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FEBS Letters

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Exploiting off-targeting in guide-RNAs for CRISPR systems for simultaneous editing of multiple genes

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(Received 9 May 2017, revised 21 July 2017, accepted 31 August 2017, available online 18 September 2017)

doi:10.1002/1873-3468.12835

Edited by Alfonso Valencia

Bioinformatics tools to design guide-RNAs (gRNAs) in Clustered Regularly Interspaced Short Palindromic Repeats systems mostly focused on minimizing off-targeting to enhance efficacy of genome editing. However, there are circumstances in which off-targeting might be desirable to target multiple genes simultaneously with a single gRNA. We termed these gRNAs as promiscuous gRNAs. Here, we present a computational workflow to identify promiscuous gRNAs that putatively bind to the region of interest for a defined list of genes in a genome. We experimentally validated two promiscuous gRNA for gene deletion, one targeting *FAA1* and *FAA4* and one targeting *PLB1* and *PLB2*, thus demonstrating that multiplexed genome editing through design of promiscuous gRNA can be performed in a time and cost-effective manner.

Keywords: computational tool; CRISPR; synthetic biology

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is a recent technology which has proven highly efficient for editing and regulating genes in diverse organisms [1–4]. This system works with a nuclease, Cas9 (CRISPR associated protein 9), which is directed to target sites in the genome by a single guide-RNA (gRNA) [5–7]. The Cas9:gRNA complex binds to a 20 base pair (bp) target sequence followed by a 3 bp Protospacer Adjacent Motif (PAM) 'NGG'. Once the complex is bound to its target, the Cas9 cleaves the DNA sequence 3 bp upstream the PAM sequence [8,9]. There has been substantial experimental and computational effort in optimizing the efficiency of Cas9:gRNA complex in editing the intended target sequence [10–14]. In particular, diverse predictive algorithms were put forward in the design of gRNA that minimize off-targeting, which occurs due to similar sequences in the genome, and optimize its specificity to the target sequence [15–20].

Here, we propose to reverse this paradigm and exploit off-targeting of gRNAs for applications where this feature is desirable. For example, when large pathways involving multiple genes are to be studied, generating individual gRNAs against each target in the pathway can be cumbersome and time consuming. Thus, the design of a gRNA capable of targeting multiple targets at once would greatly accelerate the process. Few tools available today exploit off-targeting of gRNAs. Given a list of target genes of interest, CRISPR MultiTargeter aligns the target genes' coding sequence (CDS) to the rest of the genome and retrieves similar patterns that contain a PAM sequence [19]. However, this algorithm is intended to identify a gRNA able to target all such patterns simultaneously or the output will be null.

Abbreviations
CDS, coding sequence; CPU, central processor unit; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; FFAs, free fatty acids.

Therefore, if the list of target genes is too large (> 3), there is a high chance that no such gRNA will be found. Also, CRISPR MultiTargeter does not exploit noncoding regions associated with the list of target genes, hindering the possibility of using this tool to design gRNAs for CRISPRi applications [4,21]. Similarly, CRISPR-Seek, allows to compare two sequences and retrieve gRNAs targeting to either one or two of the sequences. However, the tool is limited to a maximum of two sequences targeted [18].

In this study, we explore the idea to search for gRNAs with multiple targets and compare this to the desired list of target genes. We implemented an algorithm to identify such gRNAs—termed promiscuous gRNAs—and validated our idea to perform multiple gene deletions and insertions using a single experimental gRNA in *Saccharomyces cerevisiae*.

