TALENs-Assisted Multiplex Editing for Accelerated Genome Evolution To Improve Yeast Phenotypes

Guoqiang Zhang,[†,‡] Yuping Lin,[†] Xianni Qi,[†] Lin Li,[†] Qinhong Wang,*[,†] and Yanhe Ma[†]

[†]Key Laboratory of Systems Microbial Biotechnology, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China

[‡]University of Chinese Academy of Sciences, Beijing 100049, China

S *Supporting Information*

ABSTRACT: Genome editing is an important tool for building novel genotypes with a desired phenotype. However, the fundamental challenge is to rapidly generate desired alterations on a genome-wide scale. Here, we report TALENs (transcription activator-like effector nucleases)-assisted multiplex editing (TAME), based on the interaction of designed TALENs with the DNA sequences between the critical TATA and GC boxes, for generating multiple targeted genomic modifications. Through iterative cycles of TAME to induce abundant semirational *indels* coupled with efficient screening using a reporter, the targeted fluorescent trait can be continuously and rapidly improved by accumulating multiplex beneficial genetic modifications in the evolving yeast genome. To further evaluate its efficiency, we also demonstrate the application of TAME for significantly improving ethanol tolerance of yeast in a short amount of time. Therefore, TAME is a broadly generalizable platform for accelerated genome evolution to rapidly improve yeast phenotypes.

Multiplex Engineering for phenotypic diversity

KEYWORDS: *transcription activator-like effector nucleases, multiplex editing, genome evolution, cellular phenotypes*
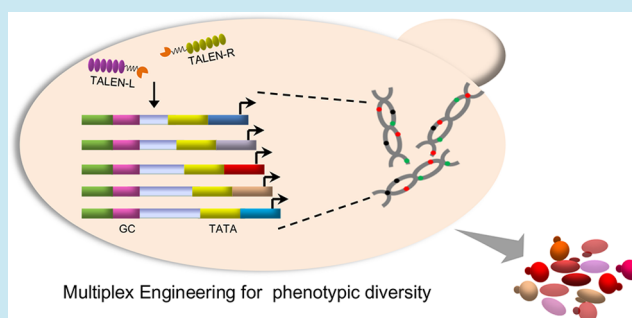
Usually, cellular phenotypes involve synergistic actions of different genes that manifest as layered gene regulatory networks and specific expression programs. The major layers consist of transcriptional regulatory elements that can integrate multiple signals and perform sophisticated, combinatorial functions during transcription.[1,2] Combinatorial regulation of transcription has several advantages, such as controlling gene expression in response to a variety of environmental signals and employing a limited number of transcriptional regulatory elements to create many combinations of regulators that are modulated by diverse sets of conditions.[3] Thus, platforms for scalable, tunable, and synergetic modulation of transcriptional regulatory elements would be capable of generating diverse phenotypes and thereby enable faster directed evolution of living cells under specific selective conditions.[4]

Traditional random mutagenesis, metabolic engineering, and adaptive evolution have created genetic variants with usefully altered phenotypes.[5−8] However, these methods are laborious, relatively inefficient, and difficult to use in parallel and for continuous modification of specific gene networks or genomes on short time scales. Recently, breakthroughs in engineering nucleases, such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guided DNA endonucleases, have offered new opportunities for multiplex engineering strategies at the genome

level aimed at allowing the evolution of biological systems.[9,10] Multiplex genome editing is performed with multiple modifications on whole genomes, thereby potentially uncovering new or greatly improved phenotypes with beneficial properties. TALENs are nonspecific DNA-cleaving nucleases fused to DNA-binding domains with a series of tandem repeats that can be easily engineered to target essentially any DNA sequence and generate desirable modifications in a wide range of cell types and organisms.[11,12] The ability of TALENs to quickly and efficiently alter DNA sequences promises to profoundly impact biological research and multiplex genome editing. Hence, multiplex genome editing based on specifically designed TALENs could be a powerful tool to induce multiple targeted modifications, thereby obtaining desired phenotypes.

Here, we report a new TALENs-assisted multiplex editing (TAME) toolbox, based on multiplex interactions of TALENs with the DNA sequences between the critical and conserved TATA box (TATAAA) and GC box (GGGCGG), developed after functional analysis of different TALENs and genomic sequence mapping via a self-programmed script. We use this toolbox to induce multiple modifications that improve the complex phenotypes of *Saccharomyces cerevisiae* rapidly and efficiently. Furthermore, we explore the prospects for extending efficient and semirational genome editing and discuss the
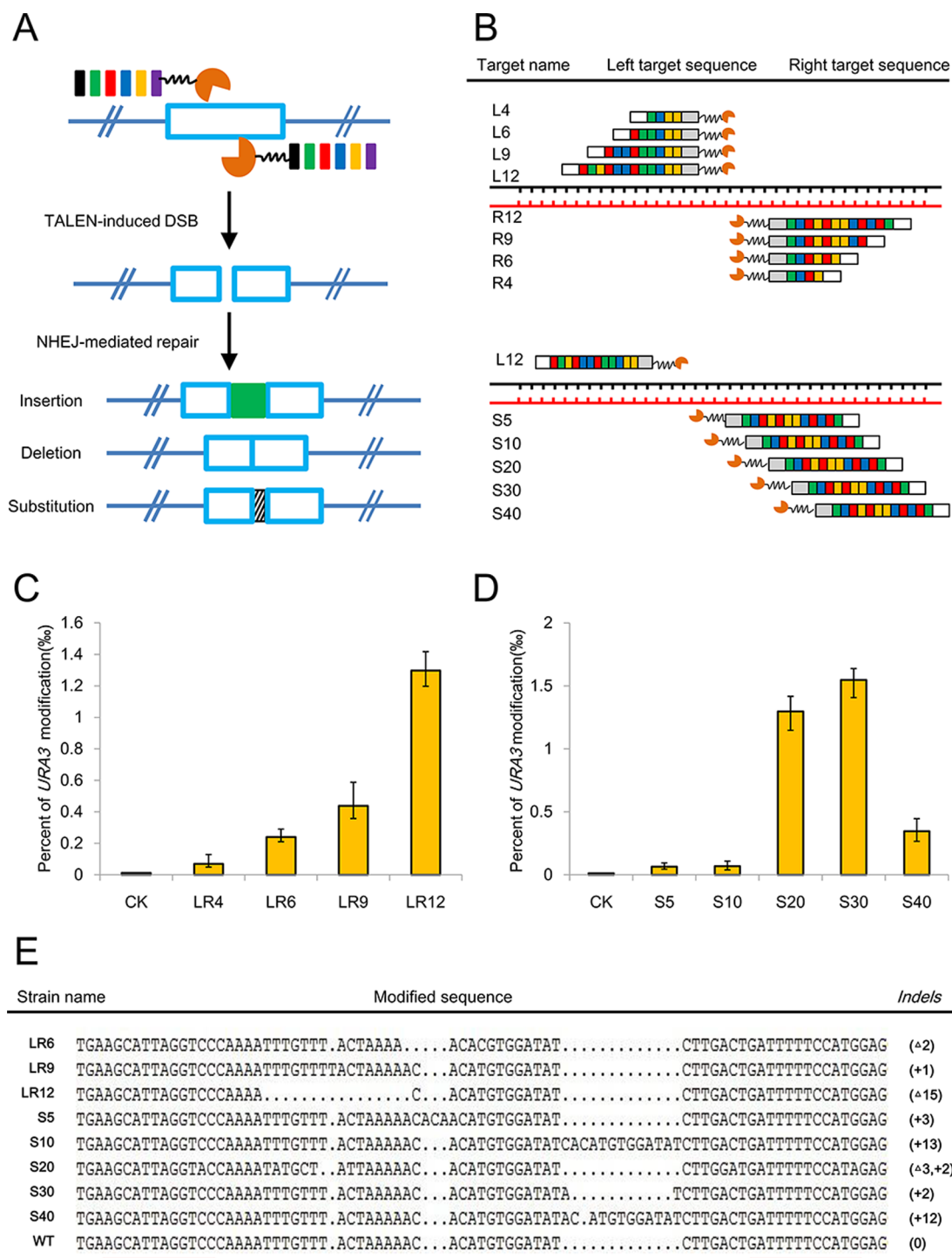
Figure 1. Evaluation and application of TALENs in *S. cerevisiae*. (A) Diagram of TALENs-induced double-strand breaks and their repair by NHEJ. (B) Diagram of the design of different TALENs used to investigate site-directed modification efficiency. L and R represent the left and right TALE binding sites, respectively, and S represents the spacer length of the TALENs. The numbers indicate the binding length or spacer length of the sequences. (C) Effect of the number of TALE repeats on *URA3* modification. (D) Effect of the TALENs spacer length on *URA3* modification. (E) *Indels* generated in the targeted region of *URA3* by different TALENs. The underlined red nucleotides represent the binding sites for *URA3* editing. All data are from at least three biological replicates and are shown as the mean ± SD.

application of continued advances toward the development of a flexibly programmable yeast chassis. Our study demonstrates a powerful and efficient TALENs-mediated toolbox for achieving multiplex genome modifications and generating genetic diversity, thereby improving complex phenotypes of living cells in short amounts of time.

