Video Article

Targeted *in Situ* Mutagenesis of Histone Genes in Budding Yeast

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Abstract

We describe a PCR- and homologous recombination-based system for generating targeted mutations in histone genes in budding yeast cells. The resulting mutant alleles reside at their endogenous genomic sites and no exogenous DNA sequences are left in the genome following the procedure. Since in haploid yeast cells each of the four core histone proteins is encoded by two non-allelic genes with highly homologous open reading frames (ORFs), targeting mutagenesis specifically to one of two genes encoding a particular histone protein can be problematic. The strategy we describe here bypasses this problem by utilizing sequences outside, rather than within, the ORF of the target genes for the homologous recombination step. Another feature of this system is that the regions of DNA driving the homologous recombination steps can be made to be very extensive, thus increasing the likelihood of successful integration events. These features make this strategy particularly well-suited for histone gene mutagenesis, but can also be adapted for mutagenesis of other genes in the yeast genome.

Video Link

The video component of this article can be found at <https://www.jove.com/video/55263/>

Introduction

The four core histone proteins H2A, H2B, H3, and H4 play central roles in the compaction, organization, and function of eukaryotic chromosomes. Two sets of each of these histones form the histone octamer, a molecular spool that directs the wrapping of ~147 base pairs of DNA around itself, ultimately resulting in the formation of a nucleosome¹. Nucleosomes are active participants in a variety of chromosome-based processes, such as the regulation of gene transcription and the formation of euchromatin and heterochromatin across chromosomes, and as such have been the focus of intense research over the course of the past several decades. A number of mechanisms have been described by which nucleosomes can be manipulated in ways that can facilitate execution of specific processes – these mechanisms include posttranslational modification of histone residues, ATP-dependent nucleosome remodeling, and ATP-independent nucleosome reorganization and assembly/disassembly^{2,3}.

The budding yeast *Saccharomyces cerevisiae* is a particularly powerful model organism for the understanding of histone function in eukaryotes. This can be largely attributed to the high degree of evolutionary conservation of the histone proteins throughout the domain eukarya and the amenability of yeast to a variety of genetic and biochemical experimental approaches⁴. Reverse-genetic approaches in yeast have been widely used to study the effects of specific histone mutations on various aspects of chromatin biology. For these types of experiments it is often preferable to use cells in which the mutant histones are expressed from their native genomic loci, as expression from autonomous plasmids can lead to abnormal intracellular levels of histone proteins (due to varying numbers of plasmids in cells) and concomitant alteration of chromatin environments, which can ultimately confound the interpretation of results.

Here, we describe a PCR-based technique that allows for targeted mutagenesis of histone genes at their native genomic locations that does not require a cloning step and results in the generation of the desired mutation(s) without leftover exogenous DNA sequences in the genome. This technique takes advantage of the efficient homologous recombination system in yeast and has several features in common with other similar techniques developed by other groups - most notably the *Delitto Perfetto*, site-specific genomic (SSG) mutagenesis, and cloning-free PCR-based allele replacement methods^{5,6,7}. However, the technique we describe has an aspect that makes it particularly well-suited for mutagenesis of histone genes. In haploid yeast cells, each of the four core histones is encoded by two non-allelic and highly homologous genes: for example, histone H3 is encoded by the *HHT1* and *HHT2* genes, and the open reading frames (ORFs) of the two genes are over 90% identical in sequence. This high degree of homology can complicate experiments designed to specifically target one of the two histone-encoding genes for mutagenesis. Whereas the aforementioned methods often require the use of at least some sequences within the ORF of the target gene to drive homologous recombination, the technique we describe here makes use of sequences flanking the ORFs of the histone genes (which share much less sequence homology) for the recombination step, thus increasing the likelihood of successful targeting of mutagenesis to the desired locus. Moreover, the homologous regions that drive recombination can be very extensive, further contributing to efficient targeted homologous recombination.

Protocol

NOTE: The experimental strategy for targeted *in situ* histone gene mutagenesis includes several steps (summarized in **Figure 1**). These steps include: (1) Replacement of the target histone gene with the *URA3* gene, (2) Generation and purification of PCR products corresponding to two partially overlapping fragments of the target histone gene using primers harboring the desired mutation(s), (3) Fusion PCR of the two partially overlapping fragments to obtain full size PCR products for integration, (4) Co-transformation of full size PCR products and backbone plasmid, and selection for marker on plasmid, (5) Screen for 5-FOA-resistant transformants, (6) Purification of 5-FOA-resistant colonies and loss of backbone plasmid, and (7) Molecular analyses to assay for proper integration of the mutant allele.