Methods

Plasmid and strain construction

Primers and repair fragments used for this study are listed in Table 1. Gene deletions were performed in the strain IMX581 using CRISPR/Cas9. IMX581 and the CRISPR plasmid backbone (pMEL13: http://www.euroscarf.de/plasmid_details.php?accno=P30782) were obtained from EUROSCARF (Frankfurt, Germany). The diagnostic primers and the repair fragments were designed using the Yeastriction web tool (http://yeastriction.tnw.tudelft.nl/) [22]. Diagnostic primers were designed to target upstream and downstream the target CDS in order to differentiate between correct and incorrect deletion *via* PCR. Repair fragments of 120 bp were designed to bind on the flanking regions (promoters and terminators) of the studied genes. DNA Oligos fragments were ordered from EUROFINS Scientific. GRNAs were cloned in pMEL13 *via* Gibson Assembly cloning with 100 bp homologous recognition sequence with the plasmid backbone. Simultaneous transformation with pMEL13-gRNA and the repair fragment resulted in the *in vivo* double-strand cut in the loci genes by the Cas9 nuclease. The double-strand break allowed integration of the repair fragments by homologous recombination. Genomic DNA extraction was performed using LiAc/SDS 1% protocol [23]. Colony PCR amplification was performed on randomly picked yeast colonies using DreamTaq polymerase (Thermo Fisher Scientific, Waltham, MA, USA). A 30-cycle PCR protocol was used as followed: (a) 95 °C for 30 s, (b) 55 °C for 30 s, and (c) 72 °C for 2 min. DNA fragments amplified were analyzed by gel electrophoresis on 1% (w/v) agarose gels TAE buffer (Thermo Fisher Scientific).

Table 1. Oligos used for this study.

Name	Primer sequence 5′→3′	Comments
01	TACGAATGACACAGGGGCAC	Binding upstream FAA1 CDS
02	AAGGAGTCAGTGCACACCAG	Binding downstream FAA1 CDS
03	TGCAACCCCTCTGAGTTGAC	Binding upstream FAA4 CDS
04	TGCATAAATGAACGTGGGCG	Binding downstream FAA4 CDS
05	GCACGGACTTCTTCAAAGCG	Binding upstream PLB1 CDS
06	TCGCCAAGCTATTGAGGACC	Binding downstream PLB2 CDS
FAA14_gRNA	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAAA TGATCGTTTGGGAAACAGTGAGAAAGTTTTAGAGCTAGAAAT AGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC	Oligo used for cloning promiscuous FAA1/4 gRNA into pMEL13 vector
PLB12_gRNA	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAG ATAAATGATCTCCCATGAACCCATTTCAAAGTTTTAGAGCTAG AAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC	Oligo used for cloning promiscuous PLB1/2 gRNA into pMEL13 vector
FAA1_repair-F	AAACTCGTTAGGATACAATAAAAACTAGAACAAACACAAA AGACAAAAAAAGACAACAATTGGATCAACATTTCCATGATAG GAAAGCCTCATCATACTAAAGCACTTTTTCAGTTTTTT	120 bp repair oligo binding both the promoter and terminator region of FAA1
FAA1_repair-R	AAAAAACTGAAAAAGTGCTTTAGTATGATGAGGCTTTCCT ATCATGGAAATGTTGATCCAATTGTTGTCTTTTTTTGTCTTTTG TGTTTGTTCTAGTTTTTATTGTATCCTAACGAGTTT	120 bp repair oligo binding both the promoter and terminator region of FAA1
FAA4_repair-F	CACATCATTTTTTTCTCTGTTCTTCACTATTTCTTGAAAAACTAA GAAGTACGC ATCAAAAGGAAGACATAGTTTTTTACTTTCCCCCCTGCCCTTC ATAAACACTACGTTTCATTTTCT	120 bp repair oligo binding both the promoter and terminator region of FAA4
FAA4_repair-R	AGAAAATGAAACGTAGTGTTTATGAAGGGCAGGGGGGAAAGTAAAAAACT ATGTCTTCCTTTTGATGCGTACTTCTTAGTTTTTCAAGAAATAG TGAAGAACAGAGAAAAAAATGATGTG	120 bp repair oligo binding both the promoter and terminator region of FAA4

Growth medium

S. cerevisiae strains with uracil auxotrophy were grown on YPD plates containing 20 g·L^{-1} glucose, 10 g·L^{-1} yeast extract, 20 g·L^{-1} peptone from casein and 20 g·L^{-1} agar. For the gene engineering processes, plasmid-carrying strains were grown on YPD plates with 200 mg·L^{-1} Geneticin® (registered trademark of Invitrogen Corp., Carlsbad, CA, USA).

Shake flask batch fermentations were carried out in minimal medium containing 5 g·L^{-1} $(NH_4)2SO_4$, 14.4 g·L^{-1} KH_2PO_4, 0.5 g·L^{-1} $MgSO_4{\cdot}7H_2O$, 20 g·L^{-1} glucose, trace metal, and vitamin solutions supplemented with 60 mg·L^{-1} uracil [24]. Cultures were inoculated, from 24 h grown precultures, into 10 mL minimal medium and cultivated at 200 r.p.m., 30 °C for 72 h. Cultures were then transferred into a falcon tube (50 mL) and centrifuged at 2000 ***g*** for 5 min.