■ RESULTS AND DISCUSSION

Evaluation of the Efficiency of TALENs-Mediated Targeted Mutagenesis in *Saccharomyces cerevisiae*. In living cells, TALENs-induced double-strand breaks (DSB) can create random sequence alterations, such as insertions, deletions, or nucleotide substitutions (collectively termed *indels*),
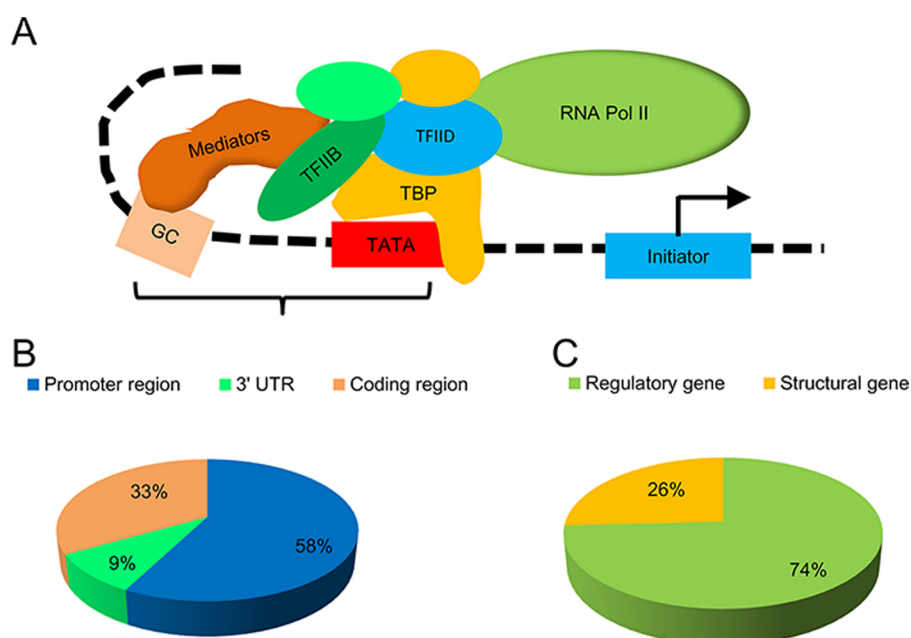
Figure 2. Potential TALENs binding sites containing the targeted GGGCGG and TATAAA sequences and related genes. (A) RNA polymerase II core promoter element. (B) Location analysis of sites containing GGGCGG and TATAAA sequences. (C) Functional analysis of related genes.

through NHEJ (nonhomologous end joining)-mediated repair (Figure 1A).[13] To achieve precise genome modifications at the targeted site, TALEs (transcription activator-like effectors) designed to recognize 12−18 bp specific sequences separated by a 13−22 bp spacer were typically required in previous studies.[14,15] However, TALENs containing 12−18 TALE repeats theoretically have very few chances to be functional at multiple sites in the genome.[16] Thus, to introduce modifications at multiple sites in a genome, TALE repeats against short sequences (e.g., less than 12 bp) would be needed.

Here, the efficiency of TALENs-mediated targeted mutagenesis induced by specific short sequences (4−12 bp) with different spacer lengths (5−40 bp) was first assessed by targeting the *URA3* gene (Figure S1, Supporting Information) and measuring 5-fluoroorotic acid (5-FOA) resistance in *S. cerevisiae* (Figure S2, Supporting Information). Cells with mutated and nonfunctional *URA3* can grow on 5-FOA plates, whereas cells with functional *URA3* are unable to grow.[17] When separately using TALEN pairs targeting specific 4, 6, 9, and 12 bp sequences with a fixed 22 bp spacer length, the TALEN pair with 12 TALE repeats showed the highest rate of site-specific mutagenesis in the *URA3* gene (Figure 1C). The rate of site-specific mutagenesis decreased as the number of TALE repeats was reduced. When using TALEN pairs with a fixed number of TALE repeats (12 repeats) and different spacer lengths (5, 10, 20, 30, and 40 bp), the TALEN pairs with 20 and 30 bp spacer lengths showed significantly higher rates of site-specific mutagenesis than those with shorter or longer spacers, consistent with previous studies.[15] Sequencing the possible mutations further proved that TALENs could play a role in site-specific modification and generate diverse *indels* when different TALE binding sequences are used (Figure 1E). For the purpose of inducing targeted multiplex editing in the genome, these results suggested that TALEN pairs targeting specific short sequences could be promising even though the modification efficiency might be not optimal.

To identify all possible TALE binding targets in yeast, we mapped the yeast genome with fixed sequence pairs of 4−9 bp

with 0−50 bp spacer lengths using a self-programmed script. The results showed that the number of TALE binding sites with more than 6 bp was limited and not suitable for multiplex editing. Thus, in this study, binding site selection and analysis based on 6 bp sequences with less than a 50 bp spacer length was used to design possible modifications through multiple genomic targeting.

Genome-Wide Computational Analysis and Selection of Multiple TALENs Modification Sites. As the canonical binding site for transcriptional binding proteins in eukaryotes, the conservative TATA box is related to the interaction between RNA polymerase and DNA and affects the transcriptional expression of about 20% of genes, which are associated with responses to stress and are highly regulated.[18−20] Moreover, the GC-rich box is another distinct pattern of nucleotides found in some eukaryotic promoter regions upstream of the TATA box. It has the consensus sequence GGGCGG and a similar function as an enhancer.[21] Therefore, altering the genomic region between TATA and GC elements at multiple loci could produce various combinations of gene expression and result in a diversity of phenotypes.[22] Thus, to develop the strategy for multiplex genome editing, these two conserved *cis*-acting regulatory elements were selected to form a complete TALEN binding site (Figure 2A).

The consensus sequence of the GC box, GGGCGG, and that of the TATA box, TATAAA, separated by 0−50 bp spacers were mapped against the *S. cerevisiae* S288c genome to identify potential TALENs-mediated modification sites using our self-programmed script. A total of 66 potential modification sites were identified, probably involving 98 genes (Supporting Information data set S1). Among these sites, more than half (about 58%) were located in promoter regions (Figure 2C). In addition, 9 and 33% of sites were located in 3′-untranslated regions (3′-UTR) and coding regions, respectively. Among the 98 related genes whose expression might be altered by TALENs-mediated modifications, 74% of these genes encode regulators of gene expression, such as transcription factors involved in response to environmental nutrients or stress (Figure 2B and
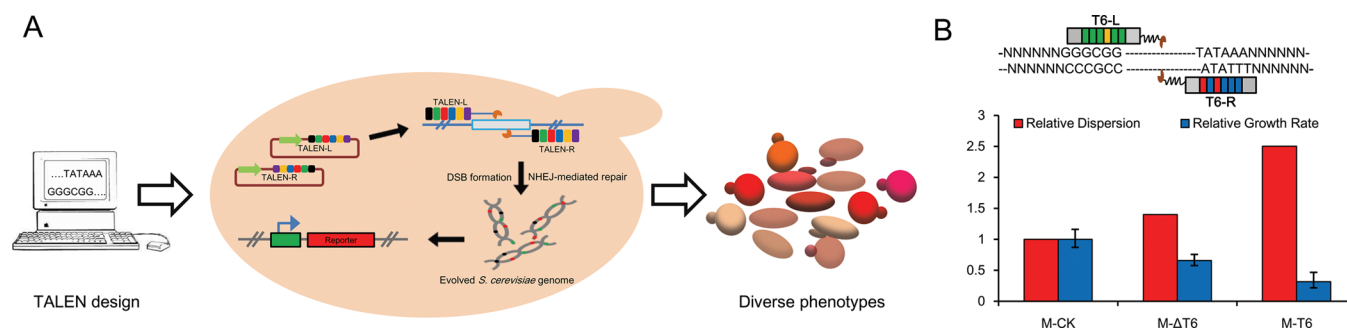
Figure 3. TALENs-assisted multiplex editing. (A) Diagram of TALENs-assisted multiplex editing (TAME) for fluorescence diversity in yeast. The double-strand breaks (DSB) induced by TALENs at defined loci can be repaired by nonhomologous end-joining (NHEJ). NHEJ-mediated repair leads to the introduction of variable length insertion, deletion, or substitution (*indels*) mutations and generates semirational genome disturbances. The *mCherry* expression cassette was constructed as a monitor to reflect the alteration of chassis cells. (B) Top: A pair of TALENs designed and constructed based on TATAAA and GGGCGG in the *S. cerevisiae* S288c genome. Bottom: Fluorescence dispersion and relative growth rate of populations M-CK, M-ΔT6, and M-T6. The relative dispersion was analyzed by flow cytometry, and variation is indicated by standard deviation (SD).

