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Multiplex metabolic pathway engineering using CRISPR/Cas9 in *Saccharomyces cerevisiae*

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ABSTRACT

CRISPR/Cas9 is a simple and efficient tool for targeted and marker-free genome engineering. Here, we report the development and successful application of a multiplex CRISPR/Cas9 system for genome engineering of up to 5 different genomic loci in one transformation step in baker's yeast *Saccharomyces cerevisiae*. To assess the specificity of the tool we employed genome re-sequencing to screen for off-target sites in all single knock-out strains targeted by different gRNAs. This extensive analysis identified no more genome variants in CRISPR/Cas9 engineered strains compared to wild-type reference strains. We applied our genome engineering tool for an exploratory analysis of all possible single, double, triple, quadruple and quintuple gene disruption combinations to search for strains with high mevalonate production, a key intermediate for the industrially important isoprenoid biosynthesis pathway. Even though we did not overexpress any genes in the mevalonate pathway, this analysis identified strains with mevalonate titers greater than 41-fold compared to the wild-type strain. Our findings illustrate the applicability of this highly specific and efficient multiplex genome engineering approach to accelerate functional genomics and metabolic engineering efforts.

1. Introduction

Metabolic pathway engineering in yeast has proven to be tremendously important for improving basic understanding of metabolism and for production of fuels, chemicals and pharmaceuticals (Van Dien, 2013). Inherent to these successes is the need to efficiently edit native genetic elements or introduce heterologous multi-enzyme pathways, typically by linking subsequent transformation events to individual selectable markers (Alani et al., 1987; Gueldener et al., 2002; Jensen et al., 2014; Tian et al., 2013). Although a substantial collection of selectable markers are available for engineering *Saccharomyces cerevisiae* and industrial yeast (Da Silva and Srikrishnan, 2012; Fang et al., 2011), the small number of dominant, counter-selectable and other auxotrophic markers still poses a bottleneck for making knock-outs of multiple genes. Moreover, recycling of markers by Cre/loxP-, endonuclease-mediated excision, or internal recombination between flanking tandem repeats requires time-consuming sequential transformation steps (Alani et al., 1987; Gueldener et al., 2002; Solis-Escalante et al., 2014), and can lead to unwanted chromosome rearrangements (Delneri et al., 2000; Solis-Escalante et al., 2014).

Apart from their use for marker excision, double-strand breaks (DSBs) mediated by site-specific endonucleases, such as I-SceI and transcription activator-like effector nucleases (TALENs), have proven to be highly efficient to enhance chromosomal integration of exogenous genetic elements (Brenneman et al., 1996; Storici et al., 2003; Wu et al., 2014). Following the DSB event, native DNA repair will be initiated through non-homologous end joining or homologous recombination in the presence of an exogenous homologous donor sequence (DiCarlo et al., 2013a, 2013b; Storici and Resnick, 2006). In mammalian cells, error-prone non-homologous end joining is a dominant repair mechanism often leading to a mutant allele at the genomic loci targeted for a DSB (Liang et al., 1998; Yang et al., 2014), whereas error-

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeat; PAM, protospacer adjacent motif; BTS1, Geranylgeranyl pyrophosphate synthase; ROX1, Heme-dependent repressor of hypoxic genes; TALEN, transcription activator-like effector nuclease; gRNA, guide RNA; DSB, double strand break; ERG9, squalene synthase; ORF, open reading frame; UAS, upstream activating sequence

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free homologous recombination is the predominant DSB repair mechanism in yeast (Pâques and Haber, 1999).

For decades knowledge of DNA repair mechanisms has been harnessed for genome engineering (Alani et al., 1987; Liang et al., 1998; Rouet et al., 1994). Currently, the orthogonality of clustered regularly interspaced short palindromic repeat (CRISPR) RNA-guided Cas9 nucleases and simplicity of the guide RNA (gRNA) design for directing Cas9-mediated DSBs has attracted significant attention (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013). Indeed, the only restriction for designing a CRISPR/Cas9 guiding sequence is a need for a protospacer adjacent motif (PAM) close to genomic target site (Cong et al., 2013; Jinek et al., 2012). For type II bacterial CRISPR/Cas9 from *Streptococcus pyogenes* (Jinek et al., 2012), any genomic loci followed by the 5′-NGG-3′ PAM sequence can thus be targeted for marker-free integration or gene disruption by designing a ≈ 20 nucleotide long gRNA matching the target (Sander and Joung, 2014). By the ease of its design, efficient CRISPR/Cas9 genome engineering has been reported in bacteria, plant, animal and fungal kingdoms (Cong et al., 2013; DiCarlo et al., 2013a, 2013b; Jiang et al., 2013; Li et al., 2013).

In *S. cerevisiae*, Dicarlo et al. (2013b) were the first to report efficient CRISPR/Cas9-mediated single gene knock-out and knock-in using double-stranded oligos (dsOligos) centered around the target PAM sequence for homology-directed DSB repair. The study showed that co-transforming a gRNA targeting a negative selectable marker *CAN1* together with a 90-bp double-stranded HR donor, including a frame-shift mutation in the targeted reading frame and premature stop codon replacing the PAM sequence, recovered almost 100% of the *CAN1* mutated cells, whereas no canavanine resistant cells were recovered when the gRNA plasmid was transformed without the HR donor (DiCarlo et al., 2013b). Other more recent reports using yeast as a host have addressed the development of self-processing ribozyme-flanked RNAs into gRNAs and the optimization of the gRNA structure (Gao and Zhao, 2014; Jacobs et al., 2014; Xu et al., 2014). Likewise, introducing a tRNA as a RNA polymerase III promoter in addition to a ribozyme at the 5′ of the gRNA, Ryan et al. (2014) were able to circumvent otherwise low genome editing efficiencies in an industrial polyploid yeast. By expressing three hepatitis delta virus (HDV) ribozyme-flanked gRNA cassettes together with Cas9, Ryan et al. (2014) also presented a one-step transformation event in a haploid laboratory yeast strain to obtain a triple knock-out of *URA3*, *LYP1* and *COX10* with an estimated efficiency of 81%. Complementary to this, Bao et al. (2014) used an intelligent CRISPR-array strategy combining HR donor and gRNA expression into a polycistronic design, which allowed for efficient triple knock-out of genes involved in an artificial hydrocortisone pathway.