Computational workflow

The workflow consists of four steps: selection of target region; extraction of gRNAs; alignment of gRNAs to all regions; analysis of promiscuous gRNAs (Fig. 1B). The workflow was implemented in the R language (v 3.3.1) under OS X 10.11.6, platform x86_64-apple-darwin13.4.0 (64-bit).

The first step loads a genome sequence and then extracts the locations of either each chromosome sequence or, for

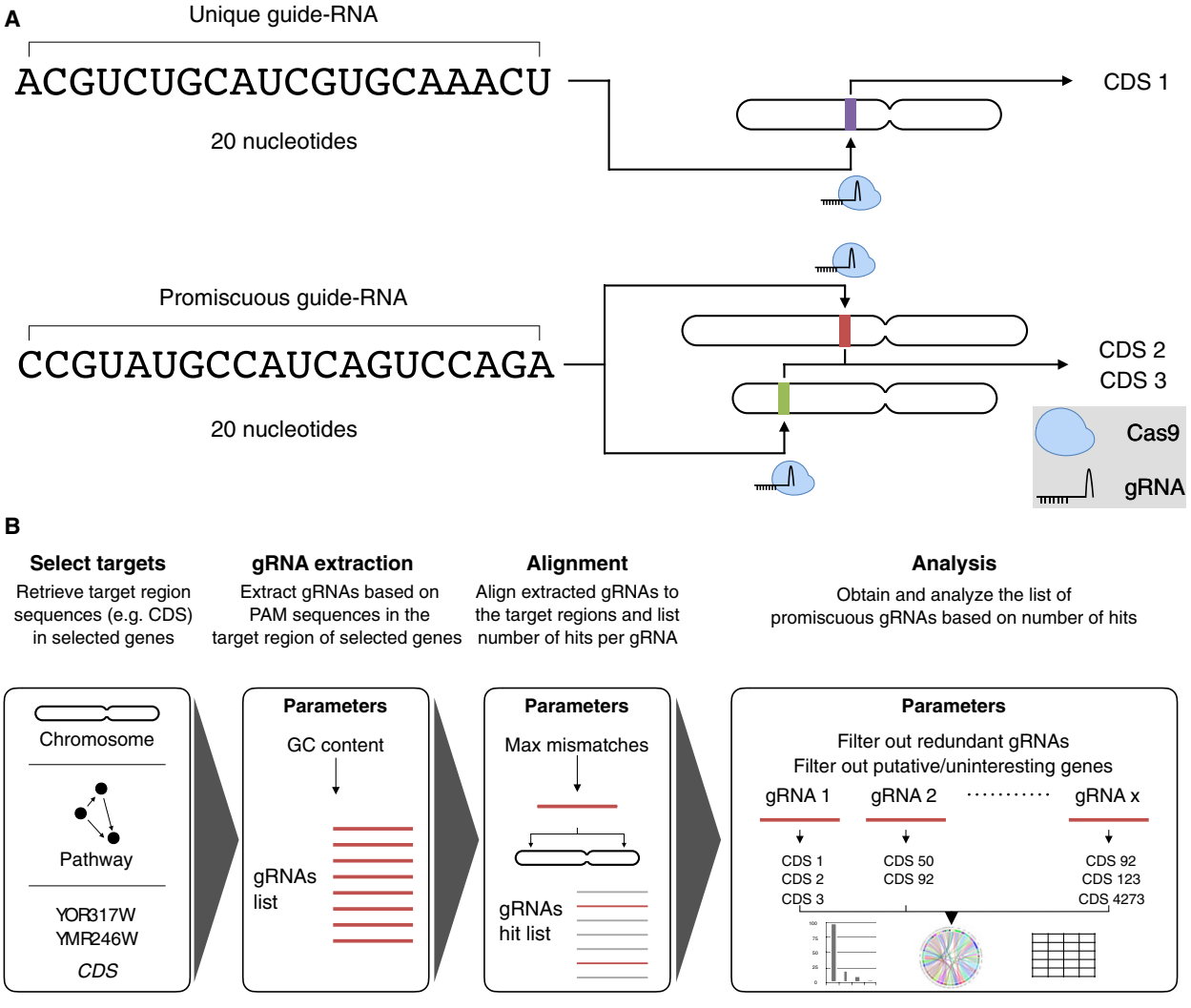


Fig. 1. Promiscuous gRNAs and the computational workflow for their identification. (A) Graphical definition of a promiscuous guide-RNA. (B) Workflow of the algorithm. In the first step, the target region of all or a selected list of genes in a genome is retrieved. Next, gRNAs are extracted from the target regions. Poor quality gRNAs (low GC content) can be filtered out. Successively, all extracted gRNAs are aligned to all regions initially retrieved and if the number of mismatches is lower than a user-defined threshold (max mismatched) than a hit is recorded. Finally, gRNAs with a number of hits greater than 1 are identified as promiscuous gRNAs and their targets are returned.

each or selected genes in the genome, the promoter region (500 bp upstream to 0 bp downstream) or the CDS (0 bp upstream to length of ORF downstream). Subsequently, the genetic sequence for each located target regions is extracted. This step was implemented for any genome available in the R library GENOMICFEATURES (Default is *S. cerevisiae* genome). The process has been parallelized using the parallel R library and utilizes the R libraries GENOMICFEATURES v1.24.5 and BSGENOME v1.44 to load the genome and locate and extract the genetic sequences.

The second step locates all PAM sequences in each target region and extracts a 20-bp gRNA upstream. gRNAs with a GC content (in %) lower than a user-defined value (default: 10%) are filtered out. This step used the R library BIOSTRINGS v2.40.2 to retrieve sequences and SEQINR v3.3-3 to compute the GC content.

The third step aligns all extracted gRNAs to all target regions. Each gRNA is aligned to each target region genetic sequence and its complement and a match is found (hit) if the alignment between sequences contained less than the user-defined maximum number of mismatches (default: 0 a.k.a. exact match). The number of hits per target region is recorded as the sum of hits in the *cis* and in the *trans* strand. This step returns a $r \times g$ matrix, where r is the number of target regions and g is the number of gRNAs and each entry is the number of hits of a certain gRNA in the *cis* or *trans* sequence of a certain target region. Note that in principle the same gRNA may match the sequence of target region of the same gene more than once, so the upper bound of each entry is greater than 1. The process can be parallelized by splitting the output matrix into n chunks corresponding to the number of cores so that in each core g/n gRNAs are aligned to the target regions. Given that g can be fairly high (> 100 000), the resulting matrix may reach a considerable size. This step was implemented using the R library Biostrings v2.40.2.