Supporting Information data set S1). For example, *MSN2* encodes a transcription activator that plays a major role in yeast general stress response by regulating gene activation and repression,[23] *YAP5* encodes an iron-responsive activator,[24] and *MET28* encodes a transcription activator of genes in sulfur-containing amino acid metabolism.[25] These *in silico* analyses suggest that simultaneous TALENs-induced modifications of multiple sites containing both GGGCGG and TATAAA sequences with a 0−50 bp spacer could potentially generate genetic diversity on a genomic scale.

Design of TALENs-Assisted Multiplex Editing. On the basis of *in silico* analysis of potential TALENs-mediated modification sites containing both GGGCGG and TATAAA sequences with a 0−50 bp spacer, we developed a novel genome editing tool kit, termed TALENs-assisted multiplex editing (TAME). The core idea of TAME is to apply designed TALENs to introduce multiplexed genetic perturbations into the genome. The typical flowchart of TAME is described in Figure 3A. First, on the basis of sequence mapping against a whole genome using a custom script, the possible TALENs modification sites containing a pair of specific short TALE binding sequences separated by spacers within a certain range are identified and located to assess the practicality of multiplex genome editing. Here, the GGGCGG and TATAAA sequences with 0−50 bp spacers, which had dozens of locations in the yeast genome, were selected to design a pair of TALENs. Then, the designed TALENs that could recognize multiple sites in the genome are introduced into yeast cells, where they trigger multiplex genome editing by generating *indels* when the expression of the designed TALENs is under the control of a suitable inducible element, such as the *GAL1* promoter. The offspring cells would harbor various genomic modifications, generating diverse genotypes and thereby producing different phenotypes. Once coupled with efficient screening methods, cells with the desired phenotype could be rapidly sorted out from the diverse mutated cell library.

As a proof of concept, we first constructed a multiplex fluorescence-based phenotype library using TAME in *S. cerevisiae* BY4741 as a model based on the interaction of TALENs with the genomic region between GGGCGG (T6-L) and TATAAA (T6-R) to induce genomic modification at multiple sites (Figure 3B, top); the inducible *GAL1* promoter was used to optimize the expression of the TALENs proteins. To establish an efficient screening method, the fluorescent protein mCherry, expressed from a plasmid, was used as a reporter to detect the phenotypic diversity of the cells. The fluorescence diversity of cells treated

with or without TAME was evaluated using flow cytometry (FCM) and a microplate reader. The TAME-treated cell library named M-T6 exhibited remarkable fluorescence diversity (Figure S3, Supporting Information). Compared to the control population (M-CK) without TAME treatment, M-T6 showed a 2.5-fold increase in the relative dispersion of fluorescence values (Figure 3B, bottom). In addition, no mutation occurred in the *mCherry*-containing reporter plasmid by DNA sequencing. The reporter plasmids isolated from the M-T6 cell library were further retransformed into a new control strain. The resulting transformants did not show variations in fluorescence (Figure S4, Supporting Information).

It has been reported that introduced DNA-binding proteins, such as artificial transcription factors[26,27] or a catalytically dead Cas9 mutant,[28] may modulate gene expression by interfering with transcriptional initiation, elongation, RNA polymerase binding, or transcription factor binding, hence inducing phenotypic variations. In order to distinguish between the effect of DNA-binding protein TALE on gene expression and that of TALEN on genome editing, which may contribute to phenotype diversity, negative control TALEN plasmids with inactive nuclease Δ*Fok*I were constructed in parallel and cotransformed with the *mCherry*-containing reporter plasmid into BY4741, resulting in strain M-ΔT6. FCM analysis showed that M-ΔT6 had a slight diversity, with a 40% increase in the relative dispersion of fluorescence values (Figure 3B and Figure S3, Supporting Information). Therefore, the fluorescence diversity of TAME-treated cells should mainly result from alterations of the cellular chassis induced by TALENs at multiple sites of the chromosomal DNA. Additionally, cell library M-T6 showed significantly inhibited cell growth under TALENs' toxicity during the TAME process. By contrast, cell library M-ΔT6 showed a slight inhibition of cell growth, which might result from the disturbance of gene expression by TALE protein (Figure 3B). Taken together, TAME-mediated genome modification may be playing the major role in the induced phenotypic diversity, although TALE DNA-binding protein might also contribute to the phenotypic diversity to an extent. Therefore, TAME successfully created genetic diversity by inducing multiplex genome disturbances and may be applied to accelerate genome evolution for improving a desired phenotype.

Accelerated Genome Evolution To Improve Phenotypes via TAME. On the basis of the multiplex modification of TAME, accelerated genome evolution to accumulate favorable mutations may be achieved under the appropriate selective
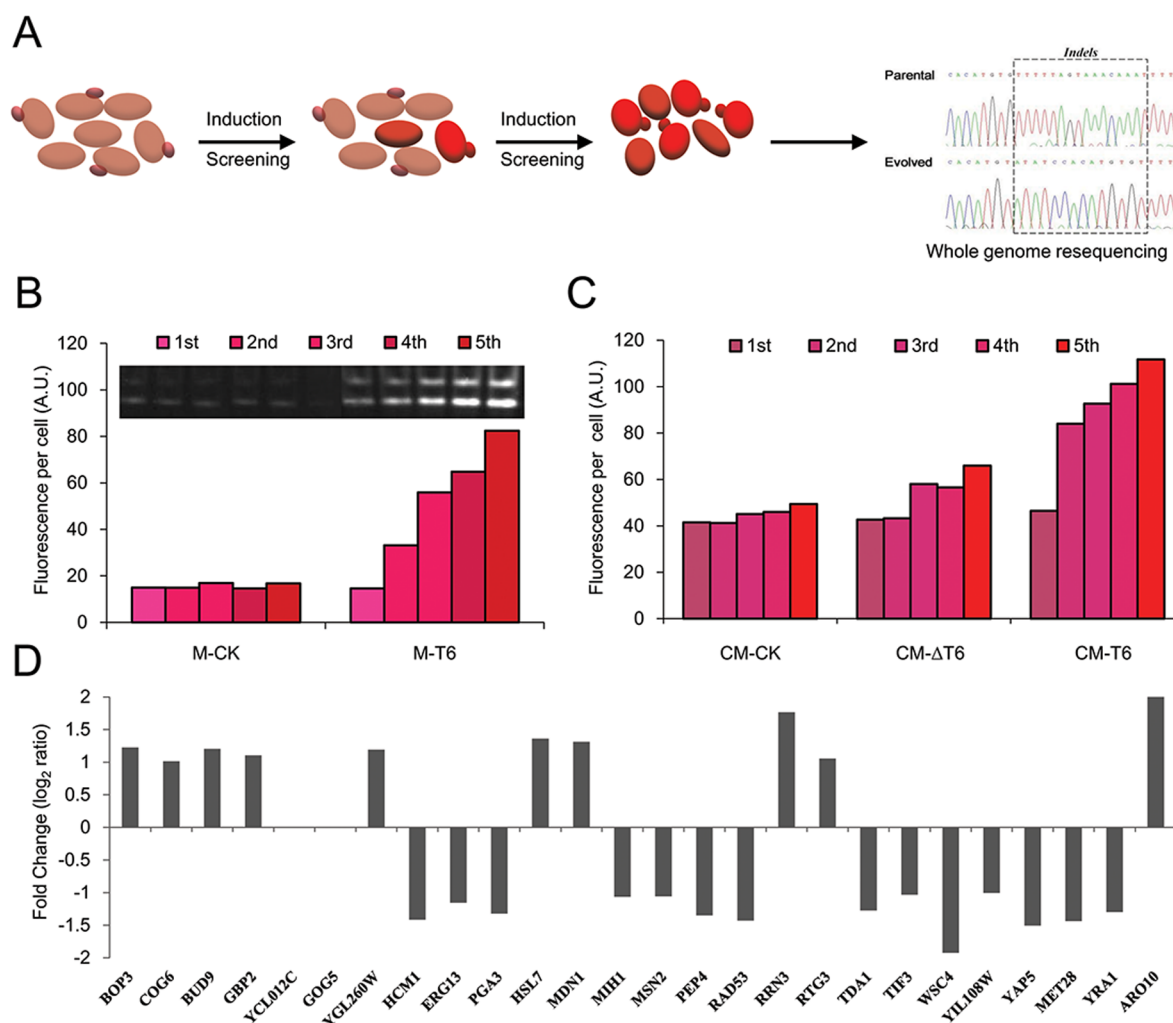
Figure 4. Accelerated fluorescent phenotype evolution of different yeast strains treated with TAME. (A) Flow diagram of the accelerated fluorescent phenotype evolution by TAME. (B) Successive passage of evolving BY4741 strains with and without TAME. The eight yeast clones with high relative fluorescent values were selected for the next passage, and the fluorescent value was the average value of the eight strains. The mCherry protein expression level was quantitatively analyzed by native gel electrophoresis and a ChemiDoc XRS system. (C) Successive passage of evolving CEN.PK-1C strains under TAME. CM-ΔT6 represents strains that carried the TALENs with inactive nuclease *Fok*I. (D) Transcriptional profiling of modified genes in the evolved strain M-T6-5. M-CK: control strain population with the fluorescent reporter. M-T6: population with the fluorescent reporter and TALEN plasmids pYES2/CT-GC/p313-GAL-TA. M-T6-5: strain with the highest fluorescence value in the M-T6 population after passaging five times.

