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Optimization of a Yeast RNA Interference System for Controlling Gene Expression and Enabling Rapid Metabolic Engineering

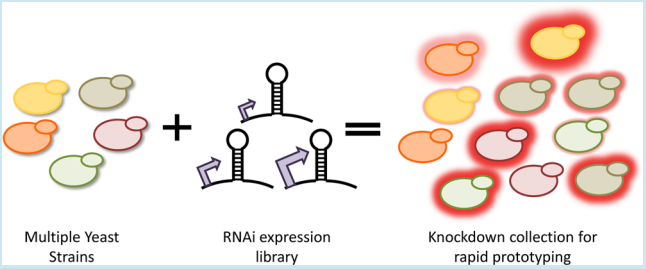
Nathan C. Crook,[†] Alexander C. Schmitz,[†] and Hal S. Alper*[,†,‡]

[†]Department of Chemical Engineering, The University of Texas at Austin, 200 East Dean Keeton Street Stop C0400, Austin, Texas 78712, United States

[‡]Institute for Cellular and Molecular Biology, The University of Texas at Austin, 2500 Speedway Avenue, Austin, Texas 78712, United States

Supporting Information

ABSTRACT: Reduction of endogenous gene expression is a fundamental operation of metabolic engineering, yet current methods for gene knockdown (*i.e.*, genome editing) remain laborious and slow, especially in yeast. In contrast, RNA interference allows facile and tunable gene knockdown *via* a simple plasmid transformation step, enabling metabolic engineers to rapidly prototype knockdown strategies in multiple strains before expending significant cost to undertake genome editing. Although RNAi is naturally present in a myriad of eukaryotes, it has only been recently implemented in *Saccharomyces cerevisiae* as a heterologous pathway and so has not yet been optimized as a metabolic engineering tool. In this study, we elucidate a set of design principles for the construction of hairpin RNA expression cassettes in yeast and implement RNA interference to quickly identify routes for improvement of itaconic acid production in this organism. The approach developed here enables rapid prototyping of knockdown strategies and thus accelerates and reduces the cost of the design−build−test cycle in yeast.



KEYWORDS: *yeast, RNA interference, metabolic engineering, itaconic acid, gene knockdown*

Applications in both synthetic biology and metabolic engineering often rely upon the ability to either partially or completely remove the activity of a gene product. Thus, knockdown and knockout strategies have been instrumental in rewiring microbial systems for the production of a wide variety of chemicals. However, it is often the case that multiple, simultaneous gene deletions are necessary to obtain maximal yields.[1−4] Among possible host organisms, yeasts have gained traction as a highly attractive system for the bioproduction of fuels and chemicals.[5] However, current methods for elimination or reduction of endogenous gene activity in yeast remain laborious and necessitate highly sequential workflows in spite of the need to test (often many) different gene knockdown/out strategies. Strain choice can also significantly influence the yield and productivity of industrial bioprocesses, but *a priori* strain selection is not always feasible.[6] Therefore, although parallel processing of multiple yeast strains during the design−build−test cycle is highly desirable, the high cost associated with each genome modification limits the amount of possible parallelization. Furthermore, if a superior wild-type strain is identified late during process optimization, it may be extremely costly and difficult to transfer genomic modifications to the new strain using the same linearized workflow. Thus, there is a strong need for a synthetic methodology to quickly and cheaply introduce gene knockdown/outs into multiple strains of yeast in order to rapidly prototype within the design−build−test cycle.

Recent reports have demonstrated the use of small regulatory RNAs as a means for rapid, facile knockdowns in the bacterial system *Escherichia coli*.[7] In higher eukaryotic systems, RNA interference (RNAi) is used to systematically target and reduce mRNA levels through the action of the RNA-induced silencing complex on double stranded RNA.[8] Specifically, double stranded RNA (dsRNA) is cleaved by Dicer to form small guide RNAs, which are then used by Argonaute to recognize and degrade the corresponding mRNA (Figure 1). A major advantage of RNAi is that it is highly portable and only requires the requisite machinery (*i.e.*, Argonaute, Dicer, and dsRNA) to be expressed; no genome engineering is explicitly required. RNAi thus enables rapid strain prototyping through synthetic import of this machinery into novel host strains. As a result, RNAi has been widely used for targeted loss-of-function studies and metabolic engineering in a wide variety of eukaryotic organisms.[9−13] Despite its utility, a functional RNAi pathway is endogenously absent from common yeast hosts such as *Saccharomyces cerevisiae*. Fortunately, the RNAi system can be introduced into *S. cerevisiae* through the heterologous expression of Argonaute and Dicer from *Saccharomyces*

