RNAi-Assisted Genome Evolution in *Saccharomyces cerevisiae* for Complex Phenotype Engineering

Tong Si,[†] Yunzi Luo,[†] Zehua Bao,[‡] and Huimin Zhao*,[†,‡,§]

[†]Department of Chemical and Biomolecular Engineering, [‡]Department of Biochemistry, [§]Departments of Chemistry and Bioengineering, Institute for Genomic Biology, University of Illinois at Urbana—Champaign, Urbana, Illinois 61801, United States

S *Supporting Information*

ABSTRACT: A fundamental challenge in basic and applied biology is to reprogram cells with improved or novel traits on a genomic scale. However, the current ability to reprogram a cell on the genome scale is limited to bacterial cells. Here, we report RNA interference (RNAi)-assisted genome evolution (RAGE) as a generally applicable method for genome-scale engineering in the yeast *Saccharomyces cerevisiae*. Through iterative cycles of creating a library of RNAi induced reduction-of-function mutants coupled with high throughput screening or selection, RAGE can continuously improve target trait(s) by accumulating multiplex beneficial genetic modifications in an evolving yeast genome. To validate the RNAi library constructed with yeast genomic DNA and convergent-promoter expression cassette, we demonstrated RNAi screening in *Saccharomyces cerevisiae* for the first time by identifying two known and three novel suppressors of a telomerase-deficient mutation *yku70Δ*. We then showed the application of RAGE for improved acetic acid tolerance, a key trait for microbial production of chemicals and fuels. Three rounds of iterative RNAi screening led to the identification of three gene knockdown targets that acted synergistically to confer an engineered yeast strain with substantially improved acetic acid tolerance. RAGE should greatly accelerate the design and evolution of organisms with desired traits and provide new insights on genome structure, function, and evolution.

KEYWORDS: *S. cerevisiae, telomere, acetic acid tolerance, RNAi screening, directed evolution*
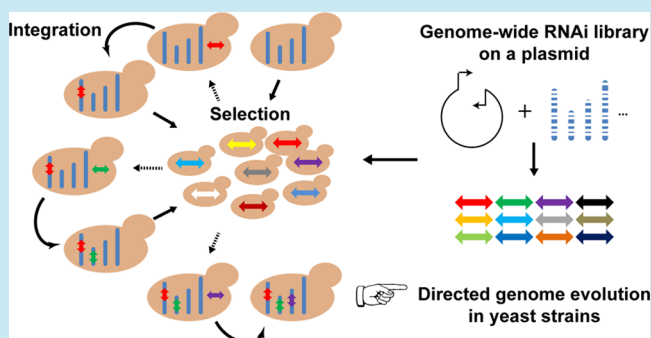
Complex phenotypes, such as inhibiter tolerance, involves synergistic actions of many genes.[1] Such complex phenotypes are often poorly understood and extremely difficult to engineer.[2,3] Adaptive engineering has been the method-of-choice to isolate evolved strains with improved inhibitor tolerance, through serial transfers with increasing inhibitor stresses in the medium.[4] Though effective, adaptive engineering is very time-consuming because the appearance of mutations is infrequent and most of these mutations are detrimental or neutral.[4] Therefore, new methods are needed to efficiently generate multiplex genetic diversity on a genome scale, as the engineering of complex traits often requires simultaneous modulation of many genes.[2,3,5,6]

The current ability to engineer a genome in multiplex is mostly limited to bacterial hosts.[3] A microbial genome can now be synthesized *de novo* which should in principle enable the ultimate genome-scale engineering, but this strategy is limited to small bacterial genomes and is also too expensive and tedious for most genome engineering applications.[7] In *Escherichia coli*, recombination-based genetic engineering (recombineering) enables generation of combinatorial genomic diversity[5] or genome-wide identification of gene targets for a certain trait.[8] However, lack of efficient tools for large-scale

DNA oligonucleotide-mediated allelic replacement hinders the application of recombineering in eukaryotes.

S. cerevisiae is not only a prominent model eukaryotic organism but also a widely used platform organism for industrial production of chemicals and fuels.[9,10] For this well-studied eukaryote *S. cerevisiae*, nonessential genes have been individually deleted to construct strain libraries for functional screening[11,12] (Figure 1A). Synthetic Genetic Array (SGA) has been developed to assay genetic interactions, whereby a query strain with a modified genetic background is crossed with a gene-deletion library to create an ordered array of haploid double mutant strains[13] (Figure 1A). Although strain libraries have provided invaluable knowledge about numerous important biological processes,[14] the tedious procedure to introduce genome-wide perturbations on a wild-type or mutated genome severely limits our ability to reprogram eukaryotic cells (Figure 1A). Besides, gene-knockout libraries are only available for certain laboratory strains of the *S. cerevisiae* species,[15] whereas different strains exhibit dramatic differences in phenotypes.[16] Therefore, effective tools to iteratively introduce genome-wide
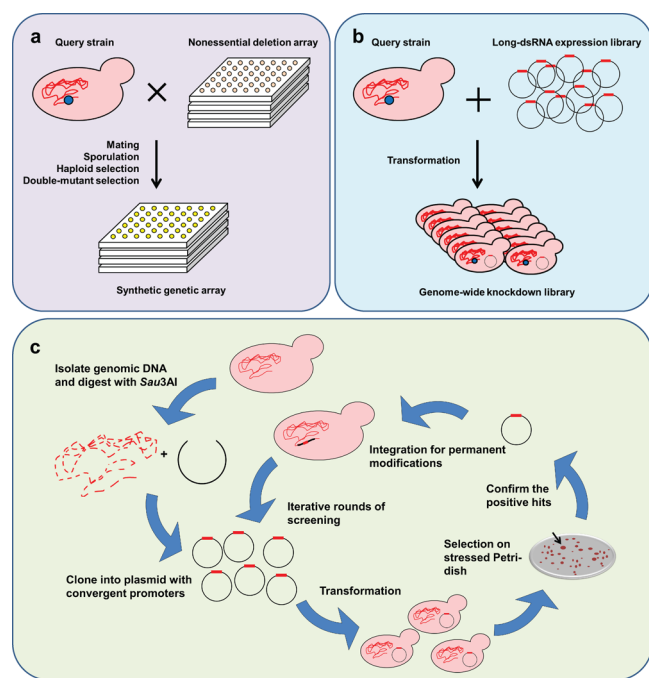
Figure 1. RNAi-assisted genome evolution enables rapid cellular reprogramming through iterative rounds of RNAi library creation and high throughput screening or selection. (A) Traditional strain libraries approach. Individual gene-knockout is performed to construct a mutant library. Synthetic Genetic Array (SGA) creates an array of double mutant strains through multiple steps of manipulation. (B) Creation of a pooled RNAi library only requires a simple step of transformation. (C) The ease of RNAi library construction enables repeated rounds of screening in an evolving genetic background (RAGE), which accumulates the beneficial modifications identified from the previous rounds of screening by integration.

modifications in customized genetic backgrounds are highly desirable for successful genome engineering practice in yeast.

Here, we report RAGE for engineering complex traits in yeast by directed genome evolution with iterative RNAi screening. Directed evolution mimics Darwinian evolution in a test tube and involves iterative rounds of genetic diversification and high throughput screening or selection, and it has achieved enormous success in tailoring biological systems ranging from single proteins to whole cells.[17,18] However, no satisfying tools exist to apply directed evolution strategy on a genome scale in *S. cerevisiae*, as the current method to introduce genome-wide mutations in an evolving yeast genome is prohibitively tedious (Figure 1A). On the other hand, the introduction of a pooled RNAi library to create genome-wide reduction-of-function modifications requires only a single step of transformation of the host cells[19,20] (Figure 1B). Such simplicity and effectiveness should enable the use of directed evolution strategy by repeating the cycles of RNAi screening to accumulate beneficial knockdown modifications (Figure 1C). RNAi is a cellular gene silencing mechanism broadly distributed in eukaryotic organisms, whereby mRNAs are targeted for degradation by homologous double-stranded RNAs (dsRNAs).[21,22] RNAi screening enables genome-wide reduction-of-function perturbations without allelic modifications and is widely used in eukaryotic functional genomics.[20,23] All known *S. cerevisiae* strains lack native RNAi machinery.[24] Recently, a heterologous RNAi pathway from *Saccharomyces castelli* was functionally reconstituted in *S. cerevisiae* to achieve

effective gene silencing.[24] Three human proteins, Ago2, Dicer, and TRBP, were also found to be sufficient to enable gene knockdown in *S. cerevisiae* by RNAi.[25] In addition, the RNAi machinery was implemented as a metabolic engineering tool to improve itaconic acid production in this yeast.[26] So far, however, no RNAi screening has been reported in this model eukaryotic organism. We first demonstrated RNAi screening in *S. cerevisiae* for suppressor analysis of a telomerase-deficient mutation *yku70Δ* and then applied iterative RNAi screening for improved acetic acid (HAc) tolerance.