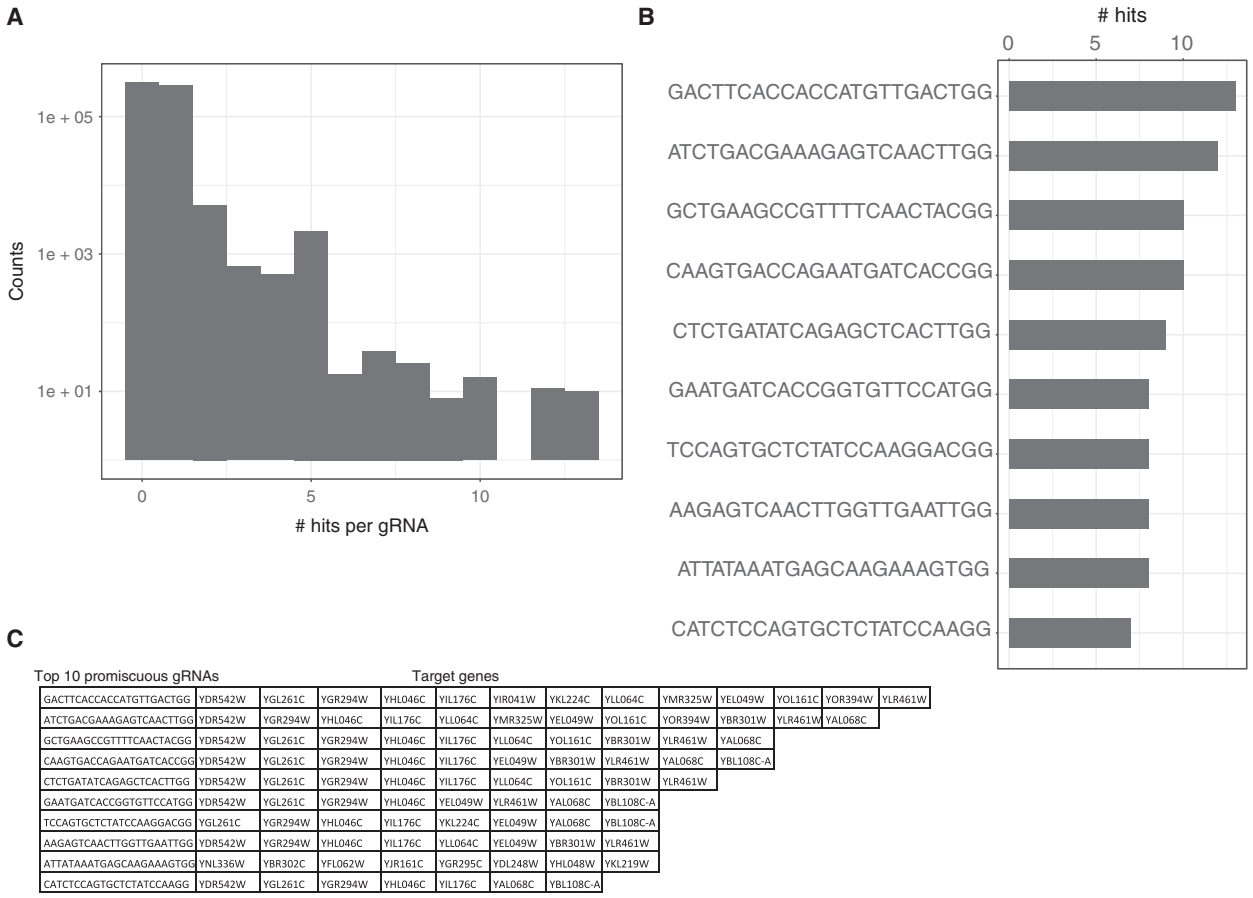
The fourth and last step analyzes the hits and returns the top gRNAs. Plots with the distribution of frequency and density of number of hits and a table with the top n promiscuous gRNAs sorted by number of hits is returned (n is user-defined, 100 by default). Before the analysis, the user can filter out target regions for which promiscuity is not desired and set the maximum number of hits per gRNA (default: 200 000). Finally, the top n gRNAs are aligned back to target regions and the corresponding genes are recorded in a table. This step was implemented using the R libraries GGPLOT2 2.2.1 and RESHAPE2 1.4.2 for plotting.

The workflow is made available through a public Github repository: https://github.com/gattofrancesco/pgRNA.

Results

We designed a computational workflow to explore the idea that off-targeting effects in gRNAs can be beneficial in applications where editing multiple genes simultaneously is desirable. The purpose of the core algorithm was to retrieve gRNAs matching more than one target, which was defined as the off-targeting effect. We refer to such gRNAs as promiscuous gRNAs (Fig. 1A). The target was defined as a region of interest in the list of target genes of interest, for example, CDS or promoter regions or chromosome sequences. Given a list of genes or a full genome sequence as input, the algorithm extracts all gRNAs by locating the 'NGG' PAM sequence in the target region (Fig. 1B). Poor-quality gRNAs, such as gRNAs with repetitive nucleotides, are filtered out by imposing a minimal GC content. In *S. cerevisiae*, a typical list of the retrieved gRNAs can vary from 195 elements for the CDS of a single gene like *FAA4* to 801,648 for all 16 nuclear chromosome coding and non-CDSs (Fig. S1). Next, each gRNA is aligned back to all the original targets, and any gRNA with a number of matches (hits) superior to 1 is recorded as promiscuous gRNA. If needed, the probability for a gRNA to find extra hits can be increased by tuning the maximum number of mismatches allowed (zero by default). Finally, the hits are analyzed, and the list with the most promising promiscuous gRNAs, for example, with highest number of hits or targeting multiple genes of interest, is returned. The workflow was implemented in R and is made available on GitHub: https://github.com/gattofrancesco/pgRNA.