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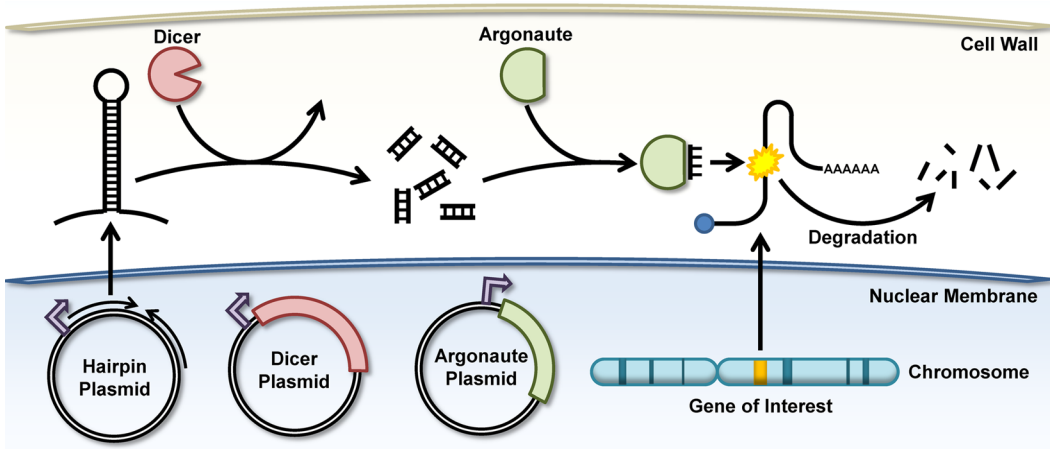

Figure 1. Schematic of a synthetic RNAi pathway in yeast. Double-stranded RNA with homology to a target gene is degraded by Dicer. The resulting guide RNAs are then used by Argonaute to recognize and cleave the mRNA of the target gene.

Table 1. Description of the Design Cycles Used in the Optimization of RNAi in Yeast

design cycle	hairpin expression	hairpin length	target copy number	hairpin copy number
0	low (pCYC1)	short (100 bp)	low (plasmid)	high (2 μm)
1	high (pTDH3)	short (100 bp)	low (plasmid)	high (2 μm)
2	high (pTDH3)	long (200 bp)	low (plasmid)	high (2 μm)
3	high (pTDH3)	long (200 bp)	single (genome)	high (2 μm)
4	high (pTDH3)	long (200 bp)	single (genome)	low (tryptophan)
5	high (pTDH3)	long (200 bp)	single (genome)	low (G418)

castelli.[14] As a result, it is possible to use RNAi for metabolic engineering and synthetic biology applications in yeast.

Here, we demonstrate the utility of a synthetic RNAi system in yeast for gene expression control. First, we elucidate key design principles for the construction of hairpin RNA expression cassettes in yeast as well as optimize expression of key components of the RNAi pathway. We then use these parameters to demonstrate the controlled regulation of a synthetic fluorescent protein. Finally, we demonstrate that this heterologous RNAi pathway can enable rapid strain prototyping by examining three industrially relevant strains of yeast (BY4741, CEN.PK2-a, and Sigma 10560-4A) to quickly identify routes for the improvement in titer of itaconic acid (a top value-added chemical from biomass[15]), thus demonstrating that this synthetic approach can speed the design−build−test cycle in yeast.