■ RESULTS AND DISCUSSION

Functional Reconstitution of RNAi Pathway. We first sought to establish RNAi screening in *S. cerevisiae*. A *Saccharomyces castellii* RNAi pathway was functionally introduced into *S. cerevisiae* recently.[24] We reconstituted this *S. castellii* RNAi pathway into our target yeast strain CEN.PK2-1c and named the resulting strain as the CAD strain (Supporting Information Figure S1). We observed repression of the expression of a reporter protein, green fluorescent protein (GFP), to various degrees by the *gfp*-silencing constructs based on either the antisense design or the convergent-promoter design (Figure 2). The reduction of GFP fluorescence was dependent on both the RNAi pathway and the RNA expression (Figure 2B and C), indicating that the imported RNAi pathway was functional. By adapting the convergent-promoter design (Figure 2A), we created a pooled long-dsRNA library from the yeast genomic DNA fragments which were generated by enzymatic digestion.[27] We randomly sequenced 50 plasmids from the library. The sequencing result (Supporting Information Figure S2) and the achieved library size (Supporting Information Table S1) suggested that a satisfying coverage of the yeast genome was achieved.

Suppressor Analysis of the yku70Δ Mutation by RNAi Screening. To verify the RNAi library, we performed a genome-wide RNAi screening to identify suppressors of the *yku70Δ* mutation (Figure 3A). Yku70 is a telomere-associated protein.[28,29] At an elevated temperature such as 37 °C, the null mutation of the *yku70* gene will lead to single stranded DNA accumulation at the telomere, which triggers DNA damage response and then cell-cycle arrest.[28] The temperature-dependent growth phenotype caused by the *yku70Δ* mutation provides a valuable model to study genetic interactions involved in DNA-repair and cell-cycle pathways.[28−30] We transformed our RNAi library plasmids into the CAD strain with the *yku70* gene deleted. The transformants were selected on solid synthetic medium at 37 °C. Colonies that grew larger than the control strain were picked up and tested for their growth capacity under challenging temperatures in liquid media. Plasmids isolated from the top growers were retransformed into fresh yeast cells, and those still conferring improved growth at 37 °C were sequenced to identify the origins of the RNAi cassettes (Figure 3B). The insets within the selected RNAi cassettes were found to be the fragments from one essential gene *ret1*, and four nonessential genes *say1, ssa1, cst6*, and *mlp2* (Supporting Information Table S2). The *mlp2* and *ssa1* genes were previously identified as suppressors of the *yku70Δ* mutation,[30,31] which confirmed the effectiveness of our RNAi library for genome-wide screening.

To eliminate the "off-target" effects, we designed a second RNAi construct targeting a different region of the same transcript for each gene (Supporting Information Table S2). The "off-target" effect often resulted from the partial homology
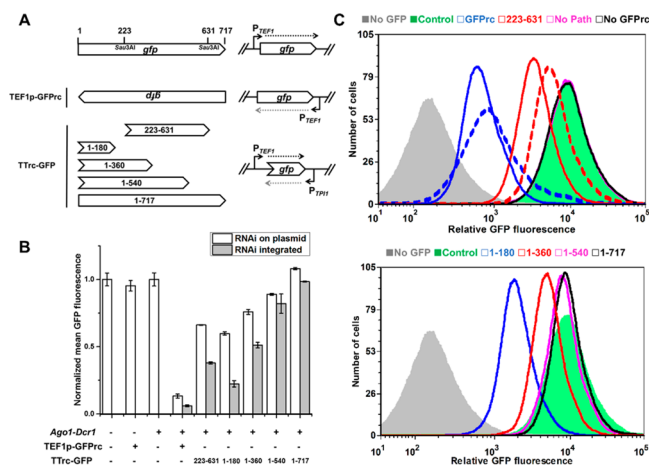
Figure 2. Repression of GFP fluorescence by RNAi constructs. The overexpression cassette of GFP was integrated into the *leu2* locus of the CAD or CEN.PK2-1c strain. (A) Scheme for RNAi expression design. The TEF 1p-GFPrc construct transcribes the full-length antisense RNA of the GFP gene, while the TTrc-GFP constructs transcribe dsRNAs derived from different regions of the GFP gene. The 223−631 bp region corresponds to the digestion product of the GFP gene by *Sau*3AI. (B) Silencing of GFP expression. The average on the mean GFP fluorescence of three biological replicates is reported. The error bars indicate standard deviations. The 100% reference of GFP signal was defined as the strain containing the integrated GFP expression cassette and the control plasmid pRS416. The RNAi cassettes are located either on a single-copy plasmid pRS416 (white bar) or in the *ura3* locus of the yeast genome (gray bar). The gene-silencing effect only showed in the presence of both the RNAi pathway and RNAi constructs. For the convergent-promoter constructs, both the position and the length of the insets will affect the repression efficiency. (C) FACS histograms showing GFP fluorescence of the yeast strains harboring different RNAi constructs. Dashed lines indicate the plasmid pRS416 is used to express the RNAi cassettes, whereas solid lines indicate the RNAi cassettes are integrated in the *ura3* locus. The *No GFP* strain is the CEN.PK2-1c strain with the pRS416-TTrc plasmid. The *Control* strain is the CEN.PK2-1c strain with an integrated GFP-overexpressing cassette and the pRS416-TTrc plasmid. The *No Path* strain is the CEN.PK2-1c strain with an integrated GFP-overexpressing cassette and the pRS416-TEF 1p-GFPrc plasmid. The *No GFPrc* strain is the CAD strain with an integrated GFP-overexpressing cassette and the empty pRS416 plasmid. The results on the strains with the convergent RNAi constructs 1−180, 1−360, 1−540, and 1−717 on the pRS416 plasmid are omitted here for clarity and reported in Supporting Information Figure S7.

to other transcripts,[32] and it was unlikely that two independent RNAi cassettes would have the common "off-target" suppression effects. All the designed RNAi constructs rescued the temperature sensitivity of *yku70Δ* at 37 °C (Figure 3B). We further estimated the knockdown efficiency by fusing the target endogenous proteins with a carboxyl-terminal GFP tag for quantification.[33] All the selected and designed RNAi cassettes showed reduction of GFP fluorescence (Figure 3C). The suppression effects of the selected nonessential genes were also validated by examining the gene knockout mutants (Figure 3B). These results confirmed that the selected genes were suppressors of the *yku70Δ* mutation. Whereas the newly identified modifiers will provide novel molecular insights on telomere biology (Supporting Information Table S3), the identification of an essential gene as a suppressor of the *yku70Δ* mutation highlighted the advantage of RNAi screening over
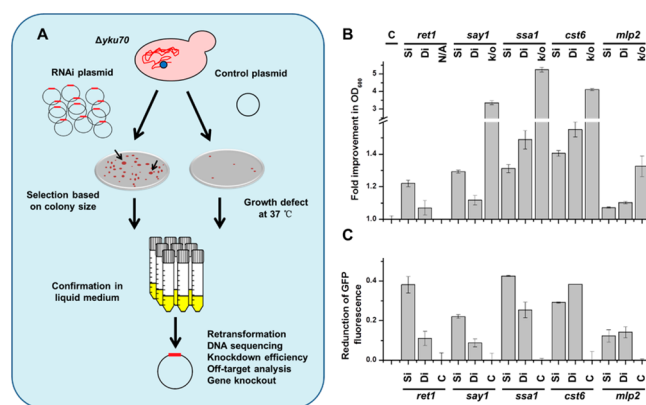