conditions by enriching for cells having the desired phenotype in diverse cell populations. To confirm this feasibility, *S. cerevisiae* BY4741 cells expressing the *mCherry* reporter and TALENs were cultivated and induced to generate a diverse mutant library as a first generation. Then, eight yeast clones showing the highest levels of red fluorescence were selected from about 200 clones for the next round of induction and selection. This procedure was repeated for five cycles, as shown in the flow diagram (Figure 4A). The fluorescence intensities of cell populations harboring the designed TALEN pair increased steadily and rapidly over five generations of fluorescence selection, whereas the control cells without the designed TALEN pair showed almost unchanged fluorescence intensities during the passage process (Figure 4B, bottom panel). At the end, the fifth-generation cell population with the designed TALEN pair showed about a 5.7-fold increase in fluorescence intensities compared to those of the control cells lacking the designed TALEN pair as well as those of the first generation of TAME-treated cells. Semiquantitative analysis of mCherry by in-gel fluorescence further confirmed that the protein expression of mCherry monomers and aggregates was

increased in the TAME-treated cells through the successive induction of TAME and fluorescence screening (Figure 4B, top panel). Since the genomic and phenotypic variation among *S. cerevisiae* isolates has been discussed previously,[29,30] another *S. cerevisiae* strain, CEN.PK-1C, was applied toward the evolution of the fluorescent phenotype to determine if TAME is a general tool for genome modification and evolution. Strain CEN.PK-1C with *mCherry* showed greater than 2-fold higher fluorescence expression than that of strain BY4741 before TAME treatment (Figure 4B,C), which confirmed the genotypic variation between these two strains. After TAME treatment, the fifth-generation population of CM-T6 cells showed around a 2.4-fold increase in fluorescence intensities compared to those of the control cells lacking the designed TALEN pair as well as those of the first-generation population of TAME-treated cells. Although the increases in fluorescence via TAME in strain CEN.PK-1C were lower than those in strain BY4741, both strains showed a significant improvement in fluorescence expression after TAME treatment. In addition, CM-ΔT6, with inactive *Fok*I, showed a slight increase (about 1.5-fold) in fluorescence intensity
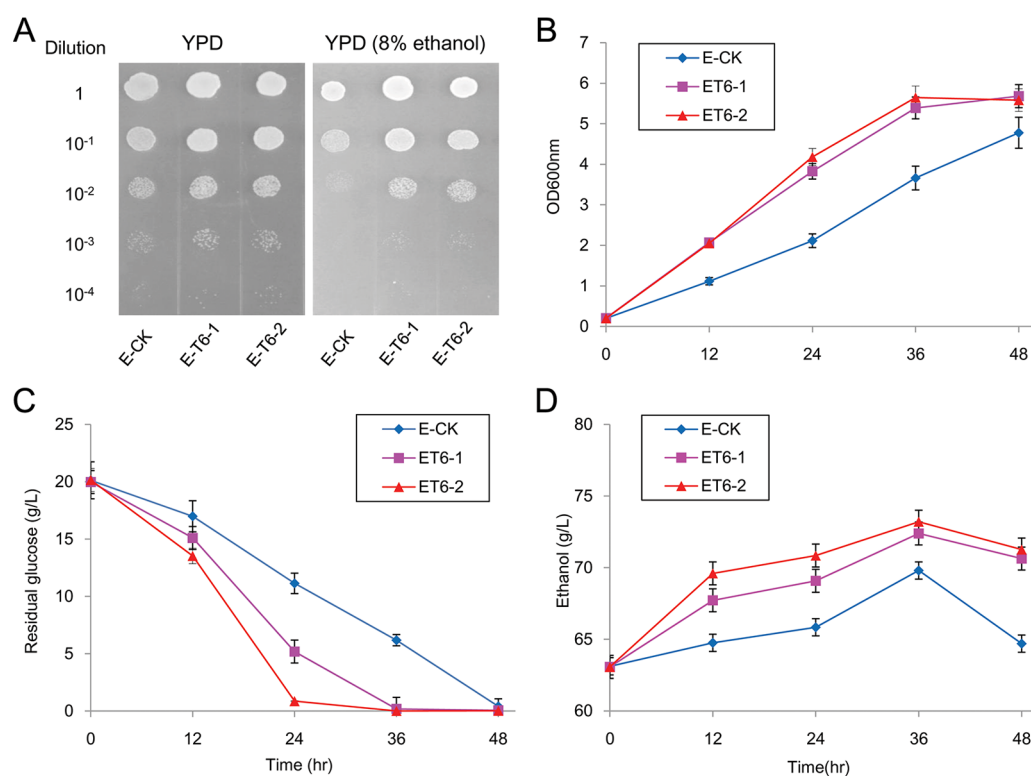
Figure 5. Accelerated evolution of ethanol tolerance in yeast via TAME. (A) Growth of wild-type E-CK and two parallel evolved strains (ET6-1 and ET6-2) evaluated on YPD plates with 8% ethanol. (B−D) Growth parameters of wild-type and evolved strains assessed in liquid YPD medium with 8% ethanol, including biomass, glucose consumption, and ethanol production. ET6 represents a yeast population evolved in an increasing concentration of ethanol assisted by T6, as described previously. The data represent the average values of three independent experiments with deviations varying between 5 and 10% of the mean.

compared to that of the control, CM-CK, which further confirmed that the TALE protein lacking genome editing activity might also contribute to the phenotypic improvement to an extent.

To uncover the underlying alterations during accelerated genome evolution via TAME, both whole-genome resequencing and transcriptional profiling by microarray were analyzed and compared between the control strain M-CK-5 (without TAME treatment) and the evolved strain M-T6-5 (with TAME treatment), which showed the highest fluorescence from the fifth cycle of selection. The whole-genome resequencing results showed that 18 sites (27.3%) of 66 preidentified potential modification sites involving 26 genes might be perturbed due to the introduction of the *indels* in the M-T6-5 strain (Supporting Information data set S2), suggesting that TAME could truly generate multiplex genome modifications. On the other hand, transcriptional profiling revealed that TAME-treated strain M-T6-5 exhibited differential expression of hundreds of genes in comparison with the wild-type strain (Figure S5, Supporting Information). The transcriptional reprogramming in the TAME-treated strain was quite broad, but it exhibited an enrichment in certain groups; in particular, 26 predefined genes were found to be modified in the TAME-treated evolved strain. Moreover, the expression levels of 24 of these 26 modified genes exhibited up- or downregulation of more than 1-fold (Figure 4C). Functional analysis revealed that these genes were mainly categorized in DNA transcription, RNA export, and protein synthesis (Supporting Information data set S2). For example, *RRN3*[31] and *MSN2*[23] encode essential transcription factors for DNA transcription; *YRA1*[32] and *GBP2*[33] are involved in mRNA export;

and *RTG3*,[34] *COG6*,[35] and *ARO10*[36] are involved in the regulation of protein synthesis and modification. Additionally, a total of 246 SNPs were found in evolved strain M-T6 by whole-genome resequencing (Supporting Information data set S3), which might also generate genomic disturbances and result in different impacts on genes expression. In general, transcriptional reprogramming of these genes might influence cell chassis on a global level, thereby indirectly increasing the expression of mCherry protein.