RESULTS AND DISCUSSION

In order to allow for rapid prototyping of yeast strains, we sought to develop a synthetic, portable version of the RNAi pathway. Previous reports have demonstrated that the RNAi machinery from *S. castelli* can be introduced into *S. cerevisiae* through genomic integration of *S. castelli* Argonaute and Dicer. This prior work demonstrated effective gene silencing during coexpression of a gene-specific hairpin.[14] Here, we sought to utilize RNAi as a portable engineering tool and required Argonaute, Dicer, and a hairpin construct to be expressed on portable plasmids (Figure 1). The effectiveness of the downregulation pathway was initially characterized through the knockdown of yellow fluorescent protein (YFP) expression. This setup enabled the elucidation of design principles influencing the extent of downregulation in yeast. Our initial design cycle (Table 1, Design Cycle 0) consisted of YFP, Argonaute, and Dicer each expressed on low copy (centromeric) plasmids, and a 100 bp hairpin RNA complementary to YFP expressed on a high copy (2 μm) plasmid. This scheme enabled us to easily change design variables by swapping out plasmids; more streamlined constructs containing Argonaute, Dicer, and a hairpin on the same plasmid may be beneficial for further downstream applications. Following from this design, we investigated the influence of hairpin expression level, hairpin length, the copy number of the target gene, and the copy number of the hairpin plasmid on the efficiency of downregulation. These parameters were then optimized and used to demonstrate the effectiveness of rapid prototyping for metabolic engineering in multiple base strains of yeast.

Increased Hairpin Expression Level Improves RNAi Efficiency. First, we investigated the effect of hairpin RNA expression level on gene knockdown efficiency. We hypothesized that increasing the expression level of the hairpin would increase the amount of dsRNA substrate available for Argonaute and Dicer, thus increasing the magnitude of downregulation. Heterologous expression of YFP was driven by either a weak (pCYC1) or strong (pTDH3) promoter. When the hairpin was expressed from a weak (pCYC1) promoter, we obtained insignificant downregulation of YFP fluorescence regardless of reporter level (Figure 2). However, we observed that increased expression of the hairpin (from a strong pTDH3 promoter, Design Cycle 1) resulted in increased knockdown capacity. Specifically, we found a 2.3-fold increase in downregulation when YFP is weakly expressed and upward of 3-fold increase in the extent of downregulation when YFP is strongly expressed (Figure 2). In total, this construct enabled up to 80% downregulation to be obtained. We additionally confirmed that the RNAi system had an insignificant effect upon growth rate (Figure S1, Supporting Information). These results confirm both that RNA interference is functional in yeast and also highlight that the absolute extent of down-

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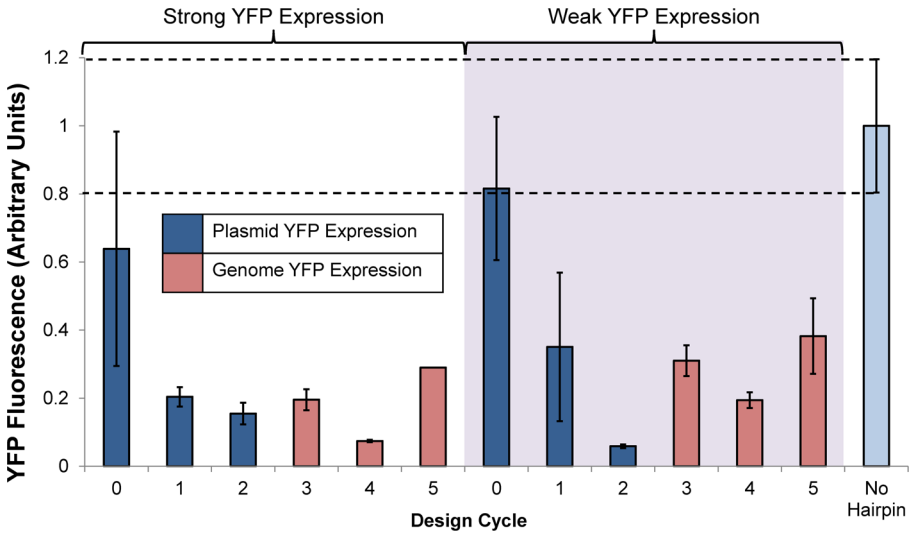