Figure 3. RNAi screening in *S. cerevisiae* to identify *yku70Δ* suppressors. (A) Scheme for suppressor screening of *yku70Δ*. (B) Comparison of growth capacity in synthetic dropout medium at 37 °C. The initial OD_{600} was 0.2, and the cell density after growing for 12 h was normalized to the CAD strain containing the control plasmid. (C) Estimation of knockdown efficiency where the expression levels of target proteins were quantified by the GFP tag.[33] The 100% reference of GFP signal was defined as the strains with the control plasmid for each target gene, respectively. Reduction of GFP fluorescence was reported as 1-(RNAi strain fluorescence/Control strain fluorescence). All the RNAi constructs were transcribed from a single-copy plasmid pRS416. Si: selected RNAi constructs. Di: designed RNAi constructs. k/o: knockout. C: control plasmid pRS416-TTrc. N/A: knockout is lethal. Error bars indicate standard deviation of three biological replicates.

gene-knockout libraries by including the genes whose null mutations are lethal.

RNAi-Assisted Genome Evolution for Improved HAc Tolerance. After establishing RNAi screening in *S. cerevisiae*, we combined it with directed evolution to rapidly engineer yeast cells for improved HAc tolerance (Figure 1C). Although bioethanol fermentation by *S. cerevisiae* from sugar cane sucrose and corn starch has been widely applied for biofuel production,[9,10] the use of lignocellulosic biomass as substrate by this yeast is highly desirable for a more sustainable biofuel process.[34] Acetic acid (HAc) is an unavoidable inhibitor either from the pretreatment step of lignocelluloses or as the byproduct during alcoholic fermentation,[35,36] and tolerance to HAc is highly desirable for commercial production of chemicals and fuels by *S. cerevisiae*.[36] Previous screening efforts to increase HAc tolerance in *S. cerevisiae* are limited to BY4741/4742 strains based on which most gene-knockout libraries are created.[36,37] Our results showed that the mutations that were reported to improve HAc tolerance in BY-strains failed to elicit growth advantage in our CEN.PK2-1c strain under HAc stress (Supporting Information Figure S3).

We first confirmed that introduction of this heterologous RNAi pathway had little impact on cellular growth and HAc tolerance of *S. cerevisiae* (Supporting Information Figure S4A), which is consistent with a previous report.[38] Following a similar procedure of the suppressor screening for *yku70Δ* (Figure 3A), four RNAi cassettes that conferred yeast cells with increased growth under 0.5% (v/v) HAc stress were identified and confirmed in the first round of RAGE (Figure 4A and B, and Supporting Information Table S4). We integrated the four selected RNAi cassettes into the yeast genome separately, and the resultant strains all exhibited better fitness than the wild-type strain under HAc stress (Figure 4C). These strains were then employed as new parent strains for the second round of
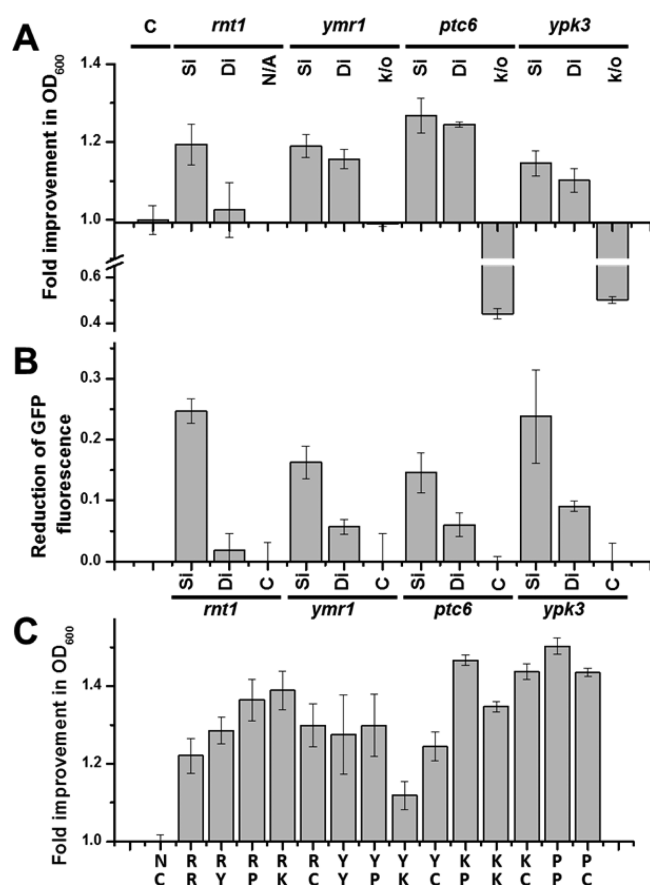
Figure 4. Knockdown targets identified by RNAi screening for improved HAc tolerance. (A) Comparison of growth capacity in the presence of 0.5% (v/v) HAc in synthetic dropout medium (pH = 4.5). The initial OD_{600} was 0.2, and the cell density after growing for 12 h was normalized to the CAD strain containing the control plasmid. (B) Estimation of knockdown efficiency where the expression levels of target proteins were quantified by the GFP tag.[33] The 100% reference of GFP signal was defined as the strains with the control plasmid for each target gene, respectively. Reduction of GFP fluorescence was reported as 1-(RNAi strain fluorescence/Control strain fluorescence). All the RNAi constructs were transcribed from a single-copy plasmid pRS416 in parts A and B. (C) HAc tolerance of strains with combinations of RNAi cassettes from the first round of RAGE. The same growth condition was employed as in part A. The names of the strains are denoted by two letters. The first letter indicates the integrated cassette in the *his3* locus, while the second letter indicates the RNAi cassette on the plasmid. R: *rnt1*_Si. Y: *ymr1*_Si. P: *ptc6*_Si. K: *ypk3*_Si. N: no integration. C: the control plasmid pRS416-TTrc.

RNAi screening (Figure 5A). With a mini-library containing the four selected plasmids from the first round, we found that the combination of the beneficial RNAi modifications did not necessarily lead to incremental improvements (Figure 4C), highlighting the necessity to perform genome-wide screening to identify targets for further engineering.

The genome-wide RNAi library was transformed into the new parent strains (Figure 5A). A higher HAc concentration (0.6% (v/v)) than the first round (0.5% (v/v)) was used to isolate mutant strains with better HAc tolerance. In the background of the strain integrated with the RNAi cassette for *ptc6* (named as the first round strain R1), the RNAi cassette for *ypr084w* resulted in the highest fitness in the second round of screening (Figure 5A). Thus, the recombinant strain integrated with both the *ptc6* RNAi and *ypr084w* RNAi cassettes (named
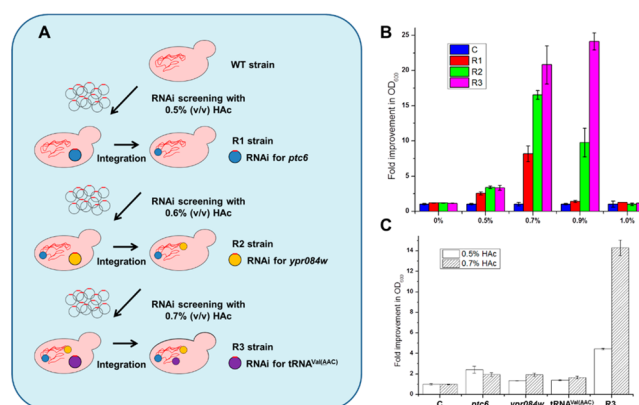