Acknowledging the importance of *S. cerevisiae* for both basic research and applied biotechnological purposes, continuous development and application of CRISPR/Cas9 for metabolic engineering purposes would allow for a significant improvement of strain construction programs for functional genomics and metabolic engineering. The aim of this study was to develop a highly efficient procedure for cloning and combinatorial expression of multiple gRNAs in a single vector, and thereby achieve multiplex genome engineering through a single transformation event. Secondly, we aimed to validate the applicability of our toolkit vector system to identify single and combinatorial genome edits for the identification of yeast strains with improved mevalonate levels, a key precursor for industrially important isoprenoid production. Here we report a facile and efficient cloning procedure to generate plasmids with single and multiple gRNA cassettes for the highly efficient genome engineering of up to 5 different loci in one transformation step. Also, we present a list of 31 yeast strains covering all possible combinations of 1–5 different genome modifications, of which 20 strains produced significantly higher mevalonate titers compared to the wild-type strain. The facile cloning procedure for multiplex vector construction, the high transformation efficiency, and the high-mevalonate producing strains should be of importance to the metabolic engineering community for improving strain construction rates and isoprenoid production.

2. Materials and methods

2.1. Strains, plasmids and media

The yeast strains used in this study were isogenic to CEN.PK2-1C. Strains and plasmids are listed in Supplementary Tables 1 and 2, respectively. Strains were grown in complete medium (YPD) with 2% glucose and synthetic complete (SC) from Sigma, supplemented with 2% glucose, minus the auxotrophic components complemented by propagated plasmids.

2.2. Selection of gRNA and plasmid construction

To select for specific gRNAs targeting *yjl064w*, *ypl062w*, *bts1*, and *rox1* all potential gRNA targets in these annotated CEN.PK113-7D genes were compared to all potential off-targets in the entire CEN.PK113-7D genome using the CRISPy tool, which is freely available at http://staff.biosustain.dtu.dk/laeb/crispy_cenpk/. From this ranking, only gRNAs without any 100%-identity to other genomic loci were selected. In CRISPy, the individual targets were linked to a pre-configured NCBI primer-blast page and PCR primers for genotyping target sites were retrieved. For targeting the *erg9* promoter we limited our search to 500-bp 5′ of the transcription start site in order to truncate the 44 bp UAS at location −172 bp.

The gRNA expression vector used by DiCarlo et al. (2013b) (Addgene reference number: 43803) was modified to replace *URA3* selection marker with *LEU2* selection marker from *Kluyveromyces lactis*. First, the gRNA expression vector was amplified with primers TJOS-22F and TJOS-22R to remove the *URA3* gene and insert restriction endonuclease sites (HindIII and XhoI). PCR product was gel purified and digested with HindIII and XhoI. Second, plasmid pUG73 (obtained from Euroscarf reference number: P30118) was linearized with HindIII and XhoI to remove and purify a fragment containing *LEU2* gene. Third, the *LEU2* selection marker was ligated to previously prepared gRNA expression vector, and the final plasmid was named pTAJAK-1.

Single gRNA expression vectors were constructed by amplifying pTAJAK-1 with primers: TJOS-20 and TJOS-6 (gRNA for *bts1*); TJOS-20 and TJOS-5 (gRNA for *ypl062w*); TJOS-20 and TJOS-36 (gRNA for *rox1*); TJOS-20 and TJOS-69 (gRNA for *yjl064w*); TJOS-20 and TJOS-71 (gRNA for *erg9* promoter). This cyclic amplification PCR resulted in the facile exchange of the 20-bp target gRNA sequence in the single gRNA expression cassette of pTAJAK-1. Amplified vectors were re-circularized by ligation.

Multiple gRNA expressing plasmids were efficiently constructed by first integrating a USER cloning site (Jensen et al., 2014) in the plasmids with single gRNA expression cassette using PCR amplification with TJOS-21 and TJOS-68. Amplified plasmids were re-circularized by ligation. Second, plasmids with single gRNA expression cassette and USER cloning site were prepared for cloning by digesting them with restriction endonucleases *Asi*SI and *Nb.Bsm*I as described in (Jensen et al., 2014). Third, gRNA expression cassettes were amplified with USER cloning compatible universal primers: TJOS-62(P1F), TJOS-63(P2F), TJOS-64(P3F), TJOS-112(P4F), TJOS-65(P1R), TJOS-66(P2R), TJOS-67(P3F), TJOS-113(P4R) (Fig. 2A). Finally, all possible combinations of double, triple, quadruple or quintuple gRNA expression cassettes were

USER cloned into an *Asi*SI-, *Nb.Bsm*I-digested vector (Fig. 2A). For a full list of all possible combinations see Supplementary Table 3. Description for detailed procedures on USER cloning can be found in Jensen et al. (2014). To construct a control plasmid without a gRNA expression cassette, pTAJAK-1 was digested with PvuII and re-ligated.

2.3. Strain construction

The strain expressing Cas9 was constructed by transforming the Cas9 expression plasmid (Addgene reference number: 43802) into CEN.PK2-1C by the lithium acetate transformation method (Gietz and Schiestl, 2007). This strain was named TC-3 and used for all further manipulations. To construct strains with single and multiple genome edits, TC-3 was transformed with appropriate plasmids by electroporation (Wu and Letchworth, 2004). For electroporation, we used 200 ng of gRNA expression plasmid and 1 nM of 90-bp long dsOligos per transformation (for *erg9* promoter engineering we used 2 μg of a 298-bp dsOligo).

2.4. Mutation efficiency

To determine gene knock-out efficiencies, 8 colonies were picked of each single target and 4–5 colonies of each strain with multiple targets for PCR of all targeted loci and subsequent Sanger sequencing. Regions of interest were amplified with primers: *bts1*: TJOS-15F; TJOS-15R, *rox1*: TJOS-81F; TJOS-81R, *ypl062w*: TJOS-82F; TJOS-82R, *yjl064w*: TJOS-72F; TJOS-72R, *erg9*: TJOS-74F; TJOS74R. Sanger sequencing was performed with primers: *bts1*: TJOS-15F; *rox1*: TJOS-81F; *ypl062w*: TJOS-82F; *yjl064w*: TJOS-72F; *erg9*: TJOS-74F. For a full list of primers used in this study see Supplementary Table 4.