Figure 2. Gene knockdowns attained by each design cycle. YFP expression was downregulated through expression of Argonaute, Dicer, and a YFP-specific hairpin using the schemes listed in Table 1 in order to elucidate design rules for RNAi in yeast. Red bars indicate the downregulation of plasmid-borne YFP, and blue bars indicate the downregulation of YFP expressed from the genome. For each condition, the knockdown was normalized to its corresponding "no hairpin" control. Bars with a white background indicate downregulation of strongly expressed (pTDH3) YFP and a purple background refers to the downregulation of weakly expressed (pCYC1) YFP. Dashed lines denote the representative range of YFP expression levels observed in cells that do not express a hairpin. Error bars represent the standard deviation observed among three biological replicates. Through iteratively improving upon our synthetic RNAi pathway, expression of genomically encoded proteins was downregulated by up to 93%.

regulation may be altered by synthetically controlling the expression of the hairpin RNA. This approach represents a significant reduction in labor compared to current genomic manipulation techniques[16] and enables metabolic engineers to quickly test the effects of multiple expression levels on a phenotype of interest. This technique also enables the capacity to simultaneously alter the extent and timing of gene downregulation by coupling the expression of the hairpin RNA to an inducible promoter[17] or a logic circuit.[18] For the remainder of this work, we optimized the synthetic RNAi system in the context of high hairpin expression, as this condition resulted in the strongest knockdown level.

Increased Hairpin Length Improves RNAi Efficiency. To further improve downregulation, we next investigated the influence of hairpin RNA length. In endogenous RNAi systems, small interfering RNA efficiency is highly sequence-dependent, with some hairpins resulting in nearly complete reduction in expression whereas others have little effect.[19] This disparate impact of RNAi is thought to result from stable secondary structures of the target mRNA occluding recognition and degradation by Argonaute.[20] While long double-stranded RNAs are known to induce the interferon response in mammalian cells,[21] no such defense mechanism exists in yeast. As a result, we hypothesized that increased hairpin length would improve downregulation efficiency by providing a greater diversity of guide RNAs and thus a greater probability that Argonaute will recognize and cleave an unstructured part of the corresponding mRNA substrate.[22] To test this hypothesis, we increased hairpin length from 100 to 200 bp (Design Cycle 2) and observed an improvement in downregulation efficiency by 30% when YFP is strongly expressed, and by nearly 6-fold when YFP is weakly expressed (Figure 2). Through this second design round, we were able to obtain inhibition levels of up to 94%, a significant improvement upon the 80% described above. It should be noted that the construction and propagation of inverted repeats of this increased length in *E. coli* were difficult, potentially because of interference with DNA replication machinery,[23] thus necessitating the use of an intron-containing spacer region to ensure plasmid stability.[24]

Decreasing Hairpin-Containing Plasmid Copy Number Improves RNAi Efficiency. The ability of RNAi to confer a useful phenotype is dependent upon the cell-to-cell variability in the extent of downregulation. Interestingly, flow cytometry analysis revealed a bimodal distribution of downregulation, with some cells almost completely downregulating YFP expression, and others exhibiting little downregulation (Figure S2A, Supporting Information). To investigate the cause of this phenomenon, we explored the effectiveness of expressing the hairpin RNA on a centromeric (low-copy) plasmid containing either an auxotrophic (TRP1) or an antibiotic resistance marker (KanMX). For this design cycle (which was performed in the context of genomic YFP expression), we observed that a low-copy auxotrophic vector (Design Cycle 4) enabled up to 93% downregulation in the fluorescence of strongly expressed YFP and 80% downregulation of weakly expressed YFP, an improvement of 2.6-fold and 1.6-fold, respectively, over Design Cycle 3. Furthermore, the population of weakly downregulated cells was significantly reduced from previous design cycles (Figure S2A, Supporting Information). While no improvements to efficiency were seen when using a vector expressing antibiotic resistance, even when exposed to a saturating, 10-fold excess of antibiotic (Design Cycle 5), these results highlight the potential to use such a construct in heterotrophic strains (Figure 2). Interestingly, the extent of knockdown did not correlate the coefficient of variation in expression levels enabled by these plasmid constructs (Figure S2B, Supporting Information), indicating that a mechanism other than copy number control is responsible for the improved knockdown observed when using low copy auxotrophic vectors. Nevertheless, these promising results inspired us to use this low-copy vector for hairpin expression in future experiments. Collectively, these design cycles were able to develop a synthetic

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RNAi system in yeast capable of efficient gene knockdown for metabolic engineering applications.