Figure 5. Yeast strains engineered by RAGE showing improved HAc tolerance. (A) Scheme of iterative RNAi screening to accumulate beneficial knockdown modifications in a yeast genome. (B) Growth capacity of the yeast strains identified from three rounds of RAGE with different levels of HAc in synthetic dropout medium (pH = 4.5). The initial OD_{600} was 0.01, and the cell density was measured after growing for 24 h (0%, 0.5%, and 0.7% (v/v)) or 48 h (0.9% and 1.0%). No cell growth was observed for the 1.0% (v/v) HAc group after 72 h. Fold improvements were compared to the CAD strain containing the control plasmid. (C) Contribution of individual knockdown modification from the R3 strain to the enhanced HAc tolerance. Plasmids harboring the selected RNAi cassettes for *ptc6*, *ypr084w*, and tRNA$^{Val(\Delta AC)}$ were transformed into the CAD strain to achieve individual gene silencing. The same growth condition was employed as in part B, except that only two HAc concentrations (0.5% and 0.7%) were tested. C: the CAD strain harboring the control plasmid pRS416-TTrc. R1–R3: the best selected strain from the first, second and third round of RAGE, respectively, with all the selected RNAi cassettes integrated (see Supporting Information Table S8 for details). The R1–R3 strains were all transformed with the control plasmid pRS416-TTrc to enable growth in the SC-U medium. Error bars indicate standard deviation of three biological replicates.

as the second round strain R2) was created and used as the parent strain for the third round of RNAi screening with 0.7% (v/v) HAc as selection pressure. The best mutant strain was found to contain an RNAi cassette with a genomic DNA fragment from tRNA$^{Val(\Delta AC)}$ as the inset (named as the third round strain R3, Figure 5A).

Superior HAc Tolerance of Engineered Strains by RAGE. The mutant strains with the highest fitness in each round of RAGE were compared in parallel with the control strain for the growth capacity in the presence of different concentrations of HAc. A general trend of R3 > R2 > R1 > control was observed under almost all the conditions, indicating the stepwise improvement of HAc tolerance (Figure 5B). The tolerance phenotype of the R3 strain was significantly increased, which accumulated greater than 20-fold more biomass under 0.9% (v/v) HAc relative to the control strain. The 100% inhibitory HAc concentration for growth was elevated from 0.8% to 1.0% (v/v).

Next, we evaluated the contribution of each RNAi cassette to the HAc tolerance of the R3 strain. The yeast strains carrying only one of the selected RNAi cassettes for *ptc6*, *ypr084w*, and tRNA$^{Val(\Delta AC)}$ were constructed and compared with the R3 strain for the growth capacity with elevated HAc levels (0.5% and 0.7% (v/v)). The results showed that each cassette led to increased biomass accumulation to some extent relative to the control, but the superior tolerance of the R3 strain cannot be readily explained by simply adding up these individual effects

(Figure 5C). The mechanism of the observed synergy cannot be deduced directly from the known functions of the targeted genes (Supporting Information Table S5). Such nonlinear interactions between the RNAi cassettes highlighted the necessity of iterative rounds of screening to accumulate the beneficial knockdown perturbations.

The fermentation performance of the R3 strain and the control strain were evaluated under an oxygen-limited condition. Three levels of HAc stress were applied as 0%, 0.7%, and 1.0% (v/v) in the synthetic minimal medium. The performance of the R3 strain was superior to the control under all the conditions in terms of glucose utilization, biomass accumulation and ethanol productivity (Supporting Information Figure S5). Specifically, in the presence of 0.7% HAc, the R3 strain exhibited a 63.1 ± 3.2% increase in ethanol productivity relative to the control strain in the period of 0−24 h. Though the final ethanol titers were similar for the two strains, the R3 strain accumulated 15.3 ± 3.5% more biomass than the control. Without the HAc stress, the R3 strain showed the same ethanol production profile as the control strain. Notably, after the glucose was depleted, the R3 strain exhibited a much faster assimilation rate of acetate than the control (Supporting Information Figure S5). The enhanced capacity to utilize acetate might provide some clues why the R3 strain had a higher level of HAc tolerance. These results confirmed that RAGE successfully improved the HAc tolerance and fermentation performance of *S. cerevisiae*.

RNAi proves to be an enabling technology with broad applications for functional analysis, therapeutics, and metabolic engineering in mammalian cells, insect cells, and plants.[39,40] Although RNAi is not a novel technique, RAGE further expands the power of RNAi by the identification and fine-tuning of multiplex gene targets and engineer yeast cells on a genome scale. The potential of employing RNAi screening for directed genome evolution offers great advantages over conventional gene-knockout strategies, such as providing a simple tool to modify a eukaryotic genome globally and iteratively, enabling fine-tuning of gene expression, including essential genes in functional screening, and identifying beneficial traits which requires synergistic genetic modifications. By targeting mRNAs to introduce reduction-of-function mutations, RAGE might be especially useful in industrial organisms for which gene deletion is extremely challenging due to polyploidy.[41]

When interpreting the results of RNAi screening, cautions need to be taken against "off-target" effect, which is mainly resulted from cross-silencing of other transcripts with partial homology.[32] The "off-target" effect is well-documented for different eukaryotic systems in literature.[20,32,42,43] The most effective way to ensure that the observed phenotypes are "on-target" is to show that these phenotypes can be generated by independent RNAi molecules, which target the same gene but contain completely distinct sequences,[19,20,23] as performed in this study by analyzing the phenotypes caused by a second designed dsRNA molecule for each gene target (Figures 3B and 4A). While it is very critical to minimize "off-target" effect when applying RNAi as therapeutics,[44] it is probably less important to achieve high specificity of RNAi in microbial strain engineering.

In this work, we constructed the RNAi library by inserting the genomic DNA fragments generated by *Sau*3AI into a pair of convergent promoters for dsRNA synthesis. Whereas this is an established method,[27] it is possible to further improve the coverage and effectiveness of the RNAi library. First, though a

library size that is big enough can ensure the complete coverage of the yeast genome, the biased fragmentation pattern determined by the recognition site of *Sau*3AI may preclude the identification of some genes in the screening. For these genes, the dsRNA molecules transcribed from the *Sau*3AI fragments may not generate observable phenotypes due to weak knockdown levels. To solve this issue, a collection of random fragmentation strategies with less inherent bias through enzymatic, chemical and mechanical means can be employed, which are originally developed to create the shotgun libraries for genome sequencing.[45] Second, the knockdown efficiency enabled by the long dsRNA molecules is moderate, which may limit the sensitivity of RNAi screening. For example, the knockout mutations of some identified *yku70*Δ suppressors led to more substantial growth advantage than the knockdown mutations (Figure 3B). Whereas the incompleteness of knockdown mutations is a well-recognized issue of RNAi screening, it is possible to improve the gene-silencing efficiency by optimizing the format of RNAi reagents. For example, in this study we observed that the full-length antisense RNA resulted in more profound knockdown effects than the dsRNA molecules transcribed by the convergent promoters (Figure 2B and Supporting Information Figure S4D). Therefore, one way to improve the sensitivity of RNAi screening is to use the full-length antisense RNAi library, which can be created by cloning of the full-length cDNA library in a reversed direction after a promoter sequence. It is noted that in a recent report, hairpin RNAi molecules were implemented for RNAi down-regulation in *S. cerevisiae*.[26] Different parameters, such as the hairpin length and expression context, were manipulated for optimized gene silencing effectiveness. It is thus possible to use hairpin RNAi design for the library construction in RAGE. For *S. cerevisiae* strains with sequenced genomes, it is also possible to design synthetic genome-wide RNAi library by computational algorithm to optimize potency and specificity.[46]

In its current form, a pooled library on an episomal plasmid coupled with an efficient selection strategy is implemented in RAGE. However, a separate integration step is needed at the beginning of each round to create a new parent strain. It should be noted that the plasmid-borne and integrated versions of the same RNAi cassette may lead to different phenotypes. For example, the integrated RNAi cassette for *ptc6* (Figure 4C, group PC) resulted in a slightly higher HAc tolerance than the plasmid-borne version (Figure 4A, group *ptc6*_Si). In addition, the *gfp*-knockdown constructs that were integrated generally led to a stronger silencing effect than their plasmid-borne counterparts (Figure 2B). The strains harboring the integrated RNAi cassettes also exhibited less population heterogeneity in GFP fluorescence profile, compared to the strains with the RNAi cassettes on the plasmid (Figure 2C and Supporting Information Figure S7). Together, these observations indicate that the different expression contexts, from a plasmid or a genomic location, may affect the kinetics of dsRNA transcription and therefore lead to different knockdown levels. This dependency of gene silencing effectiveness on RNAi expression format was also consistent with a previous report.[26] Therefore, it is critical to confirm that the improved trait is still retained upon the integration of a selected RNAi cassette. It is also possible to avoid the changes in expression contexts by direct integration of the RNAi library into the host genome for library creation, which should further speed up and automate the entire process of RAGE. While the transformation efficiency of an episomal plasmid can easily achieve a complete coverage of

the entire genome, the integration efficiency needs to be greatly improved to create a comprehensive library, possibly through the introduction of double-stranded breaks (DSBs) on a genome.[47] Recent development in creating site-specific DSBs by engineered nucleases (zinc-finger nucleases or ZFNs,[48] transcription-activator-like effector nucleases or TALENs,[49,50] and clustered regularly interspaced short palindromic repeats or CRISPR-associated nucleases[51,52]) may facilitate the automation of RAGE by direct integration.