2.5. Genome sequencing and data analysis

To investigate if there were any off-target effects we sequenced all mutated strains and the auxotrophic wild-type CEN.PK 2-1c strain on which the mutated strains were based, as well as an in-house prototropic wild type CEN.PK 113-7D using the Illumina MiSeq platform (Illumina, San Diego, CA, USA). The resulting data were 150-bp paired end reads, which were cleaned by trimming off 15 bases from the beginning of the reads and 5 bases from the end, using Trimmomatic (Bolger et al., 2014). Variants were called based on the MiSeq data using the breseq command line tool developed by the Barrick Lab (Deatherage and Barrick, 2014) with default settings for all parameters except – *b* which was set to 20. The *S. cerevisiae* CEN.PK113-7D genome (accession numbers CM001522–CM001537 and JH711449–JH711466) (Nijkamp et al., 2012) was used as reference. Variants found in all 8 strains were excluded as well as variants called on the unplaced contigs of the reference. To assess the probability of these variants being off-target effects we adopted the approach described by Ryan et al. (2014). Thus, we identified all the possible "NGG" PAM sites in the reference genome. For each PAM site, for which a variant had been identified within ± 30 bp from the site, we calculated the probability of finding equally good or better sites in the genome for binding of the gRNA upstream of the PAM site based on 10,000 random sites from the genome. This analysis was performed for each mutant strain using the corresponding gRNA sequence, and for both wild type strains as a control, using all gRNA sequences.

2.6. LC–MS analysis

Cells were first propagated in YPD medium in 24-well plates starting from OD600 = 0.1 for 144 h at 30 °C shaking incubator (Rodriguez et al., 2014). All cultures were grown as independent biological triplicates. After incubation, 1 ml of culture was taken and centrifuged to pellet the cells. Supernatant was taken and further used for LC–MS analyses.

LC–MS data were collected on an EVOQ Elite Triple Quadrupole Mass Spectrometer system coupled with an Advance UHPLC pump (Bruker, Fremont, CA). Samples were held in the autosampler at 4 °C during the analysis. 1-μL sample injections onto a Waters ACQUITY HSS T3 C18 UHPLC column, with a 1.8-μm particle size, 2.1-mm i.d. and 100-mm long, were performed at 30 °C. The solvent system used was Solvent A "Water with 0.1% formic acid" and Solvent B "Acetonitrile with 0.1% formic acid". The flow rate was 0.400 ml/min with an initial solvent composition of 100% A, 0% B held until 0.50 min, and the solvent composition was then changed following a linear gradient until it reached 5.0% A and 95.0% B at 1.00 min. This was held until 1.79 min when the solvent was returned to the initial conditions and the column was re-equilibrated until 4.00 min. The column eluent flowed directly into the heated ESI probe of the MS, which was held at 250 °C and a voltage of 4500 V. MRM Data were collected in negative ion mode and the target masses are shown in Supplementary Table 5. The other MS settings were as follows: sheath gas flow rate of 50 units, nebulizer gas flow rate of 50 units, cone gas flow rate of 20 units, cone temperature was 350 °C, and a collision gas pressure of 1.5 mTorr.

3. Results

3.1. Selection of CRISPR/Cas9 targets for metabolic pathway engineering

In order to develop and apply CRISPR/Cas9 for combinatorial pathway engineering we first selected a metabolic pathway of interest. Isoprenoids are platform metabolites for a large number of commercial products used in cancer therapy, food additives and antimalarial treatments (Engels et al., 2008; Ro et al., 2006). For all of these compounds, the mevalonate pathway in yeast has proven to be a robust production mechanism (Asadollahi et al., 2010; Scalcinati et al., 2012). We therefore decided to target the mevalonate pathway as a test bed for our combinatorial pathway engineering strategy using CRISPR/Cas9.

To explore the potential for CRISPR/Cas9-mediated metabolic pathway engineering in yeast we initially adopted the single gRNA expression strategy recently reported by DiCarlo et al. (2013b) (Fig. 1A) and selected five single loci reported to perturb flux through the mevalonate pathway (Fig. 1B–C). First, three single-gene deletions reported by Özaydın et al. (2013) to increase both mevalonate and isoprenoid levels were selected. This included *rox1*, a transcriptional regulator reported to repress genes in the mevalonate pathway and ergosterol biosynthesis (Henry et al., 2002; Montañés et al., 2011), and *ypl062w* and *yjl06w4*, two genes without any previously characterized functions, apart from improving MVA pathway flux when knocked out and especially when combined (Özaydın et al., 2013). Second, we selected *bts1*, encoding geranylgeranyl diphosphate (GGPP) synthase (Jiang et al., 1995), and *erg9*, encoding squalene synthase (Jennings et al., 1991). Overexpression of *bts1* has been shown to increase isoprenoid production 28-fold in yeast compared to native *bts1* expressing strains (Verwaal et al., 2007), whereas down-regulation of *erg9* has been shown to decrease flux towards ergosterol production and thereby increase isoprenoid production (Asadollahi et al., 2010; Paradise et al., 2008; Ro et al., 2006). Hence, *bts1* and *erg9* represent branch points in the mevalonate pathway for isoprenoid and ergosterol production, respectively (Fig. 1C). In order to test if lowering flux towards isoprenoid and/or ergosterol production would affect mevalonate levels we targeted