Figure 1: Overview of the Strategy for Targeted *in situ* Mutagenesis of Histone Genes in Budding Yeast. In this example the targeted gene is *HHT2*, but any other core histone gene can also be mutagenized using this strategy. (A) Haploid yeast cells harbor two histone H3-encoding genes (*HHT1* and *HHT2*) and two histone H4-encoding genes (*HHF1* and *HHF2*) arranged as shown in the figure (the *HHT1* and *HHF1* genes are located on chromosome II and the *HHT2* and *HHF2* genes are located on chromosome XIV - in each case, the arrows point in the direction of transcription). In the first step of the procedure, the ORF of the *HHT2* gene is replaced with the *URA3* gene, giving rise to a *hht2Δ::URA3* strain. (B) In part 1, a wild-type copy of the *HHT2* gene from a genomic DNA sample is used as template for two PCR reactions to generate the two partially overlapping fragments of the gene. The reverse primer for the first reaction includes one or more mismatched nucleotides (indicated with a red circle) that correspond to the desired mutation(s) to be introduced into the genome. The forward primer for the second reaction has the equivalent mismatch in a reverse complementary configuration (also indicated with a red circle). The two PCR products generated in part 1 (products a and b) are then used as templates for fusion PCR using two primers that anneal to products a and b in the fashion shown in part 2. This results in the generation of full-size PCR products (product c in part 3) harboring the desired mutation(s). (C) The *hht2Δ::URA3* strain is then co-transformed with the full-size PCR products and a backbone plasmid (a *HIS3*-marked plasmid in this example), and cells are selected for the presence of the plasmid (on media lacking histidine in this example). Transformants are then screened for 5-FOA resistance - resistant cells are candidates for having undergone a homologous recombination event leading to integration of the PCR product and excision of the *URA3* gene, as shown. Subsequent loss of the backbone plasmid by mitotic cell division leads to the final desired histone mutant strain. We have found that selection of the backbone plasmid followed by screening for 5-FOA resistance results in a much higher frequency of identification of correct integration events compared to direct selection on 5-FOA plates, which mostly identifies cells that have acquired spontaneous *URA3* mutations. (This figure has been modified from reference¹⁴). [Please click here to view a larger version of this figure.](#)

1. Replacement of the Target Histone Gene with the *URA3* Gene

1. Perform standard PCR-mediated one-step gene disruption replacing the ORF of target histone gene with the *URA3* gene^{8,9}.
NOTE: The use of yeast cells carrying the *ura3Δ0* is recommended as this mutation removes the entire endogenous *URA3* ORF, thus avoiding integration of the PCR product into the *URA3* locus⁸. Alternatively, the *K. lactis* *URA3* gene can be used effectively for the generation of the histone replacement in any *ura3* background as it is functional in *S. cerevisiae* but has only partial sequence homology with the *S. cerevisiae* *URA3* gene. The strain should also be auxotrophic for at least one compound that will allow for selection of the backbone plasmid in the transformation experiment (see step 4 of this protocol). This step is not necessary if a *target histone geneΔ::URA3* strain is already available.

2. Generation and Purification of PCR Products Corresponding to Two Partially Overlapping Fragments of the Target Histone Gene using Primers Harboring the Desired Mutation(s)

1. **Generate PCR products corresponding to two partially overlapping fragments of the target histone gene.**
 1. Prepare two PCR reactions as follows:
 - 1. To generate PCR products corresponding to the first half of the gene (product a in **Figure 1B**), set up the following reaction: 1 μl template DNA, 5 μl 10 μM forward primer, 5 μl 10 μM reverse primer, 0.5 μl (1.25 U) thermostable DNA polymerase, 10 μl 5x DNA polymerase buffer, 5 μl dNTP mixture (2 mM each), and 23.5 μl dH₂O.