For the engineered strains obtained by RAGE, further investigation of the mechanisms underlying the superior HAc tolerance will provide invaluable knowledge about the target traits. Based on the known functions of the selected knockdown gene targets (Supporting Information Table S5), it is noteworthy that ypk3 and ptc6 are involved in the target of rapamycin (TOR) pathway, which is responsible for the signaling of acetic acid-induced apoptosis.[53] The gene rnt1 can mediate selective mRNA degradation and thus regulate the cell wall stress response,[54] which is experienced by HAc-challenged yeast cells.[55] The gene ypk3 might also play a role in the cell wall integrity as a homologue of ypk1,[56] and the deletion of ypk1 can improve acid tolerance of the yeast cells.[57] Though the function of ypr084w is unknown, ypr084w exhibits negative interactions with the slm4 and spf1 genes,[58] which are the determinants of HAc resistance.[59] Reprogramming of tRNA modifications was reported to regulate the stress response of yeast cells,[60,61] and it will be interesting to further investigate how our selected RNAi construct for tRNA$^{Val(\underline{A}AC)}$ plays a role in such process.

It is noted that the knockout mutations of the selected genes failed to improve HAc tolerance as the knockdown mutations did (Figure 4A). The phenomenon that the performance of a knockdown mutant strain was better than both the wild-type and knockout strains has been observed previously in S. cerevisiae.[62] For some essential genes whose knockout mutations are lethal, the reduction-of-function mutants exhibit growth advantages compared to the wild-type strain.[62] We speculate that the underlying reason may be that the knockout mutations of such genes may have dual roles, which result in not only improved tolerance to a certain chemical but also impaired general fitness. Indeed, the knockout strains of the selected gene targets in the first round of RAGE grew at reduced rates compared to the CAD strain without HAc stress (Supporting Information Figure S4B), indicating the complete disruption of the normal functions of these genes may affect the general fitness of the yeast cells. Therefore, an optimal knockdown level may exist to balance the trade-off between general fitness and inhibitor tolerance. The optimal knockdown level may be determined by fine-tuning the knockdown level of a selected target, which is possible by switching among different RNAi reagent forms (antisense RNAs or dsRNAs) and varying the lengths and positions of the RNAi cassettes. These strategies successfully enabled different levels of suppression of both the gfp gene (Figure 2) and the ptc6 gene (Supporting Information Figure S4C and D).

Conclusion. Here, we show the de novo creation of an artificial RNAi regulatory mechanism tailored for the host and target trait(s) in an organism that lacks native RNAi machinery. RAGE employs a genomic DNA/cDNA-derived library[27] and thus does not require genome sequence information or a preconstructed gene-knockout library. Such strategy should be widely applicable in any host of interest with basic genetic tools and a functional RNAi pathway (native or engineered).

Although RNAi is only conserved in eukaryotes, a recent method based on synthetic small regulatory RNAs (sRNAs) was developed to modulate the expression of up to 122 chromosomal genes in E. coli.[63] Thus, it is also possible to extend the application of RAGE in bacteria by accumulating beneficial sRNAs in the E. coli genome to continuously improve a target trait. Given the versatile tools and broad applications available for both RNA interference and directed evolution, we envision RAGE will become a powerful genome-scale engineering tool for studies in biology, medicine, and biotechnology.

■ METHODS

Strains, Media, and Cultivation Conditions. S. cerevisiae strain CEN.PK2-1c (MATa ura3−52 trp1−289 leu2−3,112 his3Δ1 MAL2−8C SUC2) was purchased from EUROSCARF (Frankfurt, Germany). Zymo 5α Z-competent E. coli (Zymo Research, Irvine, CA) and NEB 5α Electrocompetent E. coli (New England Biolabs, Ipswich, MA) were used for plasmid amplification and library construction, respectively. The S. castellii strain was obtained from the ARS culture collection (NRRL number Y-12630) (Peoria, IL). S. cerevisiae strains were cultivated in either synthetic dropout medium (0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate and 0.083% amino acid drop out mix, 0.01% adenine hemisulfate, and 2% glucose) or YPAD medium (1% yeast extract, 2% peptone, 0.01% adenine hemisulfate, and 2% glucose). 10% (v/v) filtered acetic acid (HAc) solution stock was added into the above medium to make stressed medium. Before mixing, both the medium and HAc stock were adjusted to pH = 4.5. S. cerevisiae strains were cultured at 30 °C and with 250 rpm agitation in baffled shake-flasks for aerobic growth, and at 30 °C and 100 rpm in unbaffled shake-flasks for fermentation. E. coli strains were cultured at 37 °C and 250 rpm in Luria broth (LB) medium (Fisher Scientific, Pittsburgh, PA) with the supplement of 100 μg/mL ampicillin. The S. castellii strain was cultured in YPAD medium at 30 °C and 250 rpm. All chemicals were purchased through Sigma-Aldrich or Fisher Scientific.

DNA Manipulation. Plasmid cloning was mostly done by In-fusion HD cloning Kit (Clontech Laboratories, Mountain View, CA) following the manufacturer's instructions or by the DNA assembler method.[64] The complete list of the plasmids, primers and strains in this study is summarized in Supporting Information Tables S6−8. For DNA manipulations, yeast plasmids were isolated using a Zymoprep II yeast plasmid isolation kit (Zymo Research, Irvine, CA) and transferred into E. coli for amplification. QIAprep Spin Plasmid Mini-prep Kits (Qiagen, Valencia, CA) were employed to prepare plasmid DNA from E. coli. Yeast genomic DNA was isolated by Wizard Genomic DNA Purification Kit (Promega, Madison, WI). All enzymes used for recombinant DNA cloning were from New England Biolabs unless otherwise noted. The products of PCR, digestion and ligation reactions were purified by QIAquick PCR Purification and Gel Extraction Kits (Qiagen, Valencia, CA).

Reconstitution of the RNAi Machinery in S. cerevisiae. The genomic DNA of S. castellii strain was isolated and used as template to clone the ago1 and dcr1 genes. An expression cassette in which the ago1 and dcr1 genes were driven by constitutive promoters P_{TEF1} and P_{TPI1}, respectively, was assembled into a delta-integration vector by the DNA assembler method. The integration copy number of the RNAi pathway was analyzed by quantitative PCR (qPCR) with LightCycler 480 SYBR Green I Master (Roche, Indian-

apolis, IN) following the manufacturer's instructions (Supporting Information Figure S1). To test whether the RNAi machinery is functional, a reporter system based on green fluorescent protein (GFP) was devised (Figure 2A and B). The GFP fluorescence of the engineered strains was analyzed by LSR II Flow Cytometer (BD, Franklin Lakes, NJ).

