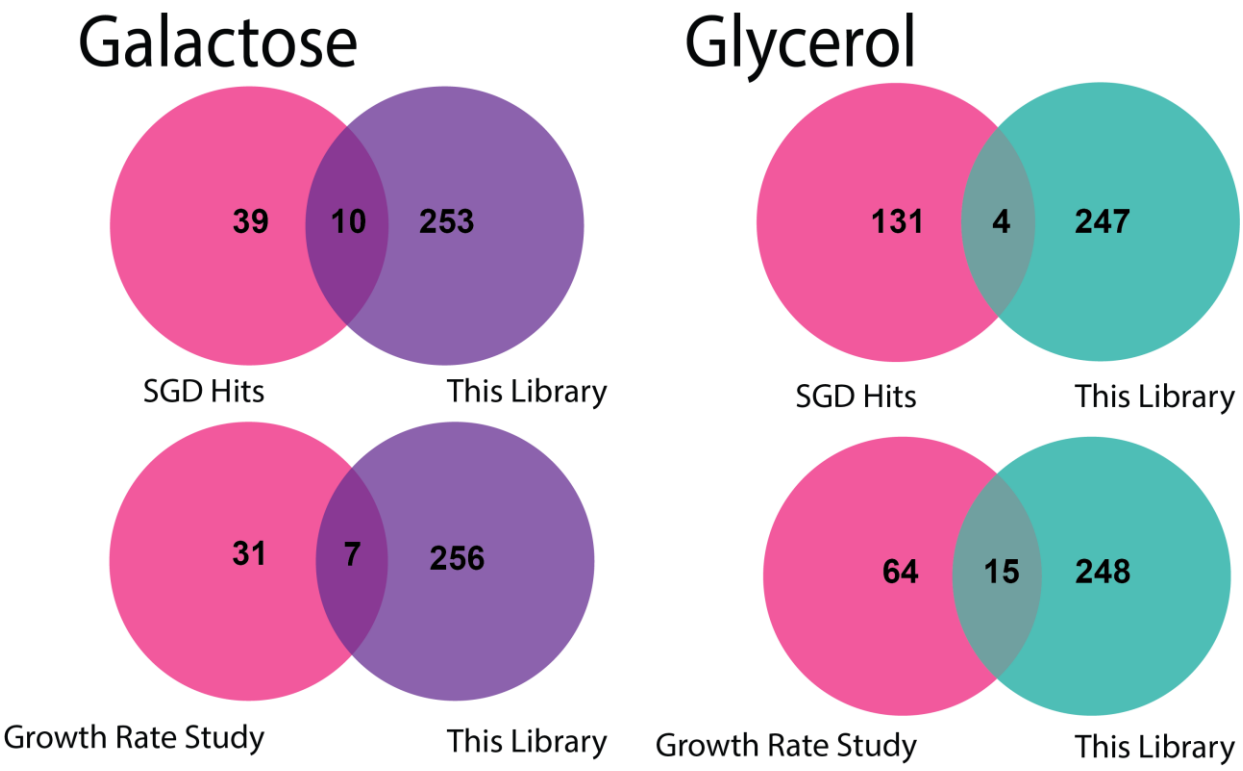


Supplementary Figure 6

Gene ontology (GO) analysis performed utilizing SGD's GO analysis tool. All shown go terms are statistically significant unless otherwise specified underneath each individual graph. This figure directly corresponds with main figure 2, with only a few clusters missing as these lacked significant GO terms. There are obvious differences in associated GO terms between different clusters, with the highest down-regulated genes identified in Glycerol being associated with sulfide metabolism and biosynthesis, as well as sugar catabolism(Glycerol Mxi1 -150). GO terms indicate a medium-level knockdown enrichment in glycerol, of genes associated with amino acid metabolism as well, while medium over expression is primarily associated with co-factor metabolism(Glycerol Mxi1 -300, Glycerol VPR -300). Medium knockdown from galactose enrichment indicates amino acid metabolism as well as carboxylic acid metabolism overrepresentation(Galactose Mxi1 80). In addition, slightly lower levels of knockdown under this condition were associated with phosphate metabolism(Galactose Mxi1 -300). GO analysis of the cluster of high overexpression resulted in the identification of a single GO-term associated with ATP biosynthesis(Galactose VPR -150).



Supplementary Figure 7
Venn diagrams of gene targets identified in this study and those that improved growth rates on alternative carbon sources from Vandersluis and colleagues as well as genes associated with improved consumption of glycerol or galactose from the *Saccharomyces cerevisiae* genome database *Yeastminer* application (yeastmine.yeastgenome.org). [25]. These analyses performed demonstrate the complementary nature of this approach. This analysis illustrates only a 10% overlap between galactose-associated targets identified in this work and deletion targets in the Saccharomyces Genome Database (SGD), highlighting the uniqueness and breadth of targets identifiable when graded expression is considered in a competitive growth environment.



Supplementary Figure 8

Pathway for the production of Betaxanthins from *S. cerevisiae.*