NOTE: The template DNA can be genomic DNA derived from a strain wild-type for the target histone gene isolated using standard procedures¹⁰. To account for variations in DNA concentration and level of impurities in different genomic preparations, it is recommended to optimize the reactions by using either undiluted DNA or different dilutions of the genomic preparations (e.g., 1:10 and 1:100). The forward primer should anneal to a region upstream of the target gene. The reverse primer should anneal within the ORF, be ~40 nucleotides in length, and contain the desired mutation(s) somewhere in the middle of it (see **Figure 1B-1** and Representative Results section for examples). The use of a high fidelity DNA polymerase is recommended in order to reduce rates of undesired mutations during the synthesis of the PCR products.

2. To generate PCR products corresponding to the second half of the gene (product **b** in **Figure 1B**), set up a reaction as indicated in 2.1.1.1 but with different primers.

NOTE: The forward primer should anneal within the ORF, be ~ 40 nucleotides in length, and contain the desired mutation(s) somewhere in the middle of it. Note that the mutation(s) in this primer is the reverse complement of the mutation(s) in the reverse primer in step 2.1.1.1. The reverse primer should anneal to a region downstream of the target gene (see **Figure 1B-1** and Representative Results section for examples).

2. Place the reactions in a thermocycler with the following settings: 94 °C 30 sec; 30 cycles of the following settings: 98 °C 10 sec, 60 °C 5 sec, 72 °C 1.5 min; and 72 °C 10 min.

Note: Optimization of PCR parameters may be required for specific primer sets and target histone gene.

2. Run 20 - 50 µl of the material from the PCR reactions on a 0.9% low melting point agarose gel in 89 mM Tris base, 89 mM boric acid, 2.5 mM EDTA (TBE) buffer.
3. Cut agarose gel sections containing the PCR products from gel using a clean scalpel or razor blade and transfer each to a 1.5 ml microcentrifuge tube. Store agarose sections containing PCR products at -20 °C until ready to use.

3. Fusion PCR of the Two Partially Overlapping Fragments to Obtain Full Size PCR Products for Integration

1. Prepare template for PCR reactions

1. Melt agarose gel sections from step 2.3 by placing the microcentrifuge tubes in a heat block set at 65 °C for 5 min (or until fully melted). Vortex tubes every 1 - 2 min to facilitate the melting process.
2. Transfer a set amount of melted agarose from each sample (e.g., 50 µl each, for a total of 100 µl) into a single microcentrifuge tube and mix by vortexing. Use this as the template in the fusion PCR reactions. Place the tube at -20 °C until ready to use.

2. Amplify a large quantity of full size PCR product (product **c** in **Figure 1B**)

1. Set up six PCR reactions, each with the following components: 2 µl template DNA, 10 µl 10 µM forward primer, 10 µl 10 µM reverse primer, 1 µl (2.5 U) thermostable DNA polymerase, 20 µl 5x DNA polymerase buffer, 10 µl dNTP mixture (2 mM each), and 47 µl dH₂O. NOTE: The number of reactions can be altered depending on the PCR efficiency. The template DNA (see 3.1.2) should be heated to 65 °C until melted, mixed by vortexing, and added last to the PCR reaction mix. Once added, mix gently but thoroughly by pipetting the solution up and down several times. To account for variations in DNA concentration in the different samples, it is recommended to first optimize the reactions by using either undiluted template or different dilutions of the template (e.g., 1:10 and 1:100). The two primers used should anneal to the two partially overlapping fragments of the target gene as illustrated in **Figure 1B-2** and be designed such that the final PCR products will have at least 40 base pairs on either side homologous to the regions flanking the *URA3* ORF that will drive the homologous recombination step (see Representative Results section for examples). The use of a high fidelity DNA polymerase is recommended in order to reduce rates of undesired mutations during the synthesis of the PCR products.
2. Place the tubes in a thermocycler with the following settings: 94 °C 30 sec; 30 cycles of the following settings: 98 °C 10 sec, 50 °C 15 sec, 72 °C 1.5 min; and 72 °C 10 min.

NOTE: Optimization of PCR parameters may be required for specific primer sets and target histone gene.