Construction of a Genome-wide RNAi Library. Two strong constitutive promoters, P_{TEF1} and P_{TPI1}, were cloned in opposite directions to drive the *in vivo* synthesis of a dsRNA molecule.[27] A *Bam*HI restriction site is engineered between the two promoters to facilitate the insertion of genomic DNA fragments generated by complete *Sau*3AI digestion. The expression cassette was placed on a single-copy plasmid pRS416. The ligation product of the genomic DNA fragments and the linearized vector was transformed into *E. coli* cells by electroporation to create a pooled plasmid library (see Supporting Information, Methods).

Construction of a Yeast Knockdown Library and Screening. In the CAD strain, the standard LiAc/ssDNA/PEG protocol[65] was used to transform 20 μg RNAi library plasmids or the control plasmid. The library DNA (20 μg) was able to achieve a library size more than 5×10^5 to ensure a nearly complete coverage (>99.92%, Supporting Information Table S1) of the yeast genome. Following the transformation, the yeast cells were recovered in 1 mL YPAD medium for 4 h, and then washed with ddH$_2$O. For *yku70Δ* suppressor screening, the transformants were spread onto 15 mm diameter Petri-dish plates of solid SC-U medium. The amount of cells was adjusted so that each plate would form about 10^4 colonies at a permissive temperature 30 °C and about 10^3 colonies when challenged with 37 °C. The library plates and the control plates were incubated at 37 °C for 3−4 days. Ninety-three colonies whose sizes were bigger than the largest colonies on the control plates were picked from the library plates. The growth performances of the selected colonies and the control strain were compared in SC-U medium at 37 °C. The initial OD$_{600}$ for all the strains were adjusted to 0.2, and the growth was monitored at 4, 8, 12, and 24 h time intervals. The RNAi plasmids from the top strains whose growth behaviors were considerably better than the control strain were isolated and amplified by *E. coli*. The selected plasmids were then individually retransformed, of which four were able to retain the enhanced HAc tolerance in a fresh background with three biological replicates. The confirmed plasmids were sent for DNA sequencing analysis. The BLAST search was used to identify the sources of the insets in the selected RNAi plasmids. For each new-identified gene target, a designed RNAi mutant and a knockout mutant were constructed for further analysis. A similar procedure was employed for HAc tolerance screening, except that 0.5% (v/v) HAc was supplemented into the growth medium and the cells was cultured at 30 °C.

Second and Third Rounds of RAGE. A similar screening procedure was employed in the subsequent rounds as in the first one. For the second round, the four selected RNAi cassettes in the first round were subcloned into the multiple cloning site of pRS403 plasmid and integrated into the *his3* locus of the CAD strain to construct the new parent strains. Then the RNAi plasmid library was transformed into the new parent strains to perform the second round of RAGE. The stress level of 0.6% (v/v) HAc was applied for the screening on the solid medium and the growth quantification in the liquid medium. For the third round of screening, the RNAi cassette for *ypr084w* was subcloned into pRS404 and integrated into the

trp1 locus of CAD_ptc6i to create the new parent strain. The stress level of 0.7% was applied. The details about all the selected and designed RNAi cassettes can be found in Supporting Information Table S4.

Characterization of Engineered Strains for HAc Tolerance. The engineered R1, R2, and R3 strains, together with the control strain, were tested for the biomass accumulation in the synthetic dropout medium SC-U containing 0, 0.5, 0.7, 0.9 and 1.0% (v/v) HAc (Figure 5B). Three biological replicates of each strain were inoculated in 3 mL SC-U to grow until saturation. Then 50 μL culture was used to inoculate 3 mL fresh SC-U to synchronize the growth phase. After 20 h, the stationary-phase cells were transferred into three culture tubes containing 3 mL SC-U medium and varying concentrations of HAc. The initial OD$_{600}$ was adjusted to 0.01 and 1 mL cell culture was taken periodically at 24, 48, and 72 h to measure the cell density. The same procedure was used to compare the R3 strain and the strains containing only one of the three RNAi cassettes from the R3 strain (Figure 5C).

The fermentation performance of the R3 strain and the control strain was compared (Supporting Information Figure S5). Three biological replicates of each strain were inoculated in 3 mL SC-U medium in 15 mL round-bottom Falcon tubes to grow until saturation. Then 1 mL culture was transferred into 20 mL SC-U medium in 125 mL baffled shake-flasks. The preparation of the seed culture was performed aerobically (30 °C and 250 rpm). After 20 h, the stationary-phase cells were transferred into 50 mL SC-U medium in unbaffled 250 mL shake-flasks. The fermentation was carried out under an oxygen-limited condition (30 °C and 100 rpm). Three levels of HAc stress were applied as 0%, 0.7% and 1.0% (v/v) in the synthetic dropout medium SC-U. Samples (1 mL) were taken at 0, 4, 8, 12, 24, and 48 h for the measurement of cell density and HPLC analysis. An HPX-87H column (BioRad, Hercules, CA) coupled with a refractive index detector (Shimadzu Scientific Instruments, Columbia, MD) was used to separate and analyze the concentrations of glucose, ethanol, and acetate in the broth following the manufacturer's instructions.

Estimation of the Gene Knockdown Efficiency. By knocking-in the GFP gene as a carboxy-terminal fusion reporter, it is possible to quantify the expression levels of the target proteins (Figures 3C and 4B). All the primers and the process to create a perfect in-frame GFP fusion protein were reported elsewhere.[33] We measured the knockdown efficiency for the four target genes found in the first round of RAGE. The cells were cultured aerobically in SC-His/Ura and subjected to the flow cytometry analysis in their log-phase. To determine the knockdown efficiency of the other two RNAi cassettes (*ypr084w* and tRNA$^{Val(\Delta AC)}$), a semiquantification assay was employed to measure the level of target RNA molecules[24] (Supporting Information Methods and Figure S6).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed methods; additional figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: 1-217-333-2631. Fax: 1-217-333-5052. Email: zhao5@illinois.edu.

■ REFERENCES

(1) Almeida, J. R. M., Modig, T., Petersson, A., Hahn-Hagerdal, B., Liden, G., and Gorwa-Grauslund, M. F. (2007) Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. *Curr. Opin. Biotechnol. 82*, 340−349.

(2) Santos, C. N. S., and Stephanopoulos, G. (2008) Combinatorial engineering of microbes for optimizing cellular phenotype. *Curr. Opin. Chem. Bio. 12*, 168−176.

(3) Woodruff, L. B. A., and Gill, R. T. (2011) Engineering genomes in multiplex. *Curr. Opin. Biotechnol. 22*, 576−583.

(4) Cakar, Z. P., Turanli-Yildiz, B., Alkim, C., and Yilmaz, U. (2012) Evolutionary engineering of *Saccharomyces cerevisiae* for improved industrially important properties. *Fems Yeast Res. 12*, 171−182.

(5) Wang, H. H., Isaacs, F. J., Carr, P. A., Sun, Z. Z., Xu, G., Forest, C. R., and Church, G. M. (2009) Programming cells by multiplex genome engineering and accelerated evolution. *Nature 460*, 894−U133.

(6) Alper, H., Moxley, J., Nevoigt, E., Fink, G. R., and Stephanopoulos, G. (2006) Engineering yeast transcription machinery for improved ethanol tolerance and production. *Science 314*, 1565−1568.

(7) Gibson, D. G., Benders, G. A., Andrews-Pfannkoch, C., Denisova, E. A., Baden-Tillson, H., Zaveri, J., Stockwell, T. B., Brownley, A., Thomas, D. W., Algire, M. A., Merryman, C., Young, L., Noskov, V. N., Glass, J. I., Venter, J. C., Hutchison, C. A., 3rd, and Smith, H. O. (2008) Complete chemical synthesis, assembly, and cloning of a *Mycoplasma genitalium* genome. *Science 319*, 1215−1220.

(8) Warner, J. R., Reeder, P. J., Karimpour-Fard, A., Woodruff, L. B. A., and Gill, R. T. (2010) Rapid profiling of a microbial genome using mixtures of barcoded oligonucleotides. *Nat. Biotechnol. 28*, 856−U138.

(9) Nevoigt, E. (2008) Progress in metabolic engineering of *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev. 72*, 379−412.