Usually, cost-effective ethanol fermentation using yeast requires tolerance to high concentrations of ethanol, which involves synergistic effects on different genes.[37,38] Therefore, it is necessary to efficiently generate multiplex modifications on a genome-wide scale in a realistic time frame. Since accelerated genome evolution via TAME seemed to be capable of building diverse and promising cellular chassis in a short amount of time, we sought to demonstrate that a model-guided approach combining TAME treatment and selective pressure could be applied to accelerate the evolution of complex traits such as ethanol tolerance. *S. cerevisiae* BY4741 with or without TAME treatment was selected as the starting population and transferred for five rounds in media containing increasing concentrations of ethanol. The results showed that the strains (E-T6) with TAME treatment manifested rapid adaptability to the high concentrations of ethanol in a short amount of time. However, the control strains remained suppressed by ethanol during the same passage process. To determine whether these evolved strains were superior to the control strains in terms of increased ethanol tolerance, the isolated wild-type and two evolved strains (named E-CK, ET6-1, and ET6-2, respectively) were transferred five

times in regular media without stress and then evaluated by measuring cell growth under conditions of 8% (v/v) ethanol (Figure 5A,B). Cell growth assays using solid and liquid media with ethanol showed that the evolved strains clearly grew faster than the control, indicating that the TAME-treated strains obtained an improved and stable trait of ethanol tolerance. Furthermore, the evolved strains also showed superiority with regard to glucose consumption and ethanol production under ethanol stress (Figure 5C,D). The control strain (E-CK) took 48 h to completely utilize 20 g/L glucose and maximally produced 6.7 g/L ethanol at 36 h. By contrast, the evolved ET6-1 and ET6-2 strains consumed 20 g/L glucose in 36 and 24 h and produced 9.3 and 10.1 g/L ethanol, respectively. We further resequenced and compared the whole genomes of evolved strain ET6-1 as well as control strain E-CK. The results showed that seven sites (10.6%) of a total of 66 potential TALENs modification sites contained the TALENs-induced *indels* (Supporting Information data set S4). These modifications could affect different genes encoding transcription polymerase, plasma membrane protein, transporter, etc. It has also been reported that the mechanism of ethanol tolerance is closely related to global transcription factors and the plasma membrane.[39,40] Overall, the TAME toolbox designed in this study could quickly improve the stress tolerance of industrial yeast by inducing multiplex genetic modifications on a genome-wide scale.

Future Considerations. Many innovative approaches for genome engineering, sometimes combined with classical strain engineering, have been developed, providing powerful platforms for multiple evolutions of complex phenotypes.[41−47] In this study, we established a new toolbox of TALENs-assisted multiplex editing (TAME) for accelerated genome evolution and successfully improved a yeast cell chassis with increased fluorescence expression and ethanol tolerance. TALENs is an emerging genome engineering method that has been broadly applied in eukaryotic organisms.[48,49] In recent studies, TALENs were functionally applied in *S. cerevisiae* to achieve effective knockout and replacement of individual genes.[15,50] However, no applications of TALENs have been reported for genome-wide multiplex editing. To our knowledge, this is the first report of TALENs designed to create multiplex and combinatorial mutations among 66 potential genomic loci in *S. cerevisiae*, which were defined by the binding sequences of a TALEN pair, including the enhancer-like GC box GGGCGG and the TATA box TATAAA. Therefore, TALENs-assisted multiplex editing could be potentially applied to accelerate the phenotypic evolution of living cells.

Since multiplex mutations during TAME treatment were created and accumulated through NHEJ (nonhomologous end joining)-mediated repair induced by TALENs, the efficiency of NHEJ repair system determines the efficiency of multiplex genome editing. Eukaryotes have evolved two distinct enzymatic DSBs repair approaches to maintain genomic integrity: homologous recombination (HR) and NHEJ. In yeast, the error-prone NHEJ efficiency is low. For example, the rate of NHEJ-based disruption of *ADE2*, *LYS2*, and *URA3* genes was in the range of 0.15−5.4%, whereas the efficiency of HR-based disruption of *URA3* was in the range of 4.5−27%.[15] Therefore, multiple rounds of TAME treatment might be helpful to accumulate *indels*. Recently, NHEJ activity was reduced by inhibiting DNA ligase IV, a key enzyme in the NHEJ pathway, to promote HR, thereby increasing the efficiency of precise genome editing with CRISPR-Cas9.[51] In reverse, promoting NHEJ at the expense of HR activity would increase the efficiency of TAME for driving the phenotypic evolution of *S. cerevisiae*.

Although the designed TALEN pair here allowed multiplex genome editing to occur, the potentially mutated sites were still limited due to the fact that only sites with consensus sequence features, including the enhancer-like GC box GGGCGG and the TATA box TATAAA as well as the appropriate spacer length, can be targeted. Thus, it is challenging to tailor the genomic target selection by TAME, whereas the selection of targets is much more versatile using other directed multiplex genome evolution tools, such as YOGE or RNAi.[45−47] In eukaryotes, the presence of multiple binding sites for different transcription factors results in combinatorial regulation and generates particular phenotypes. Recent studies have also quantified *in vitro* affinities[52] and elucidated detailed *in vivo* maps of transcription factor binding within intergenic regions in yeast.[53,54] Thus, more potential sites would be available in conjunction with genome mining. Moreover, if designed targeted sequences could be integrated into the genome, then it will be easy to precisely achieve combinatorial regulation of multiple genes by TAME, which is significant for optimizing pathways and metabolic networks.

In addition, TALENs has been reported to produce off-target effects.[55,56] Thus, TAME is also faced with the problems of off-target effects and cytotoxicity. The use of only 6 TALE repeats for multiplex and simultaneous modification in the TAME model might lead to higher off-target rates and cytotoxicity. Likewise, off-target effects are also observed using multiplex automated genome engineering (MAGE) and other genome engineering technologies and are difficult to avoid in mismatch repair-deficient strains.[57] Since TAME employed the use of efficient screening with a fluorescence reporter or selective stress, random off-target mutations might provide possible evolutionary drivers to some extent. On the other hand, TALENs-based inhibition of cell growth was observed during TAME treatment, but this could be alleviated by optimizing the expression of TALENs through a more stringent induction method.

Since fully annotated genome sequences of many important microbial organisms are publically available[58] and novel screening methods have been explored, the TAME toolbox could be applied to enhance the creation of phenotypic diversity in the adaptive laboratory evolution of many other native organisms. Furthermore, recent interest in the area of synthetic biology and genome engineering will stimulate the development of newer genome-scale engineering approaches to engineer multiple complex phenotypes for applications in a realistic time frame.

■ METHODS

Computational Analysis of Potential TALE Binding Sites on the *S. cerevisiae* S288c Genome. To identify potential TALE binding sites, custom *Perl* scripts (Table S3, Supporting Information) were used to scan the *S. cerevisiae* S288c genome (version R64-1-1, GenBank assembly ID GCA_000146045.2). When searching the potential TALE binding sites containing both GGGCGG and TATAAA sequences with a 0−50 bp spacer, the algorithm was as indicated in Figure S6. Then, the yeast genes with potential TALE binding sequences were sorted out and annotated according to the National Center of Biotechnology Information (NCBI) database. The detailed information is listed in Supporting Information data set S1.