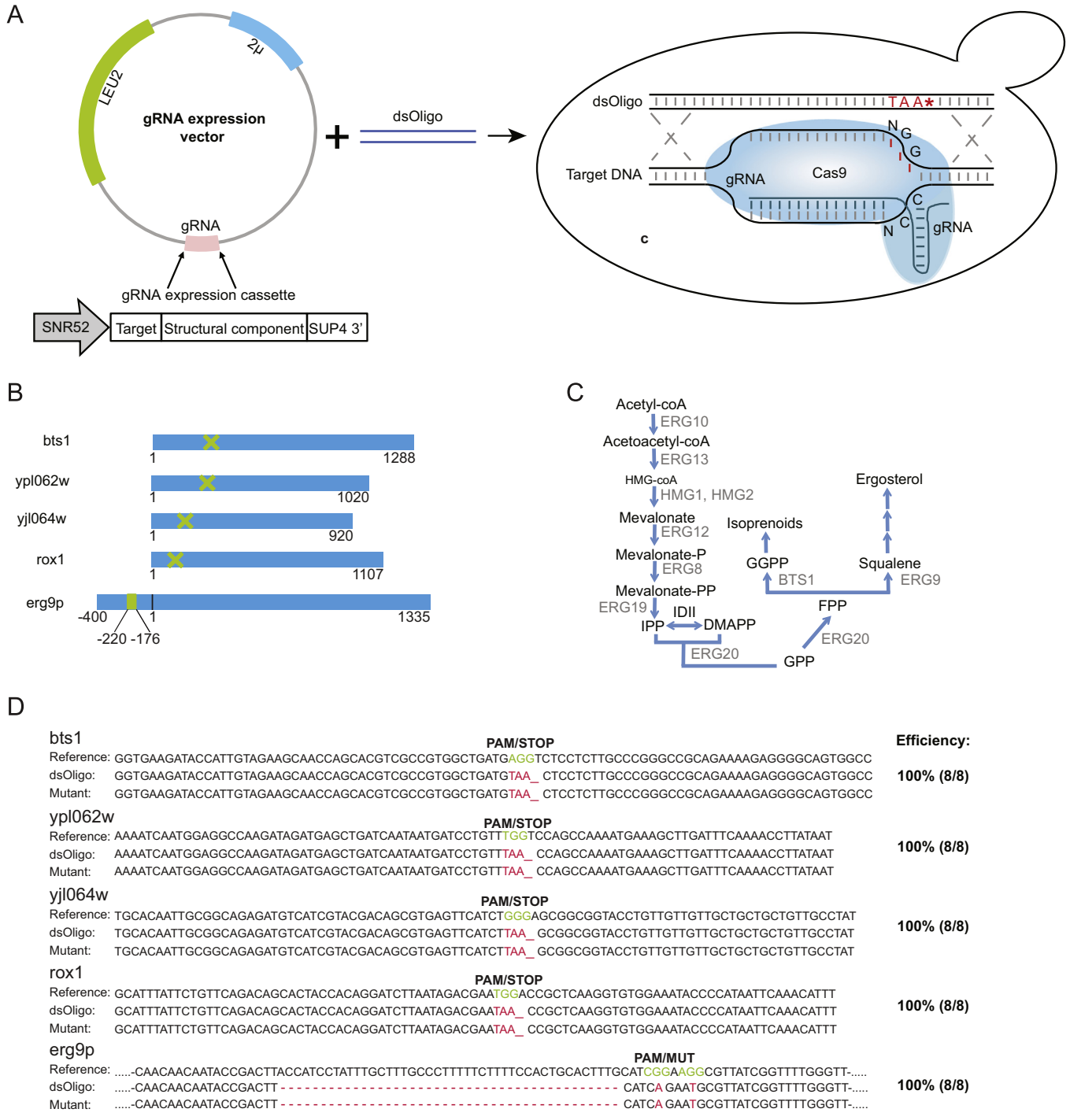

Fig. 1. Diagram of the CRISPR/Cas9 constructs and the genomic targets. (A) Design of the Cas9 and gRNA constructs according to DiCarlo et al. (2013b). (B) Schematic representation of the gRNA target sites for the 5 targeted genomic loci. (C) MVA pathway of yeast. (D) Sequence alignments of the five candidate loci compared to their respective dsOligos and representative Sanger sequencing results from Cas9 and gRNA expressing transformants. The green sequence of the wild-type reference sequence denotes the PAM site, while the red sequences indicate stop codons replacing the PAM sites and the frame shift nucleotide position. To the far right mutation efficiencies obtained from 8 representative colonies from each transformation are indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the knock-out of *bts1* and down-regulation of *erg9* in addition to the three other target genes mentioned above (Fig. 1B–C).

3.2. CRISPR/Cas9-mediated single locus editing for metabolic pathway engineering

In order to facilitate efficient design of gRNAs against the selected target genes, we developed a new version of the previously published tool CRISPy used for Chinese hamster ovary cell line engineering (Ronda et al., 2014) (http://staff.biosustain.dtu.dk/laeb/crispy_cenpk/). This tool features a new database designed specifically for finding, checking and designing PCR primers against gRNA targets of the format 20-NGG in annotated exons of the *S. cerevisiae* strain in CEN.PK113-7D, a current model for industrial biotechnology (Nijkamp et al., 2012). High-ranking gRNAs were searched for targeting exons in the 5′-end of ORFs

encoding the *bts1*, *rox1*, *ypl062w* and *yjl064w* (Fig. 1B). Additionally, by searching for 20-NGG matches in the 500-bp promoter of *erg9*, we identified a target sequence with a PAM (5′-CGG-3′) site in the upstream activating sequence (UAS) located 172 bp 5′ of the transcription start site. Truncation of this 44 bp UAS has been shown to reduce *erg9* promoter activity by 4–5 fold (Kennedy and Bard, 2001), a commonly used strategy to down-regulate squalene and ergosterol fluxes for improved isoprenoid production (Fig. 1C) (Asadollahi et al., 2010; Paradise et al., 2008; Ro et al., 2006; Scalcinati et al., 2012). Each of the identified target sequences were searched against the CEN.PK113-7D genome for potential off-targets using the CRISPy tool. Only genomic sequences matching the 13-bp sequence (Jinek et al., 2012; Ronda et al., 2014) immediately upstream of the PAM site (5′-NGG-3′) were identified as potential off-targets. For all candidate gRNAs no potential off-targets were reported from the CRISPy tool when searching for full alignment to the 13-bp sequence 5′ of the PAM, though allowing for single and double-mismatches within these 13-bp sequences retrieved between 1 and 18 mismatches per queried gRNA (Supplementary Table 6). Next, our single-gRNA expressing vectors were individually transformed into Cas9-expressing haploid yeast together with a mutant 90-bp dsOligo (knock-outs) or a 298-bp dsOligo (*erg9* promoter engineering) (Fig. 1A; Supplementary Table 7) as a template for homology-directed recombination to achieve knock-out of single target genes and truncation of the UAS in the *erg9* promoter, respectively. Data obtained by Sanger sequencing of targeted loci from 8 colonies of each of five transformations showed frame-shifts and stop codons in all the genomic contexts according to dsOligo template designs (Fig. 1D).

As no clear phenotypes were identified from any single transformants analyzed in this study, high transformation efficiency as reported here is of paramount importance when working with marker-free genome engineering. Altogether, our gRNA selection and transformation efficiencies highlight the use of the CRISPy tool for marker-free CRISPR/Cas9 genome engineering design in yeast, and supports the findings of DiCarlo et al. (2013b), showing highly efficient homology-directed repair of Cas9-mediated DSBs targeted by plasmid-based gRNAs.