4. Co-transformation of Full Size PCR Products and Backbone Plasmid, and Selection for Marker on Plasmid

1. Concentration of PCR products

1. Pool the six PCR reactions (600 µl total) from step 3.2.2 into a single microcentrifuge tube and mix by vortexing.
2. Split the sample into three 200 µl aliquots in microcentrifuge tubes. Precipitate the DNA in each tube by adding 20 µl of 3M sodium acetate (pH 5.2) and 550 µl of 100% ethanol. Mix the solution thoroughly and place on ice for at least 15 min. Collect DNA by centrifugation at ~14,000 x g for 10 min, rinse the pellet with 200 µl of 70% ethanol, and air dry.
3. Resuspend each DNA pellet into 25 µl of dH₂O, and pool into a single tube (for a total of 75 µl).

2. Yeast co-transformation

1. Prepare 10 ml of overnight culture of the strain generated in section 1 in Yeast extract Peptone Dextrose (YPD) liquid medium¹¹.
2. The following morning, inoculate 400 mL of YPD liquid medium with 8 ml of the saturated overnight culture and incubate by shaking at 30 °C for 4 - 5 h to allow cells to enter logarithmic phase of growth.
3. Collect the cells by centrifugation at ~3,220 x g for 10 min, discard the liquid medium, and resuspend the cells in 1 volume of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 M Lithium Acetate solution (TE/LiAc).
4. Collect the cells by centrifugation at ~3,220 x g for 10 min, and discard the TE/LiAc.
5. Resuspend the cells in 1 ml TE/LiAc.
6. Set up the following reaction cocktail in a microcentrifuge tube: 800 µl of cells from step 4.2.5, 40 µl of boiled 10 mg/ml salmon sperm DNA, a total of 12.5 µg of backbone plasmid DNA, and 75 µl of concentrated PCR product from step 4.1.3.

NOTE: Salmon sperm DNA should be boiled for 5 min and placed on ice for at least 5 min before use in the reaction. Total volume of backbone plasmid DNA added should be kept to a minimum (~80 µl or less). See Representative Results section for an example of a backbone plasmid.

7. Mix the cocktail tube thoroughly and aliquot evenly into eight microcentrifuge tubes (Tubes 1 - 8).
8. Set up the following two control transformation reaction tubes:
 1. Tube 9 (no PCR product control): 100 µl of cells from step 4.2.5, 5 µl of boiled 10 mg/ml salmon sperm DNA (boiled for 5 min; see step 4.2.6 Note), a total of 1.56 µg of backbone plasmid DNA and no PCR product added.
 2. Tube 10 (no DNA control): 100 µl of cells from step 4.2.5, 5 µl of boiled 10 mg/mL salmon sperm DNA (see step 4.2.6 Note), no backbone plasmid DNA added, and no PCR product added.
 3. Mix both tubes gently but thoroughly by pipetting up and down several times.
9. Incubate the ten tubes at 30 °C for 30 min.
10. To each tube, add 1.2 ml of 40% polyethylene glycol (PEG 3350) in TE/LiAc. Mix thoroughly using a P-1000 pipet until the solution is homogeneous.
11. Incubate the ten tubes at 30 °C for 30 min. Gently mix the solution by pipetting up and down and then incubate tubes at 42 °C for 15 min.
12. Collect the cells by spinning the tubes in a microcentrifuge at ~14,000 x g for 30 sec. Discard the liquid and resuspend the cells in 1 ml of sterile dH₂O.
13. Collect the cells by spinning the tubes in a microcentrifuge at ~14,000 x g for 30 sec. Discard the liquid and resuspend the cells in 500 µl of sterile dH₂O.
14. Pool tubes 1 - 8 together (total volume of 4 ml) and mix thoroughly by pipetting up and down.
15. Plate 200 µl of the above mixture on each of twenty complete minimal dropout medium plates¹¹ (plates 1-20) for selection of the backbone plasmid.
16. Plate 200 µl of the mixture from Tube 9 and 200 µl of mixture from Tube 10 each on its own selection plate (plates 21 and 22, respectively).
17. Incubate the 22 plates at 30 °C for 3 - 5 days to select for plasmid transformants.
18. Inspect transformation plates after 3 - 5 days of incubation. Approximately 5,000 colonies should be visible on plates 1-21 (see Representative Results for an example) and no colonies should be present on plate 22.

5. Screen for 5-FOA-resistant Transformants

1. Transfer cells from plates 1 - 20 (and transformation plate 21 as a control) to 5-fluoroorotic acid (5-FOA) plates¹¹ by replica-plating¹² in order to screen for loss of the *URA3* gene as a result of integration of the PCR products at the desired location.
 1. Remove the plate lid and press the plate containing colonies on a sterile velvet. Transfer the cells from the velvet to a 5-FOA plate by pressing the plate on the velvet. Incubate plates at 30 °C for 2 days.
2. Following the 2-day incubation, carefully inspect the 5-FOA plates for growth.