TALEN Plasmid Construction. According to the previously described methods by Li et al.,[15,59] specific TALEN modules

designed in this study were assembled by View Solid Biotech (Beijing, China). To limit the potential toxicity of TALEN activity during experiments, TALEN modules were inserted into the restriction sites *Hind*III and *Xba*I of pYES2/CT (*URA3*, 2MICRON, Invitrogen) under the control of the galactose-inducible *GAL1* promoter and *CYC1* terminator. The fragments expressing TALEN modules, which bind to different lengths of the left and right target sequences (4−12 bp) and have the same spacer length in the *URA3* gene (Figure S1, Supporting Information), were subcloned into the pRS423 (*HIS3*, 2MICRON) and pRS425 (*LEU2*, 2MICRON) plasmids, respectively, resulting in p423-GAL-L*x* and p425-GAL-R*x* plasmids (*x* = 4, 6, 9, or 12). The fragments expressing TALEN modules, which bind to the 12 bp right target sequences paired with the 12 bp left target sequence and different spacer lengths (5−40 bp) in the *URA3* gene (Figure S1, Supporting Information), were subcloned into the pRS425 plasmid, resulting in p425-GAL-S*y* plasmids (*y* = 5, 10, 20, 30, or 40). The fragments expressing the TALEN module that binds to the TATAAA sequence was subcloned into the pRS313 (*HIS3*, CEN) plasmid. The resulting plasmid was named p313-GAL-TA and used as a TALEN pair with plasmid pYES2/CT-GC, which contains the TALEN module that binds to the GGGCGG sequence. Control plasmids pYES2/CT-GC-Δ*Fok*I and p313-GAL-TA-Δ*Fok*I with inactive *Fok*I nucleases were constructed by enzyme digestion and disrupting the *Fok*I gene expression cassette. The primers *Eag*I-pGAL and *Xma*I-CYC1 (Table S1, Supporting Information) were used to amplify the fragments containing the TALEN modules as well as *GAL1* promoter and *CYC1* terminator. *Escherichia coli* DH5α competent cells (TransGen Biotech, Beijing, China) were used for routine cloning according to the manufacturer's instructions. *S. cerevisiae* BY4741 and *S. cerevisiae* CEN.PK-1C were used as different initial strains for TAME model construction and application. The constructed plasmids and strains are shown in Table S2, Supporting Information. All TALEN plasmids were sequenced using primers TALE-F and TALE-R (Table S1, Supporting Information) by BGI Tech (Beijing, China).

Editing the *URA3* Gene by Different TALENs in Yeast. *S. cerevisiae* strain BY4741 (MATa; *his3*Δ1; *leu2*Δ0; *met15*Δ0; *ura3*Δ0) from EUROSCARF (Frankfurt, Germany) lacks the coding sequence for the *URA3* gene. The functional *URA3* gene under its own promoter and terminator was reintroduced into BY4741 by replacing the *PDC1* gene. The resulting strain was named BY4741a. To evaluate the modification efficiency of TALENs with different numbers of TALE repeats and a fixed spacer length, p423-GAL-L*x* and p425-GAL-R*x* plasmids (Table S2, Supporting Information) expressing TALEN pairs that target the *URA3* gene were transformed into BY4741a, respectively. To evaluate the modification efficiency of TALENs with a fixed number of TALE repeats (12 repeats) and different spacer lengths, p423-GAL-L12 and p425-GAL-S*y* plasmids (Table S2, Supporting Information) expressing TALEN pairs that target the *URA3* gene were transformed into BY4741a, respectively. The empty plasmids pRS423 and pRS425 were also used to transform BY4741a as a negative control without TALEN editing. The transformants were cultivated in synthetic complete (SC) medium containing 20 g/L galactose but lacking histidine and leucine for 24 h before plating on the 5-FOA plates, which are SC medium plates containing 0.15% 5-fluoroorotic acid (5-FOA) but without histidine and leucine. The transformants were plated in parallel on SC medium plates without 5-FOA. The efficiency of *URA3* gene editing by TALENs was measured by comparing

the number of colonies on the 5-FOA plate and the plates without 5-FOA (Figure S2, Supporting Information). To identify the types of *indels* induced by TALENs, the *URA3* gene was amplified using genomic DNA extracted from those 5-FOA-resistant colonies as the PCR template and the primer pair URA3-F/URA3-R (Table S1, Supporting Information), which was sequenced using primer URA3-F.

Adaptive Evolution of Yeast Cells via TALENs-Assisted Multiplex Editing (TAME). Plasmids pYES2/CT-GC and p313-GAL-TA expressing the TALEN pair, which was designed to recognize the GGGCGG and TATAAA sequences, were used to induce multiplex editing in the yeast genome. mCherry fluorescent protein was expressed from plasmid pGAP-mCherry (*LEU2*, 2MICRON, *p*GAP) as a readout of the adaptive evolution of yeast cells via TAME. The BY4741 and CEN.PK-1C transformants containing plasmids pYES2/CT-GC, p313-GAL-TA, and pGAP-mCherry were cultivated, and TALENs were induced in the selective SC liquid medium (SC-H-L-U) containing 20 g/L galactose and lacking histidine, leucine, and uracil for 24 h; then, cells were plated on SC-H-L-U plates containing 20 g/L glucose. After 48 h, single colonies were picked and cultivated in SC-H-L-U medium (20 g/L glucose) for 12 h in 96 deep well plates. Eight cultures showing the highest relative fluorescence values were used to start the next cycle. The induction and screening process was repeated for five cycles, and about 200 colonies were picked and compared by measuring fluorescence values in each generation. The final evolved strain showing the highest relative fluorescence value was named M-T6-5 and chosen for further analysis. Empty plasmids pYES2/CT, pRS313, and pGAP-mCherry were also used to transform BY4741 and CEN.PK-1C as negative controls (M-CK and CM-CK) without TALEN editing. At the same time, plasmids pYES2/CT-GC-Δ*Fok*I and p313-GAL-TA-Δ*Fok*I were also transformed into yeast with pGAP-mCherry to test the function of TALEs. For cells showing increased fluorescence, plasmids were isolated and retransformed to revalidate the phenotypes in biological replicates.

Plasmids pYES2/CT-GC and p313-GAL-TA used in the previous model were cotransformed into BY4741 for ethanol tolerance evolution. The BY4741 transformants containing plasmids pYES2/CT-GC and p313-GAL-TA were cultivated, and TALENs were induced in selective SC liquid medium (SC-H-U) containing 10 g/L galactose, 2 g/L glucose, and 8% ethanol (v/v) and lacking histidine and uracil for 24 h; then, cells were transferred five times in 50 mL closed-top tubes starting at an OD$_{600}$ of 0.2. Following this selection phase, the evolutionary cell library was plated onto SC plates containing 20 g/L glucose and 8% ethanol to ensure single-colony isolation. Empty plasmids pYES2/CT and pRS313 were also used to transform BY4741 as a negative control (E-CK) without TALEN editing. The TAME-treated strains (ET6-1/ET6-2) and control strain were transferred to stress-free medium five times, and the resulting blank strains were identified and assayed for growth in medium containing 8% ethanol.

Fluorescence Assays. Target yeast cells carrying the *mCherry* reporter and different TALENs (or no TALEN as a control) were grown overnight in SC media supplemented with 20 g/L glucose. One aliquot of the cultures was transferred into fresh SC-L-H-U media supplemented with 20 g/L galactose and induced for 24 h. After successive passaging for three times, the culture was sorted and analyzed by a BD Influx flow cytometer (Franklin Lakes, NJ, USA). The other aliquot of cultures was used to assess the expression level of the *mCherry* reporter.

The fluorescence value was measured using a Spectramax M2 microplate reader (Molecular Devices), exciting at 587 nm and measuring emission at 610 nm, and the value was normalized to cell density by measuring the optical density of cultures at 600 nm (OD_{600}).

Genome Resequencing and Data Analysis. Yeast genomic DNA was extracted according to the method described by Philippsen.[60] Sequencing libraries were prepared and sequenced by GENEWIZ, Inc. (Illumina/Solexa Genome Analyzer II, Suzhou, China) using standard Illumina sample preparing and operating procedures. The Illumina short reads were mapped to the reference genome of *S. cerevisiae* S288c to assemble the corresponding genome of each sequenced strain. The consensus sequences and polymorphisms among the sequenced strains and S288c were delineated using SAMtools.

Semiquantitative Analysis of mCherry Protein Expression in Yeast. The selected strains with a high fluorescence value from the evolutionary process (M-T6 and control M-CK) were inoculated in SC-H-L-U medium with 20 g/L glucose for 12 h. Yeast crude extracts were prepared according to the modified method described by Kushnirov.[61] Protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit according to the manufacturer's instructions (Thermo). The same amounts of yeast crude extracts were used to separate proteins by native gel electrophoresis. The mCherry protein levels in yeast cell extracts were semiquantified using a ChemiDoc XRS system (Bio-Rad). The excitation source was blue trans-illumination, and the emission filter was a standard filter. The exposure time of intense bands was under automatic controls.