3.3. Off-target validation by genome re-sequencing of genome edited strains

Several reports have described off-target effects resulting from the use of a CRISPR–Cas9 system for genome editing (Fu et al., 2013; Hsu et al., 2013; Lin et al., 2014; Pattanayak et al., 2013). In yeast off-target mutations could potentially arise from non-homologous end-joining of endonuclease-mediated DSBs or by homologous recombination between the 90-bp donors and homologous sequences at other genomic locations similar to the targeted sites (Moore and Haber, 1996; Schiestl et al., 1993). To analyze for off-target rates in edited yeast genomes, we applied next-generation sequencing of genomes from single colonies with targeted mutations of the 5 selected candidate loci (Fig. 1D) and two control wild type strains (prototropic CEN.PK 113-7D and the auxotrophic CEN.PK 2-1c strain used as a parent strain in this study). Reads obtained from next-generation sequencing of each of the strains were mapped to *S. cerevisiae* CEN.PK113-7D draft genome (Nijkamp et al., 2012), and genomic variants (SNPs, short indels, large deletions) were called based on the mapping data (Supplementary Table 8). From this analysis, we found >800 variants in each of the strains including the wild type controls. This number does not include the variants shared between all strains. The number of variants we found in the CEN.PK strains studied is significantly higher than what Ryan et al. (2014) reported for the S288C-derived strains used in their recent study with the finished S288C reference genome used as a mapping reference. The large number of variants observed in the CEN.PK 113-7D strains is attributable to the unfinished repeat regions in the CEN.PK113-7D draft genome where the genome assembly is likely to be incorrect or incomplete. These regions could be filtered out of the analysis, but we chose not to bias the analysis by filtering out variants in specific genomic regions.

Next, we searched for candidate off-target mutations by identifying PAM sites with genomic variants 30 bp upstream or downstream of the PAM (Ryan et al., 2014). Here, we found partial matches (11–12 bp match) to gRNA sequences upstream of PAM sites close to variants in each of the CRISPR/Cas9 edited strains (Supplementary Table 9). However, we also found similarly good matches close to variants in the wild type control strains with each of the gRNAs used in this study despite the fact that these gRNAs have never been expressed in the control strains and that one of the wild type controls has never had Cas9 expressed in it either. While we cannot conclusively rule out specific off-target effects in the CRISPR/Cas9 edited strains using the same criteria used by Ryan et al. (2014)(less than 12 bp match), we got essentially the same results when we analyze variants from control wild-type strains, indicating that the results we obtained are within what one would expect to obtain randomly given the number of variants found in these strains. Taken together, these analyses show that it is unlikely that that the variants discovered in the edited strains would be due to off-target activity of Cas9.

3.4. One-step combinatorial pathway engineering using CRISPR/Cas9

We have shown above that the CRISPR/Cas9 system can mediate highly efficient site-specific mutagenesis of individual genes (Fig. 1D and Supplementary Tables 8 and 9). With the exception of multiplex automated genome engineering and yeast oligo-mediated genome engineering (MAGE and YOGE, respectively) (Dicarlo et al., 2013a, 2013b; Wang et al., 2009), existing cloning-based technologies are often confined to serial time-consuming introductions of single DNA constructs into cells. However, for di- or polyploid yeasts, for studies of epistatic relationships in metabolic pathways, or to study potentially redundant functions of gene family members, two or more knock-out events are required. For this purpose, we constructed a toolkit vector for the simultaneous expression of multiple gRNAs. To create a platform vector capable of accommodating multiple gRNA expression cassettes, we employed the USER cloning technique (Bitinaite et al., 2007; Jensen et al., 2014) for easy and simultaneous ligation of multiple DNA fragments into a single USER site of a 2-μm vector backbone (Fig. 2A). To investigate the capacity for targeted DSBs occurring from the expression of multiple specific gRNAs we first scored the transformation efficiency of a set of single, double, triple, quadruple and quintuple gRNA expressing plasmids compared to transforming an empty plasmid backbone without any gRNA cassette. From this study we observed no decrease in transformation efficiency when expressing single and double gRNA expressing cassettes compared to no gRNA (Supplementary Fig. 1). However, one-step transformation of three and four loci decreased transformation efficiency by 75% and 90%, respectively, and an even further decrease was observed when targeting five different loci in one step (Supplementary Fig. 1). Increasing the amount of gRNA plasmid resulted in a modest increase in the number of colonies obtained, indicating that the level of gRNA expression could be a bottleneck in this system. Based on the transformation efficiencies obtained from the set of single, double, triple, quadruple and quintuple gRNA expressing plasmids, we targeted the construction of vectors harboring all possible remaining combinations of double-, triple-, and quadruple-gRNA expression cassettes for targeting of our five selected loci (Supplementary Table 3). These multi-cassette plasmids were