We first sought to characterize a dry run of the computational workflow. We chose to identify promiscuous gRNAs from every known CDS based on the nuclear genome sequence of *S. cerevisiae*. We extracted 624,956 gRNAs from the CDS in 6496 genes. The bottleneck in central processor unit (CPU) time was represented by the step in which the extracted gRNAs are aligned to each CDS. The elapsed CPU time on a single 2.2 GHz core is on average 1.63 s per CDS to align all gRNAs. The resulting list of promiscuous gRNAs showed a significant number of hits in the CDS of Ty retrotransposons, tRNA, and putative proteins regions. The gRNA with the highest number of hits bound to 39 distinct transposons. Since these elements are difficult to study experimentally, we shrunk the list of targets by filtering the CDS with 5105 annotated genes. After filtering, of the initial 624 956 extracted gRNAs, 597 378 (96%) gRNAs were not promiscuous as they either had no or only one hit, 8665 (1%) had two or more targets, and 18 913 (3%) could not be aligned (Fig. 2A). The most promiscuous gRNA matched the CDS of 13 distinct genes (Fig. 2B). We observed that for the top 10 promiscuous gRNAs most hits were targeted toward paralog genes, consistent with the notion that paralogs share



Top 10 promiscuous gRNAs	Target genes												
GACTTCACCACCATGTTGACTGG	YDR542W	YGL261C	YGR294W	YHL046C	YIL176C	YIR041W	YKL224C	YLL064C	YMR325W	YEL049W	YOL161C	YOR394W	YLR461W
ATCTGACGAAAGAGTCAACTTGG	YDR542W	YGR294W	YHL046C	YIL176C	YLL064C	YMR325W	YEL049W	YOL161C	YOR394W	YBR301W	YLR461W	YAL068C	
GCTGAAGCCGTTTTCAACTACGG	YDR542W	YGL261C	YGR294W	YHL046C	YIL176C	YLL064C	YOL161C	YBR301W	YLR461W	YAL068C			
CAAGTGACCAGAATGATCACCGG	YDR542W	YGL261C	YGR294W	YHL046C	YIL176C	YEL049W	YBR301W	YLR461W	YAL068C	YBL108C-A			
CTCTGATATCAGAGCTCACTTGG	YDR542W	YGL261C	YGR294W	YHL046C	YIL176C	YLL064C	YOL161C	YBR301W	YLR461W				
GAATGATCACCGGTGTTCCATGG	YDR542W	YGL261C	YGR294W	YHL046C	YEL049W	YLR461W	YAL068C	YBL108C-A					
TCCAGTGCTCTATCCAAGGACGG	YGL261C	YGR294W	YHL046C	YIL176C	YKL224C	YEL049W	YAL068C	YBL108C-A					
AAGAGTCAACTTGGTTGAATTGG	YDR542W	YGR294W	YHL046C	YIL176C	YLL064C	YEL049W	YBR301W	YLR461W					
ATTATAAATGAGCAAGAAAGTGG	YNL336W	YBR302C	YFL062W	YJR161C	YGR295C	YDL248W	YHL048W	YKL219W					
CATCTCCAGTGCTCTATCCAAGG	YDR542W	YGL261C	YGR294W	YHL046C	YIL176C	YAL068C	YBL108C-A						

Fig. 2. Distribution of promiscuous gRNAs targeting annotated CDS in *S. cerevisiae*. (A) Frequency of the number of hits per gRNAs. (B) Top 10 gRNAs with the highest number of hits. (C) Table with the corresponding genes targeted by the promiscuous gRNAs.

high sequence similarity due to genetic duplication events thereby increasing the chance that a single gRNA binds to multiple CDS (Fig. 2C). The workflow performed a global search thereby allowing to explore if any promiscuous gRNA targets a list of genes of interest without requiring to target all of them simultaneously in contrast to CRISPR MultiTargeter. For example, if it were of interest to edit all 10 members of the SOX gene family, we identified 29 promiscuous gRNAs including a gRNA that targets four of them simultaneously (Fig. S2). In comparison, CRISPR MultiTargeter would not return any hit because there are no gRNAs that simultaneously match all 10 SOX family genes.