NOTE: A candidate integration event will be represented by a small asymmetric "squashed" colony on a 5-FOA plate - conversely, small papillae growing on 5-FOA plates are likely representative of spontaneous *URA3* mutations that arose during the growth of colonies on the transformation plates, and are thus unlikely to represent the desired integration event (see **Figure 3** in the **Representative Results** section for further elaboration on this point and for some examples).

6. Purification of 5-FOA-resistant Colonies and Loss of Backbone Plasmid

1. Using sterile toothpicks, pick the candidate colonies from the 5-FOA plates described in step 5.2 and streak for single colonies onto YPD plates. Incubate for 2 - 3 days at 30 °C.
2. Following the incubation, replica-plate each YPD purification plate to a fresh YPD plate, a drop-out plate lacking uracil to check for loss of the *URA3* gene, and a second drop-out plate to monitor for the presence or absence of the backbone plasmid. Incubate for 1 - 2 days at 30 °C.
3. Following the incubation, identify a colony from each candidate sample that is growing on the YPD plate but not growing on either drop-out plate (such a colony is expected to have lost the *URA3* gene through the recombination event and lost the backbone plasmid during mitotic cell division). Restreak such colonies on fresh YPD plates. These colonies are the integration candidates and will be analyzed further in step 7.

7. Molecular Analyses to Assay for Proper Integration of the Mutant Allele

1. Isolate genomic DNA from the candidate samples using standard procedures¹⁰.
2. **Amplify genomic region encompassing the target site.**
 1. Set up the following PCR reaction for each sample: 0.5 µl template DNA, 5 µl 10 µM forward primer, 5 µl 10 µM reverse primer, 0.5 µl (2.5 units) Taq DNA polymerase, 5 µl 10x Taq DNA polymerase buffer, 5 µl dNTP mixture (2 mM each), and 29 µl dH₂O.

NOTE: Template DNA is the genomic DNA derived from the candidate samples. It is recommended to also include two control reactions: one using genomic DNA derived from the original *histone geneΔ::URA3* strain as template and another using genomic DNA from a wild-type histone strain as template. To account for variations in DNA concentration and level of impurities in different genomic preparations, it is recommended to optimize the reactions by using either undiluted DNA or different dilutions of the genomic preparations (e.g., 1:10 and 1:100). It is important to make sure that these primers anneal to DNA sequences *outside* the region encompassed by the putatively integrated PCR product - this way, the size of the PCR products in these reactions can be used as a diagnostic tool for integration of the products at the correct genomic location (see **Representative Results** for an example).

2. Place the reactions in a thermocycler with the following settings: 94 °C 3 min; 30 cycles of the following settings: 94 °C 45 sec, 50 °C 45 sec, 72 °C 2 min; and 72 °C 10 min.
NOTE: Optimization of PCR parameters may be required for specific primer sets and target histone gene.

3. Processing of PCR products

1. Run 20 µl from each reaction on a 0.8% TBE agarose gel.
2. Assess the size of the PCR products using DNA standards as a reference to determine if the *URA3* gene has been successfully replaced by the putatively mutated histone gene (see Representative Results for an example).
NOTE: In certain cases, the desired mutation(s) introduced into the histone genes either create or destroy a restriction site. If this is the case, the presence of the desired mutation in the PCR products of the size indicative of correct integration can be assessed by subjecting the products to digestion with the corresponding restriction enzyme followed by gel electrophoresis analysis (see Representative Results for an example).
3. Subject PCR products of the size indicative of correct integration to DNA sequencing to confirm the presence of the desired mutation(s) and to ensure that no additional mutations have been introduced into the genome.

Representative Results

We describe the generation of an *hht2* allele expressing a histone H3 mutant protein harboring a substitution at position 53 from an arginine to a glutamic acid (H3-R53E mutant) as a representative example of the targeted *in situ* mutagenesis strategy.