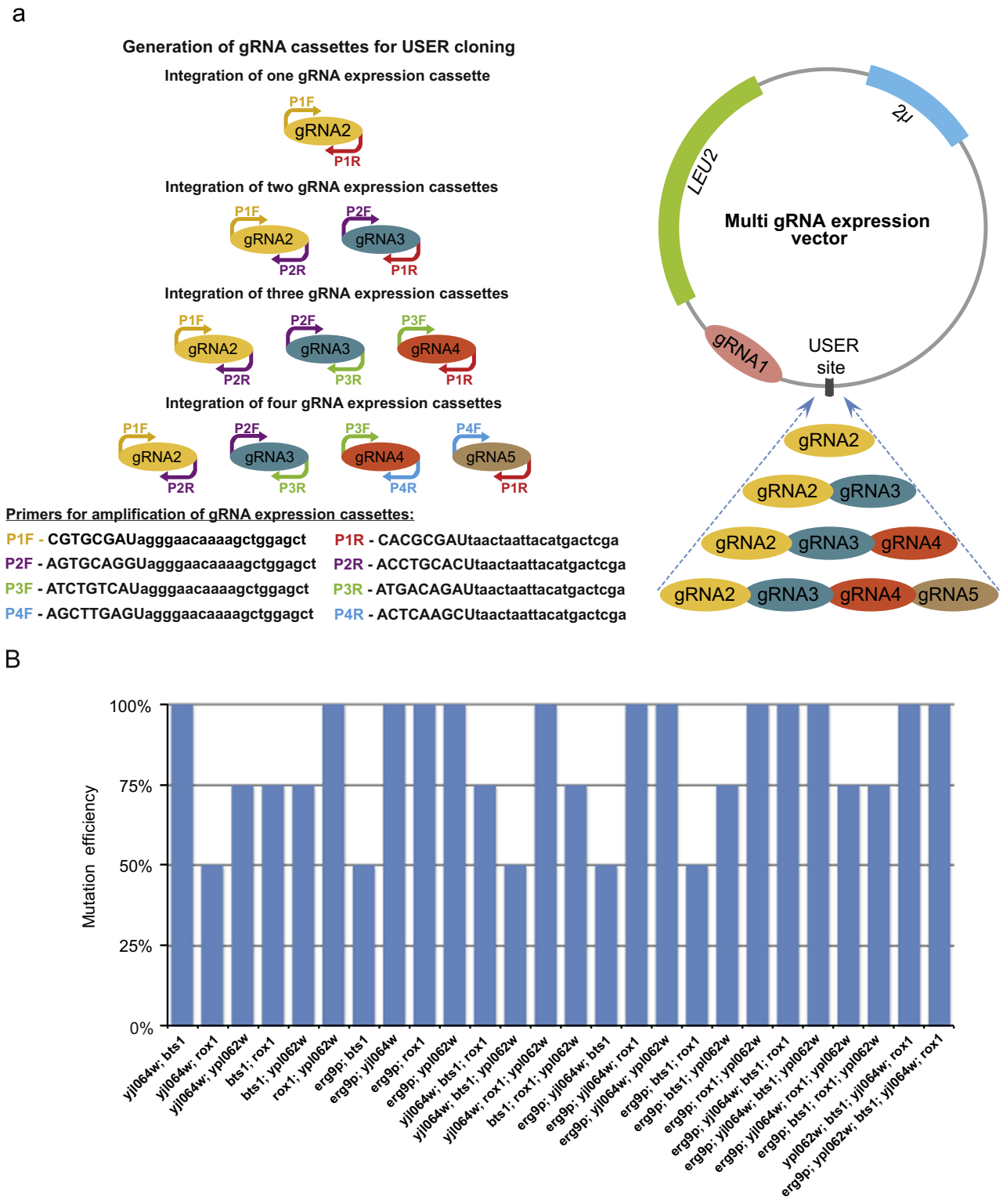

Fig. 2. Multiplex CRISPR/Cas9 toolkit vector and mutation efficiencies. (A) Schematic outline of the USER cloning design for constructing multiplex gRNA expression vectors accommodating between 2 and 5 gRNA cassettes. (B) Mutation efficiencies for 31 mutant strains (n=4–5).

individually transformed into Cas9-expressing yeast together with each variant of dsOligo templates (Supplementary Table 7) for homology-directed combinatorial genome engineering of target sites.

By sequence-based genotyping of all target sites in 4–5 colonies from each combinatorial transformation events, we observed the desired editing patterns with success rates estimated to vary from 50% to 100% (i.e., percentage of colonies with all targeted genomic edits successfully obtained) (Fig. 2B). Hence, in this analysis, picking two colonies from any of the one-step transformations for combinatorial genome engineering of 2–5 genes, one colony is very likely to encode all the targeted edits. It should also be emphasized that apart from the auxotrophic markers complemented by the Cas9 and gRNA expressing plasmids, the genomic loci edited in this study did not produce any clear phenotype compared to the wild-type strain validating the usability of this tool. Furthermore, as edits are marker-free, recycling of markers carried

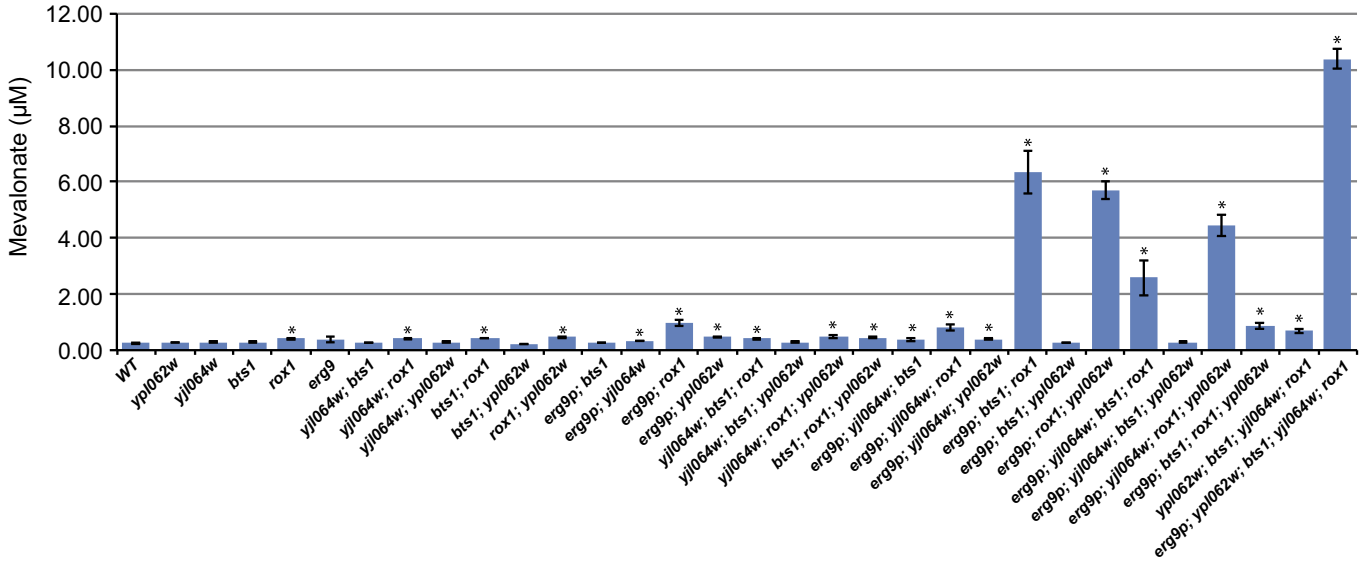

Fig. 3. Mevalonate levels of CRISPR/Cas9 genome engineered strains compared to wild-type *Saccharomyces cerevisiae*. Mevalonate levels were measured by LC–MS according to Rodriguez et al. (2014). The mevalonate data are average of 3 biological replicas with error bars representing standard deviation and * denoting mevalonate levels significantly different ($P < 0.05$) from wild-type (WT) levels.

for expression of Cas9 and gRNA(s) is easily accomplished by a single restreaking of colonies onto non-selective medium. This we tested, and found >10% of the colonies to be auxotrophic for selection markers (LEU/TRP) when transferred back to selective plates (data not shown).

In summary, our data highlight a facile vector construction pipeline suitable for highly efficient combinatorial analyses of 1–5 targeted genetic modifications in one transformation step.