We next set out to validate experimentally two sets of predicted promiscuous gRNAs. From the output list, we restricted our search to promiscuous gRNAs targeting two genes in yeast metabolism, and tested their efficacy *via* gene deletion (Fig. 3A). In a first experiment, we selected a gRNA targeting two of the fatty acid activation genes, *FAA1* and *FAA4* (Fig. 3B). Previous studies have reported that only the simultaneous deletion of *FAA1* and *FAA4* results in a deregulation of the fatty acid biosynthesis, which leads to an accumulation and secretion of free fatty acids (FFAs) [25,26]. We cotransformed a plasmid expressing constitutively one of the promiscuous gRNA together with two repair fragments of 120 bp which recombine to the flanking regions of the selected genes. The resulting transformants were randomly tested by colony PCR in order to detect the correct deletion of the genes. We observed successful deletion of both *FAA1/4* in all six tested colonies (Fig. 3C). We further observed secretion of FFAs in these transformants as opposed to the wild-type strain (Fig. 3D). This is in line with previously reported results which showed that both *FAA1* and *FAA4* must be simultaneously deleted to produce this phenotype. In a second experiment, we selected a promiscuous gRNA targeting two of the major phospholipases B genes, *PLB1* and *PLB2* (Fig. S3A) [27]. Since *PLB1* and *PLB2* are adjacently located on the same chromosome, the cuts generated by the