Implementation of RNAi in Alternate Yeast Strains. To demonstrate the generality and portability of this approach, we wished to implement RNAi in two additional commonly used strains: CEN.PK and Sigma. We expected that the portability of this system would enable rapid prototyping in multiple strains simultaneously. In order to conserve auxotrophic markers in our system and further decrease expression noise, we condensed vector design by coexpressing Argonaute and Dicer from the same low-copy plasmid. In addition, we redesigned our YFP-specific hairpin to target regions of YFP mRNA with increased variability in secondary structure, as it has been indicated that these regions are ideal targets for RNAi.[20] As a result, the length of the hairpin was increased to 240 bp. Finally, since genome modification techniques are rather inefficient for Sigma, we were unable to generate YFP-integrated versions of this strain and so tested downregulation of plasmid-borne YFP in all three strains. Across these strains, we achieved between 85 and 77% downregulation of YFP fluorescence, and between 90 and 97% downregulation of YFP mRNA (Figure 3). CEN.PK

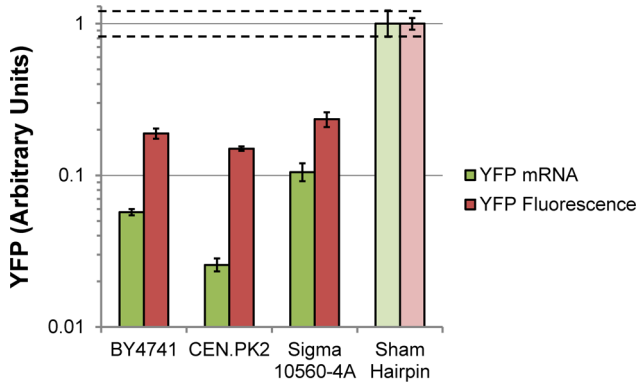

Figure 3. Gene knockdown in alternate strains of yeast. YFP expression was downregulated in BY4741, CEN.PK2-a, and Sigma 10560-4A using our synthetic RNA interference pathway. Red bars indicate YFP mRNA levels, and blue bars indicate YFP fluorescence. Error bars represent the standard deviation observed among three biological replicates. Dashed lines represent the range of transcript and expression levels characteristic of strains expressing a sham (*ADE3*-specific) hairpin. We observed strong downregulation of YFP fluorescence and mRNA in each strain, indicating that RNAi is suitable for rapid prototyping in multiple genomic contexts.

showed the highest overall downregulation competency, whereas Sigma showed the lowest. These strain-specific differences could be due to variations in the translation efficiencies of Argonaute and Dicer. In addition, although decreases in YFP fluorescence were well-correlated with decreases in YFP mRNA, knockdowns in fluorescence intensity were consistently lower than for mRNA levels. Regardless of these slight differences, these results demonstrate that our synthetic RNA interference is portable and efficient in a wide variety of strains, thus enabling reduction-of-function experiments and rapid prototyping to be easily performed in many strain backgrounds at a small marginal cost.