Microarray Analysis. Yeast mutants and controls were grown to an OD_{600} of approximately 1.0. RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). Microarray services were provided by Agilent, Inc. using the Agilent Yeast 2.0 arrays and Agilent GeneSpring data processing software. Arrays were run in triplicate with biological replicates to allow for statistical confidence in the determination of differential gene expression.

ASSOCIATED CONTENT

Ⓢ Supporting Information

Data set S1: Potential TALEN modification sites targeting the GGGCGG and TATAAA sequences with 0−50 bp spacer. Data set S2: Accumulated alterations during adapative evolution of *mCherry* via TALENs-assistant multiplex editing. Data set S3: 246 SNPs found in evolved strain M-T6 by whole-genome resequencing. Data set S4: Seven sites observed to contain TALENs-induced *indels*. Table S1: Primers used in this study. Table S2: Strains and plasmids used in this study. Table S3: The script for mapping target sequences in *S. cerevisiae* S288c genome (*Perl*). Figure S1: The coding sequence of *URA3* and the targeted sequences of different TALENs. Figure S2: The diagram of targeted *URA3* gene modification by TALENs in yeast. Figure S3: Diversity analysis of *mCherry* expressed in a population. Figure S4: Re-transformation of plasmids from evolved strains with variable fluorescence intensity. Figure S5: Transcriptional profiling of differentially expressed genes in the M-T6-5 evolved strain compared with the control. Figure S6: The flow chart of target sites mapping by computational analysis. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.5b00074.

AUTHOR INFORMATION

Corresponding Author
*Tel.: +86-22-84861950. Fax: +86-22-84861950. E-mail: wang_qh@tib.cas.cn.

Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS

REFERENCES

(1) Lelli, K. M., Slattery, M., and Mann, R. S. (2012) Disentangling the many layers of eukaryotic transcriptional regulation. *Annu. Rev. Genet.* 46, 43−68.

(2) Ellwood, K., Huang, W., Johnson, R., and Carey, M. (1999) Multiple layers of cooperativity regulate enhanceosome-responsive RNA polymerase II transcription complex assembly. *Mol. Cell. Biol.* 19, 2613−2623.

(3) Pilpel, Y., Sudarsanam, P., and Church, G. M. (2001) Identifying regulatory networks by combinatorial analysis of promoter elements. *Nat. Genet.* 29, 153−159.

(4) Chen, L. (1999) Combinatorial gene regulation by eukaryotic transcription factors. *Curr. Opin. Struct. Biol.* 9, 48−55.

(5) Eeken, J. C., and Sobels, F. H. (1983) The effect of two chemical mutagens ENU and MMS on MR-mediated reversion of an insertion-sequence mutation in *Drosophila melanogaster*. *Mutat. Res.* 110, 297−310.

(6) Joo, H., Lin, Z., and Arnold, F. H. (1999) Laboratory evolution of peroxide-mediated cytochrome P450 hydroxylation. *Nature* 399, 670−673.

(7) Zhang, Y. X., Perry, K., Vinci, V. A., Powell, K., Stemmer, W. P., and del Cardayré, S. B. (2002) Genome shuffling leads to rapid phenotypic improvement in bacteria. *Nature* 415, 644−646.

(8) Pfleger, B. F., Pitera, D. J., Smolke, C. D., and Keasling, J. D. (2006) Combinatorial engineering of intergenic regions in operons tunes expression of multiple genes. *Nat. Biotechnol.* 24, 1027−1032.

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

(10) Wood, A. J., Lo, T. W., Zeitler, B., Pickle, C. S., Ralston, E. J., Lee, A. H., Amora, R., Miller, J. C., Leung, E., Meng, X., Zhang, L., Rebar, E. J., Gregory, P. D., Urnov, F. D., and Meyer, B. J. (2011) Targeted genome editing across species using ZFNs and TALENs. *Science* 333, 307.

(11) Miller, J. C., Tan, S., Qiao, G., Barlow, K. A., Wang, J., Xia, D. F., Meng, X., Paschon, D. E., Leung, E., Hinkley, S. J., Dulay, G. P., Hua, K. L., Ankoudinova, I., Cost, G. J., Urnov, F. D., Zhang, H. S., Holmes, M. C., Zhang, L., Gregory, P. D., and Rebar, E. J. (2011) A TALE nuclease architecture for efficient genome editing. *Nat. Biotechnol.* 29, 143−148.

(12) Christian, M., Cermak, T., Doyle, E. L., Schmidt, C., Zhang, F., Hummel, A., Bogdanove, A. J., and Voytas, D. F. (2010) Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* 186, 757−761.

(13) Lieber, M. R. (2010) The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. *Annu. Rev. Biochem.* 79, 181−211.

(14) Guilinger, J. P., Pattanayak, V., Reyon, D., Tsai, S. Q., Sander, J. D., Joung, J. K., and Liu, D. R. (2014) Broad specificity profiling of TALENs results in engineered nucleases with improved DNA-cleavage specificity. *Nat. Methods* 11, 429−435.

(15) Li, T., Huang, S., Zhao, X., Wright, D. A., Carpenter, S., Spalding, M. H., Weeks, D. P., and Yang, B. (2011) Modularly assembled designer TAL effector nucleases for targeted gene knockout and gene replacement in eukaryotes. *Nucleic Acids Res.* 39, 6315−6325.

(16) Grabher, C., and Wittbrodt, J. (2007) Meganuclease and transposon mediated transgenesis in *medaka. Genome Biol. 8,* S10.

(17) Boeke, J. D., LaCroute, F., and Fink, G. R. (1984) A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol. Gen. Genet. 197,* 345−346.

(18) Stewart, J. J., and Stargell, L. A. (2001) The stability of the TFIIA−TBP−DNA complex is dependent on the sequence of the TATAAA element. *J. Biol. Chem. 276,* 30078−30084.

(19) Raser, J. M., and O'Shea, E. K. (2004) Control of stochasticity in eukaryotic gene expression. *Science 304,* 1811−1814.

(20) Basehoar, A. D., Zanton, S. J., and Pugh, B. F. (2004) Identification and distinct regulation of yeast TATA box-containing genes. *Cell 116,* 699−709.

(21) Klug, W. S., Cummings, M. R., Spencer, C. A., and Palladina, M. A. (2009) *Concepts of Genetics,* 9th ed., pp 463−464, Pearson Benjamin Cummings, San Francisco, CA.

(22) Butler, J. E., and Kadonaga, J. T. (2002) The RNA polymerase II core promoter: a key component in the regulation of gene expression. *Genes Dev. 16,* 2583−2592.

(23) Elfving, N., Chereji, R. V., Bharatula, V., Björklund, S., Morozov, A. V., and Broach, J. R. (2014) A dynamic interplay of nucleosome and Msn2 binding regulates kinetics of gene activation and repression following stress. *Nucleic Acids Res. 42,* 5468−5482.

(24) Li, L., Bagley, D., Ward, D. M., and Kaplan, J. (2008) Yap5 is an iron-responsive transcriptional activator that regulates vacuolar iron storage in yeast. *Mol. Cell. Biol. 28,* 1326−1337.

(25) Carrillo, E., Ben-Ari, G., Wildenhain, J., Tyers, M., Grammentz, D., and Lee, T. A. (2012) Characterizing the roles of Met31 and Met32 in coordinating Met4-activated transcription in the absence of Met30. *Mol. Biol. Cell 23,* 1928−1942.

(26) Park, K. S., Lee, D. K., Lee, H., Lee, Y., Jang, Y. S., Kim, Y. H., Yang, H. Y., Lee, S. I., Seol, W., and Kim, J. S. (2003) Phenotypic alteration of eukaryotic cells using randomized libraries of artificial transcription factors. *Nat. Biotechnol. 21,* 1208−1214.

(27) Park, K. S., Jang, Y. S., Lee, H., and Kim, J. S. (2005) Phenotypic alteration and target gene identification using combinatorial libraries of zinc finger proteins in prokaryotic cells. *J. Bacteriol. 187,* 5496−5499.

(28) Qi, L. S., Larson, M. H., Gilbert, L. A., Doudna, J. A., Weissman, J. S., Arkin, A. P., and Lim, W. A. (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell 152,* 1173−1183.

(29) Liti, G., Carter, D. M., Moses, A. M., Warringer, J., Parts, L., James, S. A., Davey, R. P., Roberts, I. N., Burt, A., Koufopanou, V., Tsai, I. J., Bergman, C. M., Bensasson, D., O'Kelly, M. J., van Oudenaarden, A., Barton, D. B., Bailes, E., Nguyen, A. N., Jones, M., Quail, M. A., Goodhead, I., Sims, S., Smith, F., Blomberg, A., Durbin, R., Louis, E. J. Population genomics of domestic and wild yeasts. *Nature 458,* 337−341.