We generated a strain in which the entire ORF of *HHT2* is replaced by the *URA3* gene (see step 1 of the protocol). This strain, yAAD156, also harbors a *his3Δ200* allele, which causes the cells to be auxotrophic for histidine. Following the procedure indicated in step 2 of the protocol, we then generated the two partially overlapping fragments of *HHT2*. The following primers were used for the first fragment: Forward Primer (OAD20): 5' GCGTTCATTATCGCCCAATGTG 3' and the Reverse Primer (R53Erev): 5' GTTCAGTAGATTTTGAATtcTCTAATTTCTCTCAAG 3'. The following primers were used for the second fragment: Forward Primer (R53Efor): 5' CTTGAGAGAAATTAGAgAATCCAAAAATCTACTGAAC 3' and the Reverse Primer (OAD21): 5' GCGCTTGATCAGCAGTTCATCG 3'. Note that the Reverse Primer in the first reaction and the Forward Primer in the second reaction contain the desired mutated nucleotides (in lower case) - these mutated nucleotides are nestled in between two long stretches of wild-type sequences, as this allows for efficient annealing of the primer to the template DNA despite the mismatch at the mutated positions. Also note that the mutated nucleotides in these two primers are reverse complementary to each other and cause an AG to GA mutation in the sense strand, which results in a AGA to GAA codon change, which ultimately produces the R53E mutation in the encoded protein. Results from these PCR reactions are shown in **Figure 2A**.

Using the procedure in step 3, we then generated the full-size PCR products. The primers used were Forward Primer (OAD479): 5'TATGGCTCGGTGTCAAACA 3' and Reverse Primer (OAD480): 5' CATGGTTTCTTGCCGGTTAT 3'. When designing these primers, it is important to make sure that the resulting fusion PCR products include at least 40 base pairs on either ends to drive the homologous recombination reaction (the longer the regions of homology, the more efficient and specific the homologous recombination events will be). In our experiment, the full-size PCR products contained a 195 base pair region and 220 base pair region homologous to the regions upstream and downstream of the *HHT2* ORF, respectively. A sample of these PCR products was analyzed by agarose gel electrophoresis (**Figure 2B**).

The full-size PCR products were then co-transformed along with plasmid pRS413 (a centromeric, *HIS3*-marked plasmid¹³) and transformants were selected on plates lacking histidine (SC-his plates) as described in step 4 of the protocol. His⁺ colonies were then screened for 5-FOA resistance following the procedures described in step 5 of the protocol. **Figure 3** shows an example of a transformation plate (SC-his) and 5-FOA plates following replica-plating from SC-his plates. Examples of candidate samples as well as samples unlikely to represent the desired integration events are also presented in **Figure 3**. In our experiment, we identified twelve candidate samples out of ~90,000 transformants screened. These candidates were then purified as described in step 6 of the protocol.

The twelve candidates were then subjected to the PCR analysis described in step 7 of the protocol to assess if they reflected authentic replacements of the *URA3* gene with mutant PCR products. For our experiment, we used a Forward Primer that anneals 89 base pairs upstream from the integration site of the PCR product and a Reverse Primer that anneals 302 base pairs downstream from the integration site of the PCR product. These primers generate PCR products of 1217 base pairs in size if the *HHT2* locus is occupied by *HHT2* (or mutant versions of *HHT2* harboring base pair substitutions), or PCR products of 2188 base pairs in size if the locus is occupied by the *URA3* marker gene. The sequence of the Forward Primer used (OAD476) is: 5' GAAACTATTGGCAGCCCTA and that of the Reverse Primer used (OAD477) is: 5' CCTGCGAATCAACCGATACT 3'. Of the twelve candidates we had identified, four showed integration of a PCR product at the correct location (see **Figure 4A** for an example). Finally, since the AG to GA mutation generates a new *EcoRI* restriction site, we subjected PCR products from one of the successful integration samples to an *EcoRI* digestion and were able to demonstrate that the mutant sequence had indeed been integrated into the genome (see **Figure 4B**). In this particular case we did not sequence the PCR products to ensure that additional unwanted mutations had not been incorporated into the genome: however, we have used a procedure very similar to this to generate a large number of *HHT2* mutations in a past study¹⁴, and found that the majority of the integrated mutant alleles carry no mutations other than the desired ones.