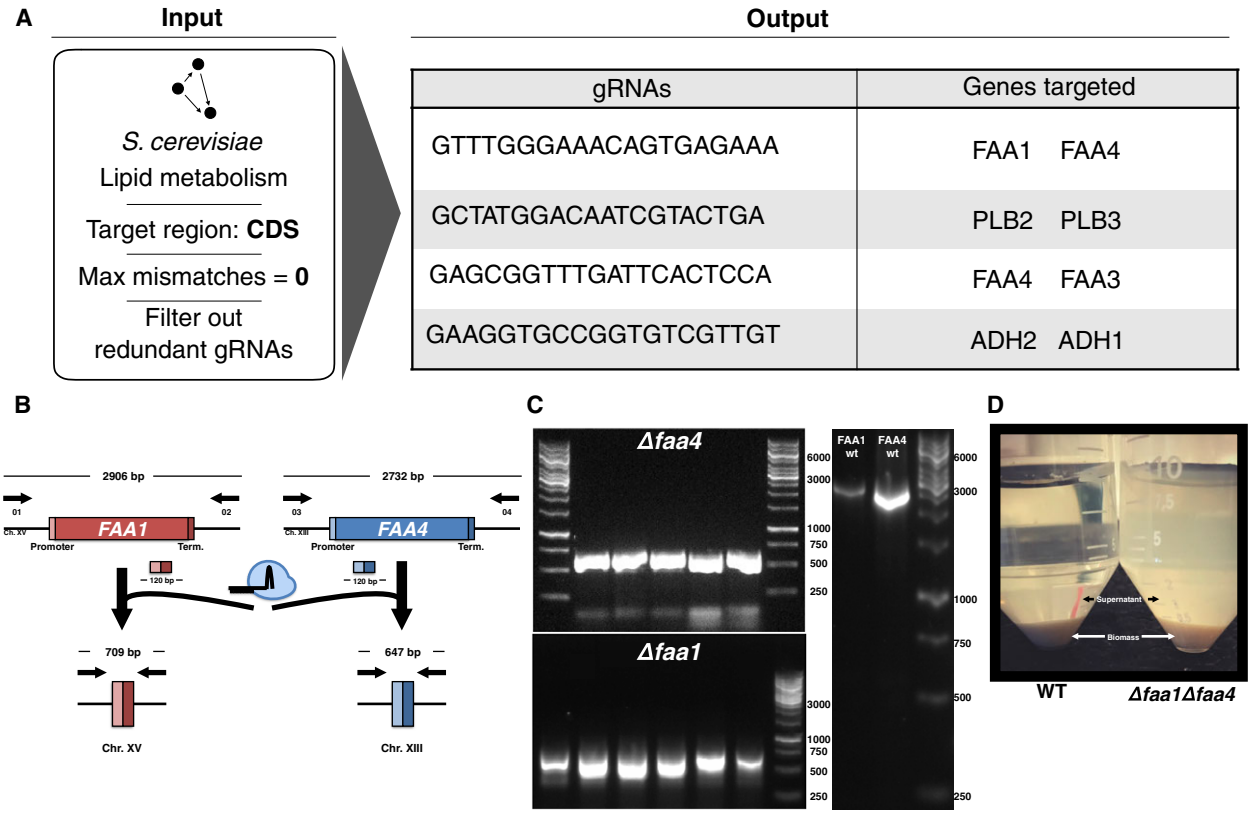


Fig. 3. Experimental validation of selected promiscuous gRNAs by gene knockouts. (A) Promiscuous gRNAs targeting the CDS region of genes in *S. cerevisiae* lipid metabolism (139 genes) with 0 mismatches were identified using the proposed workflow and gRNAs targeting genes of interest were so analyzed. (B) Experimental validation of gene knockouts with a promiscuous gRNA targeting both FAA1 and FAA4. The strain is cotransformed with the plasmid expressing the promiscuous gRNA, together with repair fragments of 120 bp matching the flanking regions of the gene targeted. (C) Confirmation by colony PCR on gel agarose. Correct deletions for *FAA1* and *FAA4* show a PCR of 709 and 647 bp, respectively. The control shows bands at 290 and 2732 bp for *FAA1* and *FAA4*, respectively. (D) Phenotypic comparison between the WT and *Δfaa1 Δfaa4* strains. The cells were grown in 10 mL minimal media for 72 h. After centrifugation, 1.5 mL of the supernatant was transferred into a vial. Here, the deletion of two of the main fatty acyl-CoA synthetases encoding genes FAA1 and FAA4 leads to a secretion of FFAs in the medium which explains the blurriness in the *Δfaa1Δfaa4* background strain.

promiscuous gRNA prevented the repair fragments to correctly loop-out the genes (Fig. S3A). Nevertheless, it led to a partial deletion of both genes by nonhomologous end joining, which we noted in all the six tested colonies (Fig. S3B).

Discussion

While most computational tools for CRISPR are primarily concerned in the design of gRNAs that minimize off-target effects, here we reversed this concept and successfully designed gRNAs efficiently targeting multiple targets simultaneously. So-termed promiscuous gRNAs have a breadth of potential applications, ranging concurrent editing of multiple genes in a pathway, investigations on phenotypic changes in untested multiple knockout strains, or minimization of redundant genetic material in a genome [28]. For example, for the synthetic yeast chromosome 2.0 (Sc 2.0) project where the objective was to remove all transposons, repetitive and redundant tRNA elements from *S. cerevisiae* genome, our algorithm predicted a single promiscuous gRNAs capable to target 148 of these elements at once [29]. The computational workflow enables flexible selection of the genome of interest, the target region for gRNA