(10) Hong, K.-K., and Nielsen, J. (2012) Metabolic engineering of *Saccharomyces cerevisiae*: A key cell factory platform for future biorefineries. *Cell. Mol. Life Sci. 69*, 2671−2690.

(11) Winzeler, E. A., Shoemaker, D. D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J. D., Bussey, H., Chu, A. M., Connelly, C., Davis, K., Dietrich, F., Dow, S. W., El Bakkoury, M., Foury, F., Friend, S. H., Gentalen, E., Giaever, G., Hegemann, J. H., Jones, T., Laub, M., Liao, H., Liebundguth, N., Lockhart, D. J., Lucau-Danila, A., Lussier, M., M'Rabet, N., Menard, P., Mittmann, M., Pai, C., Rebischung, C., Revuelta, J. L., Riles, L., Roberts, C. J., Ross-MacDonald, P., Scherens, B., Snyder, M., Sookhai-Mahadeo, S., Storms, R. K., Véronneau, S., Voet, M., Volckaert, G., Ward, T. R., Wysocki, R., Yen, G. S., Yu, K., Zimmermann, K., Philippsen, P., Johnston, M., and Davis, R. W. (1999) Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science 285*, 901−906.

(12) Giaever, G., Chu, A. M., Ni, L., Connelly, C., Riles, L., Veronneau, S., Dow, S., Lucau-Danila, A., Anderson, K., Andre, B., Arkin, A. P., Astromoff, A., El Bakkoury, M., Bangham, R., Benito, R., Brachat, S., Campanaro, S., Curtiss, M., Davis, K., Deutschbauer, A., Entian, K.-D., Flaherty, P., Foury, F., Garfinkel, D. J., Gerstein, M., Gotte, D., Guldener, U., Hegemann, J. H., Hempel, S., Herman, Z., Jaramillo, D. F., Kelly, D. E., Kelly, S. L., Kotter, P., LaBonte, D., Lamb, D. C., Lan, N., Liang, H., Liao, H., Liu, L., Luo, C., Lussier, M., Mao, R., Menard, P., Ooi, S. L., Revuelta, J. L., Roberts, C. J., Rose, M., Ross-Macdonald, P., Scherens, B., Schimmack, G., Shafer, B., Shoemaker, D. D., Sookhai-Mahadeo, S., Storms, R. K., Strathern, J. N., Valle, G., Voet, M., Volckaert, G., Wang, C.-y., Ward, T. R., Wilhelmy, J.,

Winzeler, E. A., Yang, Y., Yen, G., Youngman, E., Yu, K., Bussey, H., Boeke, J. D., Snyder, M., Philippsen, P., Davis, R. W., and Johnston, M. (2002) Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature 418*, 387−391.

(13) Tong, A. H. Y., Evangelista, M., Parsons, A. B., Xu, H., Bader, G. D., Pagé, N., Robinson, M., Raghibizadeh, S., Hogue, C. W. V., Bussey, H., Andrews, B., Tyers, M., and Boone, C. (2001) Systematic genetic analysis with ordered arrays of yeast deletion mutants. *Science 294*, 2364−2368.

(14) Forsburg, S. L. (2001) The art and design of genetic screens: Yeast. *Nat. Rev. Genet. 2*, 659−668.

(15) Scherens, B., and Goffeau, A. (2004) The uses of genome-wide yeast mutant collections. *Genome Biol., 5*.

(16) van Dijken, J. P., Bauer, J., Brambilla, L., Duboc, P., Francois, J. M., Gancedo, C., Giuseppin, M. L. F., Heijnen, J. J., Hoare, M., Lange, H. C., Madden, E. A., Niederberger, P., Nielsen, J., Parrou, J. L., Petit, T., Porro, D., Reuss, M., van Riel, N., Rizzi, M., Steensma, H. Y., Verrips, C. T., Vindeløv, J., and Pronk, J. T. (2000) An interlaboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains. *Enzyme Microb. Technol. 26*, 706−714.

(17) Arnold, F. H. (1998) Design by directed evolution. *Acc. Chem. Res. 31*, 125−131.

(18) Cobb, R. E., Si, T., and Zhao, H. (2012) Directed evolution: An evolving and enabling synthetic biology tool. *Curr. Opin. Chem. Biol. 16*, 285−291.

(19) Berns, K., Hijmans, E. M., Mullenders, J., Brummelkamp, T. R., Velds, A., Heimerikx, M., Kerkhoven, R. M., Madiredjo, M., Nijkamp, W., Weigelt, B., Agami, R., Ge, W., Cavet, G., Linsley, P. S., Beijersbergen, R. L., and Bernards, R. (2004) A large-scale RNAi screen in human cells identifies new components of the p53 pathway. *Nature 428*, 431−437.

(20) Echeverri, C. J., and Perrimon, N. (2006) High-throughput RNAi screening in cultured cells: A user's guide. *Nat. Rev. Genet. 7*, 373−384.

(21) Fire, A., Xu, S. Q., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature 391*, 806−811.

(22) Hannon, G. J. (2002) RNA interference. *Nature 418*, 244−251.

(23) Boutros, M., and Ahringer, J. (2008) The art and design of genetic screens: RNA interference. *Nat. Rev. Genet. 9*, 554−566.

(24) Drinnenberg, I. A., Weinberg, D. E., Xie, K. T., Mower, J. P., Wolfe, K. H., Fink, G. R., and Bartel, D. P. (2009) RNAi in budding yeast. *Science 326*, 544−550.

(25) Suk, K., Choi, J., Suzuki, Y., Ozturk, S. B., Mellor, J. C., Wong, K. H., MacKay, J. L., Gregory, R. I., and Roth, F. P. (2011) Reconstitution of human RNA interference in budding yeast. *Nucleic Acids Res. 39*, e43.

(26) Crook, N. C., Schmitz, A. C., and Alper, H. S. (2013) Optimization of a yeast RNA interference system for controlling gene expression and enabling rapid metabolic engineering. *ACS Synth. Biol.*, DOI: 10.1021/sb4001432.

(27) Cheng, X., and Jian, R. (2010) Construction and application of random dsRNA interference library for functional genetic screens in embryonic stem cells. *Methods Mol. Biol. 650*, 65−74.

(28) Wellinger, R. J., and Zakian, V. A. (2012) Everything you ever wanted to know about *Saccharomyces cerevisiae* telomeres: Beginning to end. *Genetics 191*, 1073−1105.

(29) Kanoh, J., and Ishikawa, F. (2003) Composition and conservation of the telomeric complex. *Cell. Mol. Life Sci. 60*, 2295−2302.

(30) Addinall, S. G., Holstein, E. M., Lawless, C., Yu, M., Chapman, K., Banks, A. P., Ngo, H. P., Maringele, L., Taschuk, M., Young, A., Ciesiolka, A., Lister, A. L., Wipat, A., Wilkinson, D. J., and Lydall, D. (2011) Quantitative fitness analysis shows that NMD proteins and many other protein complexes suppress or enhance distinct telomere cap defects. *PLoS Genet. 7*, e1001362.

(31) Vandre, C. (2008) Molecular and genetic analysis of the role of Ku in silencing and telomere function in *S. cerevisiae*. In *Cell and*

Developmental Biology, University of Illinois at Urbana−Champaign, Urbana.

(32) Jackson, A. L., Bartz, S. R., Schelter, J., Kobayashi, S. V., Burchard, J., Mao, M., Li, B., Cavet, G., and Linsley, P. S. (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol. 21*, 635−637.

(33) Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O'Shea, E. K. (2003) Global analysis of protein localization in budding yeast. *Nature 425*, 686−691.

(34) Margeot, A., Hahn-Hagerdal, B., Edlund, M., Slade, R., and Monot, F. (2009) New improvements for lignocellulosic ethanol. *Curr. Opin. Biotechnol. 20*, 372−380.

(35) Olsson, L., and HahnHagerdal, B. (1996) Fermentation of lignocellulosic hydrolysates for ethanol production. *Enzyme Microb. Technol. 18*, 312−331.