Figure 2: Generation of PCR Products Harboring Desired Mutations for Integration into the Yeast Genome. (A) PCR reaction to generate the partially overlapping fragments of *HHT2* harboring the desired mutations. Top: cartoon representation of the two PCR reactions to obtain the two *HHT2* fragments (refer to Figure 1B-1). Red circles represent the mismatched nucleotides on the primers used to introduce the AG to GA mutation in the sense strand of the gene. Bottom: gel electrophoresis analysis of PCR products *a* and *b* (lanes 2 and 3, respectively). DNA standards are shown in lane 1. (B) Fusion PCR reaction to generate full size PCR product for integration into yeast genome (refer to Figure 1B-2 and 3). Top: cartoon representation of the PCR reaction and expected PCR product (product *c*). Red circles represent desired mutation as described in (A) above. Bottom: gel electrophoresis analysis of PCR products *c* (lane 2). DNA standards are shown in lane 1. [Please click here to view a larger version of this figure.](#)

Figure 3: Representative Results from the Co-transformation Experiment and 5-FOA Screen. Left: representative SC-his plate plated with cells subjected to the co-transformation procedure after a 3-day incubation at 30°C. Approximately 5,000 colonies were counted. Middle: representative 5-FOA plate following replica-plating from an SC-his co-transformation plate after a 2-day incubation at 30°C. Samples 1 and 2 were considered candidates for having gone through a successful integration event due to their asymmetric morphologies. This is because a true replacement of the *URA3* gene with the *hht2* allele is likely to occur before (or very soon after) a transformed cell is plated on the SC-his plate and, as a result, the colony that will arise at that location will contain mostly, if not exclusively, 5-FOA-resistant cells – when such a colony is then subsequently replica-plated to a 5-FOA plate it will be squashed, giving rise to an asymmetric-looking patch of cells on the plate. Conversely, spontaneous mutations in the *URA3* gene that can arise during colony formation are more likely to be confined within a small region of a colony, and thus more likely to give rise to papillae when replica-plated to a 5-FOA plate. Right: a different representative 5-FOA plate following replica-plating from an SC-his co-transformation plate after a 3-day incubation at 30 °C. An additional candidate (sample 3) as well as two small papillae are visible on this plate (samples 4 and 5) – such papillae, which are most clearly visible after 3 or more days of incubation, are likely to represent spontaneous *URA3* mutations and not the desired integration event. [Please click here to view a larger version of this figure.](#)

Figure 4: Molecular Analyses of Candidate Integration Samples. (A) PCR assay to identify successful integration events. Top: cartoon representations of the PCR reactions used to assess successful integration of the *hht2* mutant allele into the genome. Bottom: gel electrophoresis analysis of PCR reactions using genomic DNA derived from an *HHT2* wild-type strain (used as a control, lane 2), strain yAAD156 harboring the *hht2Δ::URA3* replacement (used as a control, lane 3), a candidate integration sample (lane 4), and a 5-FOA-resistant papillae sample (and thus unlikely to represent a correct integration event, lane 5). DNA standards are shown in lane 1. Note that the sizes of the PCR products for the candidate integration sample are consistent with a successful integration event, whereas the size of the PCR products for the papillae sample is consistent with an intact *hht2Δ::URA3* locus. (B) *EcoRI* digestion to confirm presence of mutant allele. Top: cartoon representation of the expected digestion fragments derived from an *EcoRI* digestion reaction of PCR products containing either the wild-type *HHT2* gene or the mutant *hht2* allele. Bottom: gel electrophoresis analysis of digestion reactions of PCR products derived from a wild-type *HHT2* strain (lane 2; the PCR products used here were derived from the same sample as that used in lane 2 of panel A) and a candidate integration sample (lane 3; the PCR products used here were derived from the same sample as that used in lane 4 of panel A). DNA standards are shown in lane 1. Note that the digestion patterns confirm that the AG to GA mutation has been successfully introduced into the genome of the candidate integration sample. [Please click here to view a larger version of this figure.](#)

Discussion

The high level of sequence homology between the two non-allelic genes that code for each of the four core histone proteins in haploid *S. cerevisiae* cells can represent a challenge for investigators who wish to specifically target one of the two genes for mutagenesis. Previously described yeast mutagenesis methodologies, including the *Delitto Perfetto*, site-specific genomic (SSG) mutagenesis, and cloning-free PCR-based allele replacement methods^{5,6,7}, as well as more recent yeast CRISPR-based techniques¹⁵, often rely at least in part on sequences within the ORF of a target gene to recruit the recombination or repair machineries to the desired genomic site to eventually generate the final mutant allele. On the other hand, the strategy we have presented here makes use of DNA sequences flanking the targeted ORF to drive the homologous recombination event required for the integration of the mutation, and, as a result, allows for more specific targeting of a gene over another despite the two genes having highly homologous ORFs. This feature makes our strategy particularly well-suited for histone mutagenesis. However, this strategy can also be adapted for mutagenesis of other genes in the yeast genome.