Rapid Prototyping of Itaconic Acid Production in Yeasts through RNA Interference. As a final demonstration for the synthetic RNAi system in yeast, we sought to enable rapid prototyping to identify promising routes for metabolic engineering. Specifically, we undertook a combinatorial experiment whereby knockdown of a desirable gene target was simultaneously combined with various base strains. Genome scale metabolic modeling has determined that *ade3* deletions in yeast can improve the heterologous production of itaconic acid (IA) from *cis*-aconitate during *cis*-aconitate decarboxylase (CAD1) overexpression.[25] In order to rapidly determine the effect of this gene knockdown in other genomic contexts, we expressed a long hairpin specific to *ADE3* under the control of three yeast promoters (pCYC1, pTEF1, and pTDH3) that collectively span a wide range of expression. We also streamlined the RNAi system by integrating Argonaute and Dicer on the same *LEU2*-marked low-copy plasmid. This change was made because the *HIS3*-marked plasmid previously used for expression of Dicer was unavailable because of the possibility that the *ADE3* knockdown would confer histidine auxotrophy. The hairpin was maintained on a separate plasmid for modularity and ease of cloning. Next, itaconic acid production was measured upon coexpression of Dicer, Argonaute, and CAD1 in three separate strains of yeast: BY4741, CEN.PK2-a, and Sigma 10560-4A. We observed significant increases in IA production for at least one expression level of hairpin RNA in each of the three strains we tested, as indicated by a Student's *t* test (Figure 4). As a result, these experiments indicate that a gene knockdown is an adequate, quick surrogate test for genotype−phenotype linkages. Expression of a sham hairpin specific for YFP did not elicit significant improvements to IA production, indicating that the observed improvements to IA titer were not simply due to the presence of dsRNA in the cell. These results also indicate that of the tested strains, *S. cerevisiae* Sigma 10560-4A is the most advantageous for IA production, and that *ade*3 knockout is a promising strategy for improvement of titer in this strain.

We further investigated the strain-to-strain variation in the downregulation of *ade*3 in each of these strains. By measuring *ADE3* mRNA levels, we found that CEN.PK2-a downregulated *ADE3* mRNA levels to the greatest extent, followed by Sigma 10560-4A and then BY4741 (Figure S3, Supporting Information), indicating that the relatively low increases to IA production observed in BY4741 may be due to a low *ADE3* downregulation efficiency. However, it is also expected that the relationship between *ade3* knockdown and IA induction may be different for each of these three strains.

In this example, we demonstrated that rapid strain prototyping is possible in yeast through the use of a synthetic RNAi system. Unlike genomic knockouts, hairpins are generated in a facile manner, thus enabling RNA interference to be a potent tool for the rapid screening of knockdown strategies in multiple organisms. Moreover, for more difficult to use strains (such as Sigma), gene expression tools are more mature than genome editing tools. As a result, RNAi systems enable a wide range of expression control with more ease than genome modifications. These experiments also suggest that Sigma 10560-4A would be the best strain for itaconic acid production out of the yeasts we tested. In this regard, this work demonstrates the potential of RNAi to significantly expedite the design−build−test cycle.

Conclusions. In this work, we have demonstrated that RNA interference is an effective tool for expediting the design−build−test cycle and enabling rapid prototyping of engineered yeast strains. We have uncovered several important design principles influencing knockdown level and have used an optimized scheme to demonstrate the effectiveness of RNAi