3.5. Optimizing mevalonate pathway activity using CRISPR/Cas9

Following off-target analysis and successful combinatorial genome engineering of our five selected target sites, we analyzed mevalonate levels in wild-type and all 31 mutant strains (Supplementary Table 3) (Rodriguez et al., 2014). From this analysis we identified 20 strains with significantly higher mevalonate levels (average between 0.34 and 10.38 μM) compared to the wild-type strain (0.25 μM) (Fig. 3). Increases ranged from 1.4- to 41.5-fold, whereas no significant decreases compared to the wild-type strain were observed in any of the 31 mutant strains. In accordance with Özaydın et al. (2013), single deletion of *rox1* significantly affected mevalonate levels. In fact all 16 strains having the *rox1* deletion showed significantly increased mevalonate levels (Fig. 3). Similarly, though deletion of the 44-bp UAS of the *erg9* promoter only caused a minor insignificant increase in mevalonate levels, 12 out of 15 strains having the UAS-deleted *erg9* promoter in combination with other single or multi-gene disruptions showed significantly increased mevalonate levels (Fig. 3). Interestingly, combinatorial effects on mevalonate levels identified from multiple knock-outs were also observed. For instance, the quadruple mutant *erg9*;*yjl064w*;*rox1*;*ypl062w* showed higher mevalonate levels compared to the triple mutant *erg9*;*yjl064w*;*rox1* (4.46 vs 0.81 μM), whereas the *erg9*;*bts1*;*rox1* triple mutant showed much higher mevalonate levels compared to the quadruple mutant *erg9*;*bts1*;*rox1*;*ypl062w* (6.33 vs 0.88 μM). This indicates that *ypl062w* can account for both synergistic and antagonistic effects on mevalonate levels depending on genetic background. Likewise, both *erg9;bts1;rox1* and *erg9;yjl064w;bts1;rox1* show higher mevalonate levels compared to *erg9;rox1* and *erg9;yjl064w;rox1* (approx. 6- and 3-fold, respectively) (Fig. 3). However, though the single knock-out of *bts1* restrains flux towards isoprenoid production (Özaydın et al., 2013) (Fig. 1c), single *bts1* knock-out strains did not show increased mevalonate levels.

In conclusion, the mevalonate levels in the 31 mutant strains generated in this study, especially triple, quadruple and quintuple edited strains, validate our exploratory analyses for high-mevalonate producing strains and identifies combinatorial genome edits suitable for further metabolic engineering to improve bio-based production favored by high mevalonate levels and/or pathway flux.

4. Discussion

In this study we present a powerful tool for multiplex genome engineering using CRISPR/Cas9. We have demonstrated our combinatorial approach to (i) be a facile and efficient cloning procedure for vectors expressing single and combinatorial gRNA cassettes, (ii) be highly efficient for specific targeting of up to 5 different loci in one transformation step, and (iii) be applicable for combinatorial engineering of metabolic pathways targeting both gene knock-outs and promoter engineering. With respect to the latter, this study included the marker-free generation of 31 yeast strains with all possible combinations of single, double, triple, quadruple and quintuple genomic edits.

From our list of engineered strains, we identified a set of high-mevalonate producing strains, with the highest mevalonate titers obtained in strains with 3 or more genomic edits (Fig. 3). Though our genome engineering targets were all selected based on previous studies reporting the importance of these targets to perturb isoprenoid production (Asadollahi et al., 2010; Paradise et al., 2008; Ro et al., 2006; Scalcinati et al., 2012; Verwaal et al., 2007; Özaydın et al., 2013), combining all of the edits as outlined in this study would have been tedious by use of conventional marker-assisted sequential gene disruptions and promoter engineering. Specifically, though Özaydın et al. (2013) did identify four single gene deletion strains with increased isoprenoid levels, only 7 strains having all possible combinations of single, double and triple gene disruptions were targeted using time-consuming marker-assisted integration of PCR-amplified knock-out cassettes. Using the procedure outlined in this study, going from gRNA expressing vectors to the generation of the 31 pathway engineered

Table 1
Comparison of different methods for multiple gene disruption using CRISPR/Cas9.

	HI-CRISPR (Bao et al., 2014)	CRISPRm (Ryan et al., 2014)	This study
Genes disrupted	1 and 3	1, 2, and 3	1, 2, 3, 4 and 5
Best efficiencies reported (in parenthesis number of colonies screened)	1: 100% (16) 3: 100% (7)	1: 100% (16–18) [a] 2: 86% (97) [b] 3: 81% (108) [b]	1: 100% (8) 2: 100% (4) 3: 100% (4) 4: 100% (4) 5: 100% (5)
Time spent	4 days	2 days [c]	5 days [d]
Avrg time/gene	1.33–4 days	0.66–2 days	1–5 days
Labor intensity for efficient cloning of array and multiple gRNA cassettes	Low	Medium	Low
Other constraints	Golden gate target site dependency One plasmid-born marker	Both restriction-free and non-directional restriction-based ligation cloning One plasmid-born marker Co-transformation of donor DNA	USER-cloning dependent Two plasmid-born markers Co-transformation of donor DNA

[a] Also reported for a single genomic locus of polyploid yeast.
[b] In diploid strains these numbers were 43% and 19%, for two- and three-loci efficiencies, respectively.
[c] Replica plating onto phenotype-selective media not included.
[d] This also includes time allocated for single transformation of the Cas9 expressing plasmid prior to transformation of gRNA expressing plasmid. If a Cas9 expressing strain is already present, 1-5 genomic edits can be accomplished in 2 days.

strains is amenable within a single week. In support of this genome engineering procedure, the highest mevalonate accumulation obtained by Özaydın et al. (2013) was approx. 4-fold (30–35 mg/L) higher compared to the parental strain having *erg9* expressed from the weak *cyc1* promoter (7–8 mg/L), and expressing the tHMG1 along with an AgBIS∼ERG20 fusion. Though not directly comparable, our highest mevalonate titers were >41-fold higher (approx. 1.5 mg/L) than the wild-type strain (approx. 0.03 mg/L), and >26-fold higher than our *erg9* promoter engineered strain (approx. 0.06 mg/L) (Fig. 3). This information is useful for further engineering of high performing isoprenoid producing strain. For instance, we showed that the quintuple engineered strain *erg9;yjl064w;rox1;ypl062w;bts1* accumulated in excess of two-fold more mevalonate than the *erg9;yjl064w;rox1;ypl062w* quadruple mutant strain. This could indicate that *bts1* gene disruption could be a valid approach to redirect flux from GGPP towards for instance bisabolene production, an isoprenoid with diesel-like properties (Peralta-Yahya et al., 2011; Özaydın et al., 2013). Testing a subset of our high mevalonate strains for improved isoprenoid production is currently being carried out in our laboratory.