(36) Mira, N. P., Teixeira, M. C., and Sa-Correia, I. (2010) Adaptive response and tolerance to weak acids in *Saccharomyces cerevisiae*: A genome-wide view. *OMICS: J. Integr. Biol. 14*, 525−540.

(37) Mira, N., Palma, M., Guerreiro, J., and Sa-Correia, I. (2010) Genome-wide identification of *Saccharomyces cerevisiae* genes required for tolerance to acetic acid. *Microb. Cell Fact. 9*, 79.

(38) Drinnenberg, I. A., Fink, G. R., and Bartel, D. P. (2011) Compatibility with Killer explains the rise of RNAi-deficient fungi. *Science 333*, 1592−1592.

(39) Hebert, C. G., Valdes, J. J., and Bentley, W. E. (2008) Beyond silencing—Engineering applications of RNA interference and antisense technology for altering cellular phenotype. *Curr. Opin. Biotechnol. 19*, 500−505.

(40) Dorsett, Y., and Tuschl, T. (2004) siRNAs: Applications in functional genomics and potential as therapeutics. *Nat. Rev. Drug Discovery 3*, 318−329.

(41) Le Borgne, S. (2012) Genetic engineering of industrial strains of *Saccharomyces cerevisiae*. In *Recombinant Gene Expression: Reviews and Protocols*, 3rd edition (Lorence, A., Ed.), pp 451−465, Humana Press Inc, Totowa.

(42) Birmingham, A., Anderson, E. M., Reynolds, A., Ilsley-Tyree, D., Leake, D., Fedorov, Y., Baskerville, S., Maksimova, E., Robinson, K., Karpilow, J., Marshall, W. S., and Khvorova, A. (2006) 3′ UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nat. Methods 3*, 199−204.

(43) Ma, Y., Creanga, A., Lum, L., and Beachy, P. A. (2006) Prevalence of off-target effects in *Drosophila* RNA interference screens. *Nature 443*, 359−363.

(44) Bumcrot, D., Manoharan, M., Koteliansky, V., and Sah, D. W. Y. (2006) RNAi therapeutics: A potential new class of pharmaceutical drugs. *Nat. Chem. Biol. 2*, 711−719.

(45) Shendure, J., and Aiden, E. L. (2012) The expanding scope of DNA sequencing. *Nat. Biotechnol. 30*, 1084−1094.

(46) Horn, T., Sandmann, T., and Boutros, M. (2010) Design and evaluation of genome-wide libraries for RNA interference screens. *Genome Biol.*, 11.

(47) Storici, F., Durham, C. L., Gordenin, D. A., and Resnick, M. A. (2003) Chromosomal site-specific double-strand breaks are efficiently targeted for repair by oligonucleotides in yeast. *Proc. Natl. Acad. Sci. U.S.A. 100*, 14994−14999.

(48) Urnov, F. D., Rebar, E. J., Holmes, M. C., Zhang, H. S., and Gregory, P. D. (2010) Genome editing with engineered zinc finger nucleases. *Nat. Rev. Genet. 11*, 636−646.

(49) Sun, N., Liang, J., Abil, Z., and Zhao, H. M. (2012) Optimized TAL effector nucleases (TALENs) for use in treatment of sickle cell disease. *Mol. Biosyst. 8*, 1255−1263.

(50) Joung, J. K., and Sander, J. D. (2013) INNOVATION TALENs: a widely applicable technology for targeted genome editing. *Nat. Rev. Mol. Cell Biol. 14*, 49−55.

(51) Cong, L., Ran, F. A., Cox, D., Lin, S. L., Barretto, R., Habib, N., Hsu, P. D., Wu, X. B., Jiang, W. Y., Marraffini, L. A., and Zhang, F. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science 339*, 819−823.

(52) DiCarlo, J. E., Norville, J. E., Mali, P., Rios, X., Aach, J., and Church, G. M. (2013) Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. *Nucleic Acids Res. 41*, 4336−4343.

(53) Almeida, B., Ohlmeier, S., Almeida, A. J., Madeo, F., Leão, C., Rodrigues, F., and Ludovico, P. (2009) Yeast protein expression profile during acetic acid-induced apoptosis indicates causal involvement of the TOR pathway. *Proteomics 9*, 720−732.

(54) Catala, M., Aksouh, L., and Abou Elela, S. (2012) RNA-dependent regulation of the cell wall stress response. *Nucleic Acids Res. 40*, 7507−7517.

(55) Mira, N. P., Teixeira, M. C., and Sa-Correia, I. (2010) Adaptive response and tolerance to weak acids in *Saccharomyces cerevisiae*: A genome-wide view. *OMICS: J. Integr. Biol. 14*, 525−540.

(56) Schmelzle, T., Helliwell, S. B., and Hall, M. N. (2002) Yeast protein kinases and the RHO1 exchange factor TUS1 are novel components of the cell integrity pathway in yeast. *Mol. Cell. Biol. 22*, 1329−1339.

(57) Kawahata, M., Masaki, K., Fujii, T., and Iefuji, H. (2006) Yeast genes involved in response to lactic acid and acetic acid: acidic conditions caused by the organic acids in *Saccharomyces cerevisiae* cultures induce expression of intracellular metal metabolism genes regulated by Aft1p. *FEMS Yeast Res. 6*, 924−936.

(58) Costanzo, M., Baryshnikova, A., Bellay, J., Kim, Y., Spear, E. D., Sevier, C. S., Ding, H., Koh, J. L. Y., Toufighi, K., Mostafavi, S., Prinz, J., St. Onge, R. P., VanderSluis, B., Makhnevych, T., Vizeacoumar, F. J., Alizadeh, S., Bahr, S., Brost, R. L., Chen, Y., Cokol, M., Deshpande, R., Li, Z., Lin, Z.-Y., Liang, W., Marback, M., Paw, J., San Luis, B.-J., Shuteriqi, E., Tong, A. H. Y., van Dyk, N., Wallace, I. M., Whitney, J. A., Weirauch, M. T., Zhong, G., Zhu, H., Houry, W. A., Brudno, M., Ragibizadeh, S., Papp, B., Pál, C., Roth, F. P., Giaever, G., Nislow, C., Troyanskaya, O. G., Bussey, H., Bader, G. D., Gingras, A.-C., Morris, Q. D., Kim, P. M., Kaiser, C. A., Myers, C. L., Andrews, B. J., and Boone, C. (2010) The genetic landscape of a cell. *Science 327*, 425−431.

(59) Mira, N. P., Palma, M., Guerreiro, J. F., and Sa-Correia, I. (2010) Genome-wide identification of *Saccharomyces cerevisiae* genes required for tolerance to acetic acid. *Microb. Cell Fact. 9*, 79.

(60) Chan, C. T. Y., Pang, Y. L. J., Deng, W., Babu, I. R., Dyavaiah, M., Begley, T. J., and Dedon, P. C. (2012) Reprogramming of tRNA modifications controls the oxidative stress response by codon-biased translation of proteins. *Nat. Commun. 3*, 937.

(61) Preston, M. A., D'Silva, S., Kon, Y., and Phizicky, E. M. (2012) tRNAHis 5-methylcytidine levels increase in response to several growth arrest conditions in *Saccharomyces cerevisiae*. *RNA 19*, 1−14.

(62) Breslow, D. K., Cameron, D. M., Collins, S. R., Schuldiner, M., Stewart-Ornstein, J., Newman, H. W., Braun, S., Madhani, H. D., Krogan, N. J., and Weissman, J. S. (2008) A comprehensive strategy enabling high-resolution functional analysis of the yeast genome. *Nat. Methods 5*, 711−718.

(63) Na, D., Yoo, S. M., Chung, H., Park, H., Park, J. H., and Lee, S. Y. (2013) Metabolic engineering of *Escherichia coli* using synthetic small regulatory RNAs. *Nat. Biotechnol. 31*, 170−174.

(64) Shao, Z., Zhao, H., and Zhao, H. (2009) DNA assembler, an *in vivo* genetic method for rapid construction of biochemical pathways. *Nucleic Acids Res. 37*, e16.

(65) Gietz, R. D., and Schiestl, R. H. (2007) High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc. 2*, 31−34.