Additional features of our strategy include the facts that the lengths of the regions that drive homologous recombination of the mutant genes can be designed to be very extensive, thus increasing the efficiency of integration at the target genomic location, and that besides the desired mutation(s) no additional DNA sequences are introduced into the genome. An additional advantage of this system is that once a strain harboring replacement of a specific histone gene with *URA3* has been constructed, it can be used for the generation of any desired mutant version of that particular histone gene. Thus, for example, strain yAAD156, which is available to the research community upon request, can be used to make any desired *hht2* mutation without the need of constructing a *de novo hht2Δ::URA3* allele. Finally, by using properly designed PCR primers, this strategy can also be used to generate histone alleles with internal deletions or with mutations at multiple codons, as long as they are relatively near each other (for example, we have generated an *hht2* allele encoding an H3-K56R,L61W double mutant protein using this strategy).

The ability to specifically target one of two highly homologous histone genes for mutagenesis could be useful in a number of different experimental settings. For example, since the *HHT1-HHF1* genes are expressed at different levels compared to the *HHT2-HHF2* genes¹⁶, it could be of interest to determine whether a particular H3 or H4 mutation confers different phenotypes depending on which gene it is expressed from. Another example is a scenario in which an investigator wishes to generate haploid cells expressing a particular histone mutant from both corresponding histone genes - this could be achieved by first mutagenizing each gene independently in two different strains, and then obtaining double mutant haploid cells through a cross and subsequent isolation of the desired meiotic products. Yet another example relates to mutagenesis of histones H2A and H2B: given the fact that two slightly different isoforms of each protein are encoded by the corresponding non-allelic gene set, an investigator may want to assess the effects of a specific H2A or H2B mutation in the context of either isoform. When

designing experiments to mutagenize the *HTA1* and *HTB1* loci (encoding H2A and H2B, respectively), investigators should be aware of recent findings showing that strains carrying an (*hta1-htb1*) Δ are viable only if they have amplified the *HTA2-HTB2* locus (and nearby *HHT1-HHF1* locus as well) through the generation of a small circular chromosome¹⁷, as this could complicate the interpretation of the results.

We have recently used a version of this histone mutagenesis strategy to generate histone H3 proteins expressing all possible amino acid substitutions at position 61, which is normally occupied by the amino acid leucine¹⁴. For those experiments, instead of using yAAD156 as the starting strain for the mutagenesis, we used strain yADP106, which harbors a replacement of the *HHT2* ORF with both the *URA3* and *TRP1* nutritional markers (instead of just *URA3*). The presence of both marker genes facilitated the identification of candidates for integration of the mutant PCR products following the 5-FOA screen and purification step (steps 5 and 6 of the protocol), as such candidates became phenotypically Ura⁻ and Trp⁻, whereas spontaneous *URA3* mutation resulted in cells with a Ura⁻ Trp⁺ phenotype. yADP106 is also available upon request, as is the sequence of the *URA3-TRP1* cassette, which could be amplified as a unit and used for mutagenesis of other histone genes using the strategy described herein.

Inability to obtain the desired histone mutants using this strategy could be attributable to a number of possible reasons, including insufficient amount of full-size PCR products used for the integration step or an insufficient number of transformants following the co-transformation step. The former problem could be addressed by pooling together larger sets of PCR reactions or by designing different primer sets that produce a higher yield of product, whereas the latter problem could be solved by using higher amounts of backbone plasmid in the co-transformation experiment. Although, as indicated earlier, one can use this procedure to generate histone mutants harboring two or more amino acid substitutions, the targeted amino acids have to be relatively close to each other since the respective codons need to be located within the same primer molecule. Thus, this strategy cannot be easily adapted for the generation of histones with multiple mutations located distantly from each other across the length of the proteins.

Disclosures

The authors declare that they have no competing financial interests.

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