Another interesting finding from our study is the regulatory potential of the two uncharacterized proteins encoded by *ypl062w* and *yjl064w*. For instance, the triple mutant strain *erg9p;rox1;ypl062w* had the third highest accumulation of mevalonate of all strains tested, whereas only minor increases were observed from either the *erg9p;rox1*, *erg9p;ypl062w* or the *rox1;ypl062w* double mutant strains (Fig. 3). The *ypl062w* mutant was previously reported to have very low levels of glycogen (Wilson et al., 2002), and it has been speculated that when less carbon is sequestered in the form of glycogen, this could increase the acetyl-CoA availability for the mevalonate pathway (Özaydın et al., 2013), especially in combination with derepressed mevalonate pathway flux (*rox1*) and potential re-direction from squalene and ergosterol synthesis (*erg9p*). With respect to *yjl064w* no reports exist for its functional annotation apart from the increased carotenoid and bisabolene levels observed in *yjl064w* knock-out strains (Özaydın et al., 2013). We also consider it relevant to mention that the chromosome annotation of *yjl064w* completely overlaps the *dls1* gene, encoding a subunit of the chromatin-remodeling complex, ISW2/yCHRAC (Iida and Araki, 2004). Further studies will be needed to improve our understanding of the effect on mevalonate accumulation in *yjl064w* mutant strains.

During preparation of this manuscript, two other CRISPR/Cas9 studies in yeast were published addressing the efficiency of targeting up to three different loci by CRISPR/Cas9 genome engineering (Bao et al., 2014; Ryan et al., 2014). In the CRISPRm method published by Ryan et al. (2014) single and triple gene disruptions were obtained in haploid *S. cerevisiae* strain S288C with efficiencies of 100% and 81%, respectively (Table 1), whereas Bao et al. (2014) reported 100% gene disruption efficiency targeting both single and triple sites (Table 1). Interestingly, in these two studies, two different approaches were used to stabilize and multiplex gRNA cassette expression. For CRISPRm, Ryan et al. (2014) used a self-cleaving HDV ribozyme fused to the gRNA to control cellular levels of correctly folded gRNAs, whereas as Bao et al. (2014) made use of an ingenious design fusing HR donor and gRNA into a single commercially ordered expression cassette (spacer) which could then be assembled in vitro with more spacers to construct a CRISPR array for targeting up to 3 loci from a single HI-CRISPR vector. Compared to the CRISPRm and HI-CRISPR methods, our facile USER-based cloning procedure allows for a complementary design of single gRNA expression cassettes (Fig. 2A) able to target up to 5 different loci in a comparable amount of time (Fig. 3, Supplementary Fig. 1 and Table 1). Most importantly, the results obtained in our study highlight the possibility to increase the number of genomic targets amenable to one-step CRISPR/Cas9 engineering in yeast. The application of this possibility is evident, as 5 out of 6 of our quadruple and quintuple edited strains had significantly higher mevalonate levels compared to wild-type levels (Fig. 3).

Though our current study is limited to five simultaneous genome edits, seven simultaneous genomic edits have been reported in mice and insect cells (Liu et al., 2014; Sakuma et al., 2014; Wang et al., 2013; Yu et al., 2013). In yeast, DiCarlo et al. (2013b) observed that selection of cells containing both gRNA and constitutively expressed Cas9 resulted in a reduction of transformation frequency as compared with cells with only Cas9 expressed (DiCarlo et al., 2013b). In addition to this, we also observed fewer colonies when targeting multiple sites compared to single and double targeting (Supplementary Fig. 1). Even though we recovered sufficient numbers of colonies with desired modifications, efficiently targeting more than five different loci simultaneously could possibly cause insufficient numbers of colonies with desired modifications indicating the effects of genome instability and/or toxicity by constitutively expressed Cas9.

Finally, one concern encountered when applying this homology-directed genome engineering approach mediated by CRISPR/Cas9 is

the cost of 90-bp dsOligo synthesis for individual targets (approx. 100 USD/dsOligo). As metabolic engineering and synthetic biology designs become more and more complex there is an inherent need to reduce genome engineering costs. To this end it would be advisable to test the transformation efficiency of various dsOligo lengths, as it is well-known that the recombination frequency is determined by the length of the homology regions (Matsubara et al., 2014). Furthermore, the recently reported use of microarray-based oligo library synthesis used for MAGE in *Escherichia coli* (Bonde et al., 2014) should also be considered as a cost-effective alternative to traditional column-based synthesis.

However, as DNA synthesis prices are continuing to drop, the application of our CRISPR/Cas9-mediated tool significantly advances the state-of-the-art in the currently applied marker-based sequential strain engineering step of the design-build-test cycle adopted in modern metabolic engineering (Van Dien, 2013). With the marker-free and multiplex option reported in this study, the effect of model-guided and/or rationally designed genomic edits can be assessed much faster. Combined with high-throughput screening assays, all potentially beneficial genomic edits could be screened and allow for a much faster turn-around time of the design-build-test cycle. Thus, our toolkit opens the doors for drastically downsizing the workload of strain engineering and we therefore anticipate our genome engineering platform to have major applicability within the yeast metabolic engineering and functional genomics communities.

5. Conclusions

Here we demonstrate an easy vector tool allowing for single and multiplex genome editing using targeted CRISPR/Cas9-mediated DSB and homology-directed repair. Our one-step marker-free genome editing approach allows for efficient gene disruption and promoter engineering of 1–5 target loci. Also, by re-sequencing genomes with single edits in 5 different loci we conclude that the homology-directed repair of CRISPR/Cas9-mediated DSBs is highly unlikely to leave off-target edits in viable transformants. In order to apply CRISPR/Cas9 for metabolic engineering we performed an exploratory analysis of mevalonate levels by assessing the combinatorial effects of multiplex genome edits. This revealed a >41-fold increase for the highest mevalonate producing strain, obtained without any further heterologous expression of genes in the mevalonate pathway. Our work offers not only an efficient vector construction and multiplex genome engineering pipeline, but also a set of high-mevalonate mutant strains ready for further metabolic engineering towards production of industrially relevant isoprenoid-derived chemicals and fuels.

Contributions

T.J., M.K.J. and J.D.K conceived and designed the experiments. T.J., S.H. and M.K. performed the experimental work. S.H., I.B and M.H. analyzed the LC–MS and MiSeq data. M.K.J. and T.J. wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2015.01.008.

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