binding, and degree of exactness in gRNA alignment. The workflow uses any DNA sequence as input to discover promiscuous gRNAs, and it is therefore designed to be species-independent thus enabling applications. In this study, we validated that the approach was effective to design promiscuous gRNAs that bind to the coding region of multiple genes in *S. cerevisiae* metabolism. We envision that promiscuous gRNAs would be valuable for CRISPR *in vivo* gene editing in more complex organisms, such as mammalian cells, where usually only one

gRNA can be expressed due to restrained DNA packing capacity of viral delivery systems [30].

The key limitation of this approach lies on the impossibility to know *a priori* if a desired list of genes can be edited using a promiscuous gRNA. This trivially depends on the presence of identical or highly similar sequences among the genes of interest that bind to one (or more) of the extracted gRNAs, which explains why most of the multiple targets here found for *S. cerevisiae* belong to the same gene family. Nevertheless, the proposed workflow permits to control the maximum number of mismatches allowed in the alignment between each gRNA and the target regions to increase the chances to find interesting promiscuous gRNAs. However, if the intention is to derive a gRNA that matches exactly a list of genes of interest, then it would be more meaningful and time-effective to adopt other tools like CRISPR MultiTargeter.

In conclusion, this study proposes a computational approach to exploit off-targeting effects of gRNAs to consent multiplexed genome editing in a time and cost-effective fashion.

Acknowledgements

The authors acknowledge the Novo Nordisk Foundation, the Knut and Alice Wallenberg Foundation, and the Swedish Foundation for Strategic Research for funding support.

Authors' contributions

The project was conceived by RF and supervised by FG and JN. RF performed the experiments. RF and FG designed and implemented the algorithm. FG deployed the software. RF and FG drafted the paper. All authors read and approved the final manuscript.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Promiscuous gRNAs targeting coding and noncoding sequences in *S. cerevisiae* nuclear chromosomes.

Fig. S2. Promiscuous gRNAs targeting CDS of SOX1—10 genes in *S. cerevisiae*.

Fig. S3. Experimental validation of *PLB1* and *PLB2* knockouts.