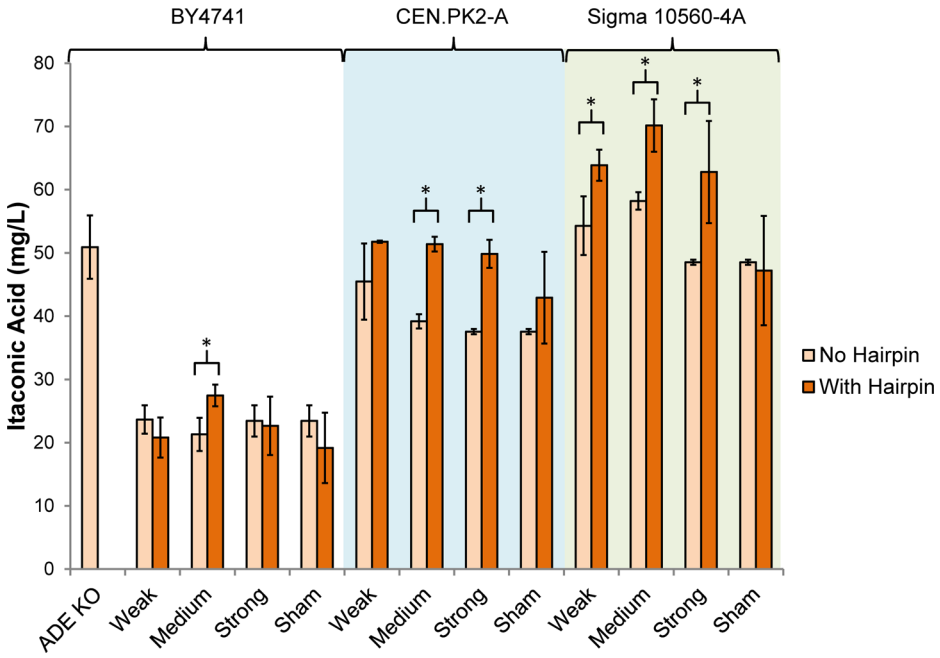

Figure 4. Rapid prototyping of gene knockdowns conferring increased itaconic acid (IA) production in multiple yeast strains. Argonaute, Dicer, *cis*-aconitate decarboxylase, and an *ADE3*-specific hairpin were coexpressed in BY4741, CEN.PK2-a, and Sigma 10560-4A to rapidly identify promising engineering targets for IA production. Blue bars indicate IA production in strains without hairpin expression, and red bars indicate IA production in strains expressing a hairpin. Error bars represent the standard deviation observed among three biological replicates. Asterisks represent a statistically significant ($p < 0.05$) difference in IA production as calculated by a one-tailed Student's *t* test. In this experiment, sham hairpins were specific to YFP. Significant increases to IA production were observed for at least one hairpin expression level in each strain we tested, indicating the potential of the *ade3* gene knockout, identifying Sigma 10560-4A as the most promising base strain for IA production, and confirming that RNAi enables rapid prototyping of engineering strategies in yeast.

through testing a putative genetic target for improved itaconic acid production. The portable nature of this approach (only requiring heterologous expression of Argonaute and Dicer) can enable rapid prototyping of both previously engineered and unsequenced industrial strains (esp. where polyploidy may be a substantial hurdle to genome engineering). Because of the linkage between downregulation capacity and hairpin RNA expression, it is possible to develop more advanced control of this system through the use of inducible promoters,[17] sophisticated logic circuits,[18] or oscillators.[26] Finally, this work has the potential to be multiplexed (*i.e.*, coexpressing many hairpin cassettes simultaneously) to investigate the impact of multiple gene knockdowns or streamlined by integrating all components necessary for RNAi (Dicer, Argonatue, and the hairpin) on the same vector. Thus, this work opens the door for metabolic engineering in yeast using RNA interference, which enables wider exploration of knockout targets, more finely tuned control of knockdown level, and greater flexibility in strain evaluation, resulting in an expedited design–build–test cycle.

METHODS

Strains and Media. Yeast expression vectors were propagated in *Escherichia coli* DH10*β*. Yeast strains are listed in Tables S2 and S7 (Supporting Information). *E. coli* strains were routinely cultivated in LB medium[27] (Teknova) at 37 °C with 225 rpm orbital shaking. LB was supplemented with 100 μg/mL of ampicillin (Sigma) when needed for plasmid maintenance and propagation. Yeast strains were cultivated on a yeast synthetic complete (YSC) medium containing 6.7 g of Yeast Nitrogen Base (Difco)/liter, 20 g of glucose/liter, and a mixture of appropriate nucleotides and amino acids (CSM, MP Biomedicals, Solon, OH). All media were supplemented with 1.5% agar for solid media.

For *E. coli* transformations, 25 μL of electrocompetent *E. coli* DH10*β*[27] were mixed with 30 ng of ligated DNA and electroporated (2 mm Electrporation Cuvettes (Bioexpress) with Biorad Genepulser Xcell) at 2.5 kV. Transformants were rescued for one hour at 37 °C in 1 mL of SOC Medium (Cellgro), plated on LB agar, and incubated overnight. Single clones were amplified in 5 mL of LB medium and incubated overnight at 37 °C. Plasmids were isolated (QIAprep Spin Miniprep Kit, Qiagen) and confirmed by sequencing.