(30) Wohlbach, D. J., Rovinskiy, N., Lewis, J. A., Sardi, M., Schackwitz, W. S., Martin, J. A., Deshpande, S., Daum, C. G., Lipzen, A., Sato, T. K., and Gasch, A. P. (2014) Comparative genomics of *Saccharomyces cerevisiae* natural isolates for bioenergy production. *Genome Biol. Evol. 6,* 2557−2566.

(31) Stepanchick, A., Zhi, H., Cavanaugh, A. H., Rothblum, K., Schneider, D. A., and Rothblum, L. I. (2013) DNA binding by the ribosomal DNA transcription factor rrn3 is essential for ribosomal DNA transcription. *J. Biol. Chem. 288,* 9135−9144.

(32) Kashyap, A. K., Schieltz, D., Yates, J., III, and Kellogg, D. R. (2005) Biochemical and genetic characterization of Yra1p in budding yeast. *Yeast 22,* 43−56.

(33) Windgassen, M., and Krebber, H. (2003) Identification of Gbp2 as a novel poly(A)$^+$ RNA-binding protein involved in the cytoplasmic delivery of messenger RNAs in yeast. *EMBO Rep. 4,* 278−283.

(34) Dilova, I., and Powers, T. (2006) Accounting for strain-specific differences during RTG target gene regulation in *Saccharomyces cerevisiae. FEMS Yeast Res. 6,* 112−119.

(35) Kudlyk, T., Willett, R., Pokrovskaya, I. D., and Lupashin, V. (2013) COG6 interacts with a subset of the Golgi SNAREs and is important for the Golgi complex integrity. *Traffic 14,* 194−204.

(36) Vuralhan, Z., Morais, M. A., Tai, S. L., Piper, M. D., and Pronk, J. T. (2003) Identification and characterization of phenylpyruvate decarboxylase genes in *Saccharomyces cerevisiae. Appl. Environ. Microbiol. 69,* 4534−4541.

(37) Bai, F. W., Chen, L. J., Zhang, Z., Anderson, W. A., and Moo-Young, M. (2004) Continuous ethanol production and evaluation of yeast cell lysis and viability loss under very high gravity medium conditions. *J. Biotechnol. 110,* 287−293.

(38) van Voorst, F., Houghton-Larsen, J., Jønson, L., Kielland-Brandt, M. C., and Brandt, A. (2006) Genome-wide identification of genes required for growth of *Saccharomyces cerevisiae* under ethanol stress. *Yeast 23,* 351−359.

(39) 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.

(40) Lam, F. H., Ghaderi, A., Fink, G. R., and Stephanopoulos, G. (2014) Engineering alcohol tolerance in yeast. *Science 346,* 71−75.

(41) Patnaik, R. (2008) Engineering complex phenotypes in industrial strains. *Biotechnol. Prog. 24,* 38−47.

(42) Sandoval, N. R., Kim, J. Y., Glebes, T. Y., Reeder, P. J., Aucoin, H. R., Warner, J. R., and Gill, R. T. (2012) Strategy for directing combinatorial genome engineering in *Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 109,* 10540−10545.

(43) 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,* 894−898.

(44) Wang, H. H., Kim, H., Cong, L., Jeong, J., Bang, D., and Church, G. M. (2012) Genome-scale promoter engineering by coselection MAGE. *Nat. Methods 9,* 591−593.

(45) DiCarlo, J. E., Conley, A. J., Penttilä, M., Jäntti, J., Wang, H. H., and Church, G. M. (2013) Yeast oligo-mediated genome engineering (YOGE). *ACS Synth. Biol. 2,* 741−749.

(46) Dalia, A. B., McDonough, E., and Camilli, A. (2014) Multiplex genome editing by natural transformation. *Proc. Natl. Acad. Sci. U.S.A. 111,* 8937−8942.

(47) Si, T., Luo, Y., Bao, Z., and Zhao, H. (2014) RNAi-assisted genome evolution in *Saccharomyces cerevisiae* for complex phenotype engineering. *ACS Synth. Biol. 4,* 283−291.

(48) Zhang, Y., Zhang, F., Li, X., Baller, J. A., Qi, Y., Starker, C. G., Bogdanove, A. J., and Voytas, D. F. (2013) Transcription activator-like effector nucleases enable efficient plant genome engineering. *Plant Physiol. 161,* 20−27.

(49) Huang, P., Xiao, A., Zhou, M., Zhu, Z., Lin, S., and Zhang, B. (2011) Heritable gene targeting in *zebrafish* using customized TALENs. *Nat. Biotechnol. 29,* 699−700.

(50) Aouida, M., Piatek, M. J., Bangarusamy, D. K., and Mahfouz, M. M. (2014) Activities and specificities of homodimeric TALENs in *Saccharomyces cerevisiae. Curr. Genet. 60,* 61−74.

(51) Maruyama, T., Dougan, S. K., Truttmann, M. C., Bilate, A. M., Ingram, J. R., and Ploegh, H. L. (2015) Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of non-homologous end joining. *Nat. Biotechnol. 33,* 538−542.

(52) Zhu, C., Byers, K. J., McCord, R. P., Shi, Z., Berger, M. F., Newburger, D. E., Saulrieta, K., Smith, Z., Shah, M. V., Radhakrishnan, M., Philippakis, A. A., Hu, Y., De Masi, F., Pacek, M., Rolfs, A., Murthy, T., Labaer, J., and Bulyk, M. L. (2009) High-resolution DNA-binding specificity analysis of yeast transcription factors. *Genome Res. 19,* 556−566.

(53) Harbison, C. T., Gordon, D. B., Lee, T. I., Rinaldi, N. J., Macisaac, K. D., Danford, T. W., Hannett, N. M., Tagne, J. B., Reynolds, D. B., Yoo, J., Jennings, E. G., Zeitlinger, J., Pokholok, D. K., Kellis, M., Rolfe, P. A., Takusagawa, K. T., Lander, E. S., Gifford, D. K., Fraenkel, E., and Young, R. A. (2004) Transcriptional regulatory code of a eukaryotic genome. *Nature 431,* 99−104.

(54) MacIsaac, K. D., Wang, T., Gordon, D. B., Gifford, D. K., Stormo, G. D., and Fraenkel, E. (2006) An improved map of conserved regulatory sites for *Saccharomyces cerevisiae. BMC Bioinf. 7,* 113.

(55) Hockemeyer, D., Wang, H., Kiani, S., Lai, C. S., Gao, Q., Cassady, J. P., Cost, G. J., Zhang, L., Santiago, Y., Miller, J. C., Zeitler, B., Cherone, J. M., Meng, X., Hinkley, S. J., Rebar, E. J., Gregory, P. D., Urnov, F. D., and Jaenisch, R. (2011) Genetic engineering of human pluripotent cells using TALE nucleases. *Nat. Biotechnol. 29*, 731−734.

(56) Mussolino, C., Morbitzer, R., Lütge, F., Dannemann, N., Lahaye, T., and Cathomen, T. (2011) A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. *Nucleic Acids Res. 39*, 9283−9293.

(57) Isaacs, F. J., Carr, P. A., Wang, H. H., Lajoie, M. J., Sterling, B., Kraal, L., Tolonen, A. C., Gianoulis, T. A., Goodman, D. B., Reppas, N. B., Emig, C. J., Bang, D., Hwang, S. J., Jewett, M. C., Jacobson, J. M., and Church, G. M. (2011) Precise manipulation of chromosomes *in vivo* enables genome-wide codon replacement. *Science 333*, 348−353.

(58) NCBI Resource Coordinators (2014) Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res. 42*, D7−D17.

(59) Li, T., Huang, S., Jiang, W. Z., Wright, D., Spalding, M. H., Weeks, D. P., and Yang, B. (2010) TAL nucleases (TALNs): hybrid proteins composed of TAL effectors and *FokI* DNA-cleavage domain. *Nucleic Acids Res. 39*, 359−372.

(60) Philippsen, P., Stotz, A., and Scherf, C. (1991) DNA of *Saccharomyces cerevisiae*. *Methods Enzymol. 194*, 169−182.

(61) Kushnirov, V. V. (2000) Rapid and reliable protein extraction from yeast. *Yeast 16*, 857−860.