For yeast transformations, 50 μL of chemically competent *S. cerevisiae* BY4741 were transformed with 1 μg of each appropriate purified plasmid according to established protocols,[16] plated on the appropriate medium, and incubated for three days at 30 °C. Single colonies were picked into 1 mL of the appropriate medium and incubated at 30 °C. All yeast strains generated through plasmid transformation are listed in Table S8 (Supporting Information). YFP-integrated yeast strains were generated through homologous recombination-mediated gene replacement[16] and are listed in Table S7 (Supporting Information). Yeast and bacterial strains were stored at −80 °C in 15% glycerol.

Cloning Procedures. Restriction enzyme-based plasmid construction schemes are detailed in Table S5 (Supporting Information). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). PCR reactions were performed with Q5 Hot-Start High-Fidelity DNA Polymerase from New England Biolabs (Ipswich, MA) according to manufacturer specifications and the schemes listed in Table S4 (Supporting Information). Digestions were performed according to manufacturer's (NEB) instructions, with diges-

tions close to the end of a linearized strand running overnight and digestions of circular strands running for 1 h at 37 °C. PCR products and digestions were cleaned with a QIAquick PCR Purification Kit (Qiagen). Phosphatase reactions were performed with Antarctic Phosphatase (NEB) according to manufacturer's instructions and heat-inactivated for 20 min at 65 °C. Ligations (T4 DNA Ligase, Fermentas) were performed for 18 h at 22 °C followed by heat inactivation at 65 °C for 20 min.

Homologous recombination-based plasmid construction schemes are detailed in Table S6 (Supporting Information). All assembly reactions were performed according to standard procedures.[28]

Flow Cytometry. Yeast colonies were picked in triplicate from glycerol stock, grown in the appropriate medium to midlog phase, and analyzed (LSRFortessa Flow Cytometer, BD Biosciences. Excitation wavelength: 488 nm. Detection wavelength: 530 nm). Day-to-day variability was mitigated by analyzing all comparable transformants on the same day. An average fluorescence and standard deviation was calculated from the mean values for the biological replicates. Flow cytometry data was analyzed using FlowJo software.

RT-PCR Assay. For each tested variant, the replicate yielding the most typical fluorescence measurement or itaconic acid yield was grown to an optical density of 0.5 and its RNA was extracted (Quick-RNA Miniprep, Zymo Research Corporation). 2 μg of RNA was reverse-transcribed (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems) and quantified in triplicate (SYBR Green PCR Master Mix, Life Technologies) immediately after RNA extraction. Transcript levels were measured relative to that of a housekeeping gene (ALG9) (Viia 7 Real Time PCR Instrument, Life Technologies). Primers used for quantification are listed in Table S3 (Supporting Information).

Itaconic Acid Production. Strains of interest were precultured for 3 days in the appropriate selective medium, and 30 μL of this culture were used as inoculum for a 3 mL culture in the same medium, which was grown for 3 days in a rotary drum incubator at 30 °C. This culture was then pelleted down (4 min at 1600*g*), and the supernatant was filtered using a 0.22 μm syringe filter (Corning). 2.0 μL of filtrate was analyzed with a HPLC Ultimate 3000 (Dionex) using a Zorbax SB-Aq column (Agilent) in a mobile phase composed of 99.5% potassium phosphate buffer (pH = 2.0) and 0.5% acetonitrile at 30 °C. Flow rate was maintained at 1.25 mL/min, and absorption was measured at 210 nm.

Growth Rate Analysis. Strains of interest were precultured for 3 days in the appropriate selective medium, and 1 μL of this precultured was used as an inoculum for a 250 μL culture in the same selective medium. Growth rate measurements were then obtained using a Bioscreen C (Growth Curves USA).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Details regarding the plasmids and strains used in this study, as well as growth rates, RNA levels, and plasmid expression variability data. This information is available free of charge *via* the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: (512) 471-4417. Fax: (512) 471-7060. E-mail: halper@che.utexas.edu.

Notes

The authors declare no competing financial interest.

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